Original Article

Antibacterial and Antibiofilm Activity of *Ziziphora clinopodioides* Essential Oil: An In Vitro Study

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Abstract

Objective: The present study examined the antibacterial and antibiofilm efficacy of *Ziziphora clinopodioides* (Z. clinopodioides) essential oil (EO) against the primary biofilm colonizers, such as *Streptococcus sanguinis* (S. sanguinis), *Streptococcus mitis* (S. mitis), and *Streptococcus oralis* (S. oralis).

Methods: The hydrodistillation process was used for the essential oil extraction, and its components were analyzed using gas chromatography-mass spectrometry (GC-MS). Antibacterial effect against ATCC strains of S. sanguinis, S. mitis, and S. oralis was assessed using agar well diffusion. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were investigated using the broth macrodilution method. Qualitative tube methods were used to evaluate the antibiofilm efficacy of the Z. clinopodioides EO. 0.12% Chlorhexidine (CHX) served as a positive control.

Results: GC–MS recognized 24 elements with menthol (29.85%), (R)-carvone (10.53%), and eucalyptol (9.88%) as main constituents. Z. clinopodioides EO demonstrated dose-dependent inhibition zones among species and mixtures; generally, inhibition zones were smaller than CHX. The MICs for S. mitis and S. oralis were 2.34 µL/mL, while for S. sanguinis and the three-species mixture were 6.25 µL/mL; The MBCs were 4.68 and 12.5 µL/mL, respectively. The essential oil exhibited weak (S. sanguinis) to moderate (S. mitis, S. oralis, and the mixed culture) antibiofilm activity.

Conclusions: Z. clinopodioides EO demonstrated antibacterial and antibiofilm effectiveness against primary biofilm colonizers; however, its effectiveness was lower than that of chlorhexidine.

Keywords: *Z. clinopodioides EO; Antibacterial efficacy; Antibiofilm activity; Primary biofilm colonizers.*

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Introduction

Periodontitis is an inflammatory disease of microbiological origin in the oral cavity. Dysbiosis between the oral bacteria and host immune response dysregulation enhances the pathogenicity from periodontally healthy states to disease condition[1].

An extensive variety of microorganisms have been found to be pathogenic agents, sometimes called periopathogens[2]. *S. oralis, S. mitis, and S. sanguinis* are considered commensal bacteria that participate in the complex bacterial biofilm formation as primary biofilm colonizers on the tooth surface[3]. Initial colonizers are crucial, creating attachment substrates for later colonizers, including *Porphyromonas gingivalis* and *Fusobacterium nucleatum,* eventually affecting the future phases of biofilm formation and periodontal disease[3-6]. It is advised that, in addition to following good oral hygiene, initiatives are required to limit the development of dental biofilm, periodontal disease, and tooth cavities by targeting the primary colonizers at the first stages of dental biofilm formation[7,8].

Herbal medicines comprise active substances derived from plant parts or other plant parts considered to possess therapeutic properties. Herbal products are favored over traditional pharmaceuticals because of their extensive biological efficacy, enhanced safety characteristics, and reduced expenses. Moreover, conventional medications are recognized for inducing several adverse effects, and prolonged use has led to antibiotic resistance.

Consequently, natural products are being utilized as an alternative for combating or preventing prevalent diseases, including those impacting the oral cavity[9-11].

Ziziphora is a genus within the *Lamiaceae* family, and *Z. clinopodioides* is one of this group's most recognized aromatic and edible species. It is extensively dispersed in Asia and Europe, particularly in Turkey and Iran[12].

In traditional medicine, it has been utilized as a decoction for multiple purposes, including a stomach tonic, sedative, carminative, and antipyretic agent[13]. *Z. clinopodioides* is a potent treatment for many diseases, such as cough, the common cold, bronchitis, headache, viral infections, diarrhea, nausea, typhus, inflammation, edema, cardiovascular disorders, sleeplessness, diabetes, and asthma[14,15]. Additionally, this plant is utilized as a spice to enhance the flavor of food and beverages[15]. Essential oils (EO) consist of volatile compounds derived from aromatic plants. These essential oils have notable biological capabilities, encompassing antibacterial, antifungal, and antioxidant activities[16,17].

Numerous studies have demonstrated the antibacterial activity of *Z. clinopodioides* against *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella enterica Typhi*[15,18-20]. Furthermore, the antibacterial and antibiofilm properties of *Z. clinopodioides* EO against early biofilm colonizers, such as *S. oralis, S. mitis, and S. sanguinis*, have not been studied before, which is critical during the initial phases of plaque formation. Investigating the efficacy of this botanical compound in inhibiting bacterial proliferation and biofilm development may provide a novel preventive strategy in oral healthcare. This study investigated the antibacterial and antibiofilm properties of *Z. clinopodioides* EO against *S. sanguinis, S. mitis, and S. oralis*. The results may facilitate the creation of diverse plant-derived products to prevent early biofilm formation and reduce the risk of dental biofilm-associated diseases.

Materials and methods

Ziziphora clinopodioides was collected in late Spring from East Kurdistan (Sanandaj governorate). A plant taxonomist from the College of Agriculture at the University of Sulaimani confirmed the identity of plants. The current study was registered and approved by the Ethical Committee of the College of Dentistry at the University of Sulaimani (Approval number 24/252, dated 16 December 2024).

Essential oil extraction

Essential oil (EO) extraction was conducted using hydrodistillation at the Bahar factory in Sulaymaniyah, according to the recognized methodology[21]. Hydrodistillation, using water to extract volatile compounds, is regarded as a gentle technique that reduces the destruction of sensitive ingredients. An appropriate quantity of plant material was positioned in the Novin stainless steel distillation machine (Tehran, Iran), a distillation machine specifically engineered for the factory's requirements. Water was added according to the quantity of the plant, 5 liters for every 1 kilogram of the plant, and upon boiling for 8 hours, the produced steam was passed through the plant. The steam transports the volatile components, and the resulting vapor mixture of steam and oil flows into the condenser, where it is cooled by circulating water, turning the vapor into a liquid mixture of essential oils and water before finally entering the oil separator. Because essential oil has a lower density than water, it separates and floats above the watery layer. The oil was gathered and kept in dark, securely sealed containers at room temperature, resulting in 2–3% essential oils per 100 grams.

GC-MS analysis of the extracted EO

The EO components were recognized using a GC-MS system involving an Agilent (7890A) gas

chromatography-mass spectrometry system (Santa Clara, CA, USA).

Bacterial strains preparation

The bacterial strain was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), including reference strains of *S. mitis* (ATCC 49456), *S. oralis* (ATCC 35037), and *S. sanguinis* (ATCC 10556). Following ATCC guidelines, the lyophilized cultures were rehydrated and revived under sterile circumstances.

Thereafter, each strain was cultured in Blood Agar and Brain Heart Infusion (BHI) broth (HiMedia, India). The agar plates and broth were incubated for 24 hours at 37°C in a 5% CO_2 environment generated by a candle inside the jar. The stock cultures were stored in 20% glycerol (Unimedic Pharma, Sweden) at -80 °C for future use (Figure 1).

Essential oil preparation and dissolution

An emulsifying agent was used, which was composed of 10% dimethyl sulfoxide (DMSO) (Merck, Germany) and 0.5% Tween 80 (Biochem-France)[22], due to EO being poorly soluble in water.

Figure 1:(A) *S. sanguinis* (ATCC 10556), (B)*S. mitis* (ATCC 49456), (C) *S. oralis* (ATCC 35037)

Assessment of the minimal inhibitory and minimum bactericidal concentrations

The broth macro dilution method was used to find the MIC of the tested EO[23]. A 400 µL/mL stock solution of *Z. clinopodioides* EO was prepared using 10% DMSO (Merck, Germany) and 0.5% Tween 80 (Biochem, France) as solvents. CHX 0.12% (KIN, SA, Spain) was the positive control, and MHB (HiMedia, India) was the negative control to allow bacterial growth. Two-fold serial dilutions were performed in ten sterile glass test tubes containing 900 µL of Mueller-Hinton broth, supplemented with 100 µL of bacterial inoculum, for MIC detection. Using a loop, a single fresh bacterial colony from a previous 24-hour culture of each species was collected and added to sterile MHB (HiMedia, India) in the tubes. The mixture was then vortexed and standardized to a 0.5 McFarland turbidity standard, equal to 1.5×10^8 CFU/mL. The final volume in each tube was 1 mL, starting with 200 - 0.390 µL/mL for *S. sanguinis* and the three species, and 150 - 0.29 µL/mL for *S. mitis* and *S. oralis*. To more accurately replicate the natural polymicrobial environment, the essential oil MICs were evaluated for each bacterial species individually and in combination. Cotton plugs were used to seal the inoculation tubes, which were then incubated for 24 hours at 37 °C in a candle jar. The MIC was defined as the lowest amount of the essential oil that significantly inhibited the growth of bacteria. After incubation, a series of dilution tubes were checked for bacterial growth, usually as turbidity or a layer of microorganisms at the bottom of the tube. Any tube in the set or dilution series that showed no bacterial growth was the MIC of the essential oils[24].

The MBC was determined by finding concentration that did not allow bacterial growth during the MIC test. A sample was taken from the chosen tubes using a sterile wire loop, placed on agar plates, and kept in a candle jar at 37 °C for 24 hours[25]. It was then determined whether or not bacteria were growing.

Antibiofilm efficacy evaluation

A qualitative tube adhesion approach was used to evaluate the efficacy of the extracts against the biofilms[26]. The inoculated broth used to test the MIC of different EOs was thrown away correctly. To remove planktonic or nonadherent bacteria, the test tubes were washed three times with phosphate-buffered saline (PBS) (Central Drug House, India), pH 7.3. After that, they were turned upside down for 45 minutes to ensure they were completely dry. Next, each tube was given 1 mL of 1% crystal violet and left at room temperature for 15 minutes. Rinsing the tubes three times with sterile distilled water eliminated the rest of the dye. The biofilm formation was evaluated qualitatively by observing (examiner's vision) the violet color on the wall and bottom of the tubes, then classifying it based on the intensity of the violet color as nonadherent (0), weakly adherent (+), moderately adherent (++), or firmly adherent (+++)[27]. All tests were done three times. The same protocols for antibiofilm activity were used with three different species at the same time.

Statistical analysis

The results are shown as mean ± standard deviation and were analyzed using SPSS software version 27 (SPSS Inc., Chicago, IL, USA). The independent t-test was employed for comparisons, with $p \leq 0.05$ indicating statistical significance.

Results

Ordering specific bacterial strains

Colonies of *S. sanguinis* are usually small and can be grayish or colorless; however, *S. mitis* and *S. oralis* are classified as alpha-hemolytic *streptococci*, signifying that they generate a greenish color around the colonies on blood agar (Figure 1).

GC-MS Examination of Bioactive Compounds

GC-MS analysis of *Z. clinopodioides* EO

The GC-MS analysis of the *Z. clinopodioides* EO identified 24 compounds. The main compound was menthol (29.85%), (R)-Carvone (10.533%), Eucalyptol (9.883%), L-β-pinene (6.278%), and p-Menthan-3-one (5.259%), as shown in Table 1.

Antibacterial Activity

The Antibacterial Efficacy of *Z. clinopodioides* EO

As shown in Table 2 and Figure 2, the essential oil of *Z. clinopodioides* exhibited concentration-dependent effects, characterized by an increase in inhibition zones corresponding to rising EO concentrations. It worked against *S. mitis* and *S. oralis* at 30%, 40%, and 50% concentrations, against *S. sanguinis* and all three species together at 40% and 50% concentrations. The inhibition zones for *S. mitis* were between 9.55 ± 1.0 mm and 17.99 ± 1.0 mm, and for *S. oralis*, they were between 7.0 ± 1.0 mm and 8.32 ± 0.3 mm. At 30% EO, *S. sanguinis,* and three species together showed no inhibition, but they did show zones of 8.92 ± 0.8 mm and 9.88 ± 0.5 mm at 40% and 50%, respectively, 7.8 ± 0.4 mm at 40% and 8.92 ± 1.5 mm at 50% against three species together. The agar well diffusion assay controls, which had 10% dimethyl sulfoxide and 0.05% Tween-80, did not have an inhibition zone. CHX, on the other hand, had much

bigger inhibition zones: 21.51 ± 1.0 mm for *S. mitis*, 18.89 ± 0.9 mm for *S. sanguinis*, 19.61 ± 0.6 mm for *S. oralis*, and 16.38 ± 1.4 mm for the three species together. CHX generally had a larger inhibition zone than the tested EO, as shown in Table 2.

MIC and MBC evaluation of *Z. clinopodioides* EO against the bacterial strain

The broth macro dilution method was used for the evaluation of the MICs of *Z. clinopodioides* EO against bacterial strains, which were 2.34 µL/mL for *S. oralis* and *S. mitis*, but 6.25 µL/mL for *S. sanguinis* and the three species together. The MBCs, illustrated in Figure 3, were 4.68 µL/mL for *S. oralis* and *S. mitis*; however, 12.5 µL/mL for *S. sanguinis* and the three species, as shown in Table 3.

Antibiofilm properties of *Z. clinopodioides* EO

The tube assay results showed that *Z. clinopodioides* EO affected how bacteria stuck to surfaces and formed biofilms. The amount of violet crystal deposition on the inner tube surfaces was used to measure biofilm formation. Darker staining meant that there was more biofilm present. The oil significantly affected biofilm production, even at the lowest concentration tested. The oil decreased biofilm adherence at its MIC, showing weak activity against *S. sanguinis* and moderate inhibitory effects on *S. mitis, S. oralis*, and the three species. In contrast, 0.12% CHX completely stopped biofilm growth for *S. sanguinis* and *S. oralis*. The negative control tubes, which only had bacterial inoculum in MHB, showed a lot of biofilm buildup, as shown in Table 4 and Figure 3.

Table 1: Results of GC-MS analysis of Z. clinopodioides EO.

Peak	R. T	Area%	MW	MF	CAS#	Compound name
1	4.16	1.643	100	C6H12O	928-96-1	3-Hexen-1-ol, (Z)-
2	4.395	1.254	136	C10H16	7785-70-8	1R-α-Pinene
3	5.423	6.278	136	C10H16	18172-67-3	L-β-pinene
4	6.217	0.045	136	C10H16	99-86-5	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-
5	6.289	0.191	130	C8H18O	589-98-0	3-Octanol
6	6.434	1.882	136	C10H16	5989-27-5	D-Limonene
7	6.827	9.883	154	C10H18O	470-82-6	Eucalyptol
8	7.08	0.319	136	C10H16	99-85-4	γ-Terpinene
9	7.55	0.015	136	C10H16	586-62-9	Terpinolen
10	8.034	0.402	154	C10H18O	78-70-6	Linalool
11	9.033	0.232	154	C10H18O	89-79-2	L-isopulegol
12	9.208	1.006	156	C10H20O	491-01-0	Neomenthol
13	9.361	5.259	154	C10H18O	10458-14-7	p-Menthan-3-one
14	9.584	29.85	156	C10H20O	2216-51-5	Menthol
15	10.373	0.083	170	C10H18O2	53404-49-2	Bicyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl
16	10.956	2.161	198	C12H22O2	20777-45-1	Isomenthol acetate
17	11.222	10.533	150	C10H14O	6485-40-1	(-)-(R)-Carvone
18	11.782	1.719	150	C10H14O	499-75-2	Carvacrol
19	13.852	0.057	204	C15H24	483-76-1	δ-Cadinene
20	13.943	0.083	154	C10H18O	562-74-3	4-Terpinenol
21	14.078	0.126	154	C10H18O	10482-56-1	α-Terpineol
22	15.164	0.287	222	C15H26O	511-67-1	Epiglobulol
23	15.885	0.075	220	C15H24O	1139-30-6	Caryophyllene oxide
24	16.126	0.054	220	C15H24O	1139-30-6	β-Caryophyllene oxide

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

Table 2: Mean and standard deviations of inhibition zones and p-values of CHX and *Z. clinopodioides* EO at different concentrations against S. mitis, S. sanguinis, S. oralis, and the three species together.

Oil%	Inhibition zone (*S. mitis*)			Inhibition zone (*S. sanguinis*)		
	***Z. clinopodioides*EO**	**CHX0.12%**	***p*-value**	***Z. clinopodioides*EO**	**CHX0.12 %**	**p-value**
30%	9.55±1.0	21.77±1.0	0.00			
40%	13.77±3.3	21.0±1.0	0.02	8.92±0.8	18.24±0.2	0.00
50%	17.99±1.7	21.77±1.0	0.03	9.88±0.5	19.55±1.6	0.001
	Inhibition zone (*S. oralis*)			Inhibition zone (three species)		
30%	7.0±1.0	20.33±0.8	0.00			
40%	7.99±1.3	18.51±0.4	0.00	7.8±0.4	16.44±1.3	0.00
50%	8.32±0.3	19.99±0.6	0.00	8.92±1.5	16.33±1.5	0.004

The t-test was used for comparisons, and $p \leq 0.05$ was considered statistically significant.

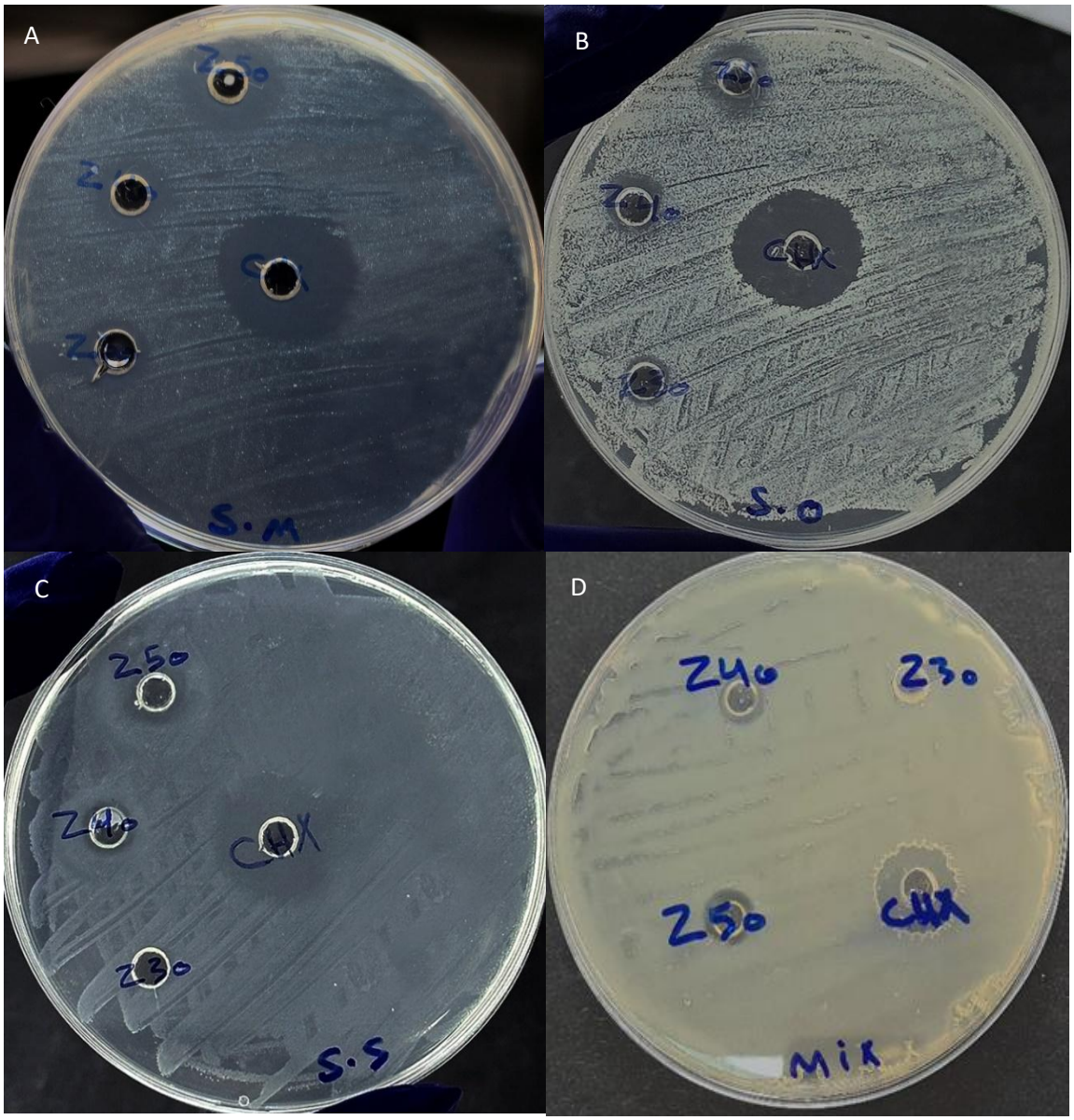

Figure 2: Antibacterial susceptibility with different concentrations (30%, 40% and 50%) for *Z. clinopodioides* EO by agar well diffusion method against (A) *S.mitis*, (B) *S. oralis*, (C) *S.sanguinis*, and (D) the three species together. Z30: *Z. clinopodioides* 30%, Z40: *Z. clinopodioides* 40%, Z50: *Z. clinopodioides* 50%, CHX: Chlorohexidine 0.12%

Table 3: The MIC and MBC values of *Z. clinopodioides* EO on bacterial strains.

Bacteria	*Z. clinopodioides* EO	
	MIC	MBC
S. oralis	2.34 µL/mL	4.68 µL/mL
S. sanguinis	6.25µL/mL	12.5µL/mL
S. mitis	2.34µL/mL	4.68µL/mL
Three species together	6.25µL/mL	12.5µL/mL

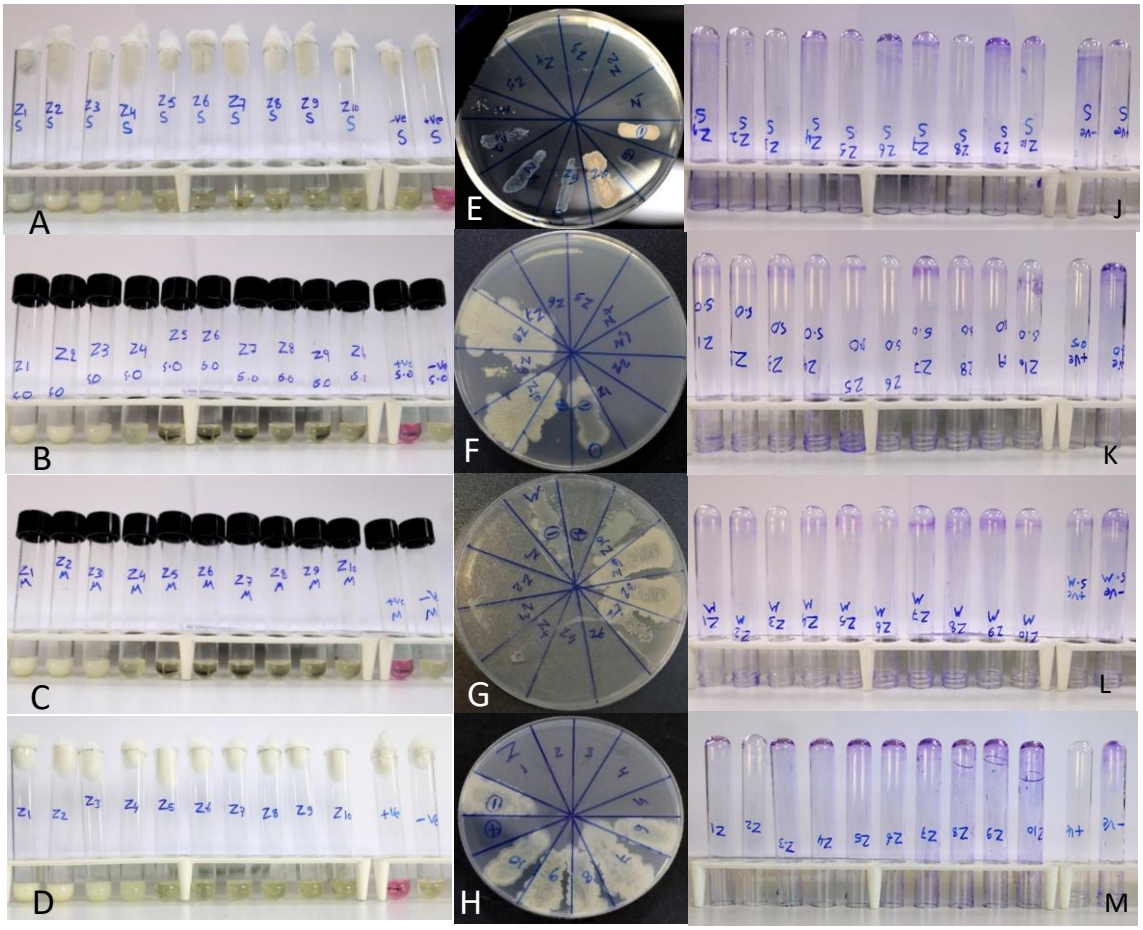

Figure 3*:* A, B, C, and D: Broth macro dilution method to determine MIC values, E, F, G, and H: Agar culture method to confirm MBCs and I, J, K, L: Qualitative antibiofilm assay using the tube method to assess the anti-biofilm activity of a two-fold dilution series of *Z. clinopodioides* EO on *S. sanguinis, S. oralis, S. mitis,* and the three species together, respectively.

Table 4: Qualitative biofilm formation of the *Z. clinopodioides* EO.

Bacteria	Z. clinopodioidesEO	CHX (positive control)	MHB (negative control)
S. oralis	++	0	+++
S. sanguinis	+	0	+++
S. mitis	++	+	+++
Three species together	++	0	+++

Discussion

Periodontitis is a complicated inflammatory disease with multiple contributing factors. One of the most critical factors that affects the development and progression of periodontal disease is an increase in the number of pathogenic bacteria in dental biofilm, which triggers a massive immune response[28]. The current study displays that Ziziphora EO prevents growth and lessens biofilm development of primary colonizers such as *S. sanguinis, S. mitis*, and *S. oralis*. These strains are the essentials and form the base for the attachment of secondary colonizers, "red-complex" bacteria, linking initial colonization to periodontal disease progression[1,4]. Natural EOs could contribute to biofilm control within preventive approaches by targeting these initial colonizers.

Using this natural product as a preventive or therapeutic agent is a good alternative to chemical and traditional antibacterial agents that have many adverse effects, such as tooth discoloration, taste alterations, burning sensations, and the emergence of bacterial resistance[29,30]. This *in vitro* study evaluated the antibacterial and antibiofilm effectiveness of *Z. clinopodioides* EO against primary biofilm colonizers, specifically *S. oralis, S. mitis,* and *S. sanguinis*, obtained from ATCC. The GC-MS analysis was used to determine the main compounds of the *Z. clinopodioides* EO, namely menthol (29.85%), (R)-Carvone (10.533%), Eucalyptol (9.883%), which enhance the *Z. clinopodioides* EO bioactivity as antibacterial and antibiofilm agent. Lamiaceae species are recognized for membrane-active monoterpenes that escalate cell-membrane permeability and can harm exopolysaccharide production and bacterial quorum sensing[16,17]. The detected growth inhibition zones and qualitative antibiofilm are consistent with such properties.

The findings demonstrated that *Z. clinopodioides* EO exhibits antibacterial efficacy against bacterial strains, as assessed by agar well diffusion and broth macro dilution techniques, with a significant difference in the average diameter of the inhibitory zone at various doses of *Z. clinopodioides* EO. Therefore, it may be stated that clarifying the impact of the oil increases with higher concentration. The comparison of the inhibition zone of *Z.clinopodioides* EO with that of a positive control group revealed that bacterial growth in the presence of chlorhexidine (CHX) was lower than that of *Z. clinopodioides* EO at concentrations of 30%, 40%, and 50%, for *S. oralis,* and *S. mitis,* 40%, and 50%, for *S. sanguinis,* and three species together. Moreover, the *Z. clinopodioides* EO exhibited diminished efficacy against the three bacterial strains collectively, when bacteria grow together, forming a polymicrobial, more complex, and more protective biofilm that may minimize the antibacterial effect of the EO[31].

In the present study, the EO demonstrated bacteriostasis/bactericidal effects, with mean MICs of 2.34 µL/mL for *S. oralis and S. mitis*, and 6.25µL/mL for *S. sanguinis, and* the polymicrobial mixture, MBCs were 4.68 and 12.5 µL/mL, respectively. The three-species mixture and S. sanguinis's higher MIC/MBC compared to the other two strains are due to intrinsic differences in the sensitivity and protective influence of the polymicrobial community. Antimicrobial tolerance in mixed communities can be enhanced by metabolic cooperation, physical shielding, and changed gene expression[31]. These results highlight the necessity of testing potential treatments in multispecies models that more closely resemble clinical plaque in addition to single strains. Other investigators reported a MIC against other non-oral pathogens that was equivalent to 3 µg/mL, 67 µg/mL, 33 µg/mL, 33 µg/mL, and 67 µg/mL for *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella enterica Typhi*, respectively[18]. However, no previous studies on periodontal pathogens were available to compare with the current findings.

The discrepancies in MIC values documented by various studies across regions are primarily linked to the chemical composition and concentration of active constituents in essential oils, which are substantially affected by plant genotype and environmental factors, including geographical conditions, soil properties, temperature, harvesting season, and, importantly, the oil extraction technique. Additionally, other factors may influence the MIC values, including variations in antibacterial testing methodologies, such as the formulation of the growth medium, incubation parameters, and the emulsifying agents or solvents utilized, which may modify the bactericidal efficacy of the essential oils. Furthermore, variations in the cell wall architecture across various strains of the same bacterial species may also affect MIC outcomes[32].Moreover, *Z. clinopodioides* EO demonstrated antibiofilm efficacy against bacterial strains, as assessed by the qualitative tube method. Methods interrupting any stage of biofilm formation may be beneficial in treating biofilm-related periodontal diseases[33].

The analysis of the results showed that *Z. clinopodioides* EO had a weak antibiofilm effect against *S. sanguinis*; however, it had a moderate antibiofilm effect against *S. mitis, S. oralis,* and the three species together. Several studies reported the antibiofilm potential of *Z. clinopodioides* EO[34,35]; however, no previous investigation has demonstrated the antibiofilm activity of the *Z. clinopodioides* EO on periodontal pathogens.

The present study offers significant findings into the antibacterial and antibiofilm effectiveness of *Z. clinopodioides* EO against primary biofilm colonizers using ATCC bacterial strains. As anticipated, 0.12% of CHX inhibited biofilm formation totally and performed better than EO. The gold standard CHX remains the clinical standard[10]. Concerns regarding ecological effects on the microbiome, tooth discoloration, taste disruption, and mucosal irritation may limit the long-term use of CHX. For these reasons, there is interest in botanical adjuncts that may enable dose-sparing or intermittent use.

However, certain limitations should be acknowledged. Standard laboratory strains may not represent the phenotypic diversity, resistance mechanisms, or biofilm-forming abilities of clinical or environmental isolates. Furthermore, the *in vitro* experimental framework does not replicate the intricate biological environments found *in vivo*, where aspects such as host immune responses, nutritional gradients, and surface properties can significantly influence biofilm formation and antimicrobial efficacy. Further research is required regarding the activity of the *Z. clinopodioides* EO against other oral bacteria, especially periodontal pathogens, and additional investigation about the active compounds of *Z. clinopodioides* extracts in different places and seasons.

Conclusion

Z. clinopodioides EO, abundant in menthol, (R)-Carvone, and other monoterpenes, demonstrated antibacterial and antibiofilm effectiveness against key primary colonizers such as *S. sanguinis, S. oralis, and S. mitis*. However, it was still less effective than chlorhexidine.

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