Original Article

Antibacterial and Antibiofilm Profiles of *Thymus Vulgaris* Essential Oil on Clinically Isolated *Porphyromonas Gingivalis* and *Prevotella Intermedia*: An *in vitro* Study

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Abstract

Objective: To investigate the antibacterial and antibiofilm effect of the essential oil extracted from *Thymus vulgaris* (TV) against clinically isolated *Porphyromonas gingivalis* (P. gingivalis) and *Prevotella intermedia* (P. intermedia).

Methods: Subgingival plaque samples were collected from periodontitis patients with a probing pocket depth of at least 6mm. Conventional microbiological tests and molecular techniques were used to isolate and confirm *P. intermedia*. In addition, previously isolated *P. gingivalis* was obtained from the microbiology department of the college of dentistry, Sulaimani University. The hydrodistillation method was used for the extraction of the essential oil. The antibacterial activity of TV against the confirmed clinical strains has been determined by disc diffusion and broth dilution methods to determine the minimum inhibitory (MIC) and bactericidal (MBC) concentrations. In addition, the antibiofilm activity of the essential oils was evaluated by a qualitative tube method.

Results: The essential oil extracted from TV revealed comparable antibacterial activity with inhibition zones ranging from 9.55±0.30mm to 15.85±0.30mm for *P. gingivalis* and 10.55±0.25mm to 16.85±0.40mm for *P. intermedia*. The MIC of the tested oil were 1.56µL/mL and 0.78µL/mL, while the MBC were 3.125µL/mL and 1.56 µL/mL against P. gingivalis and P. intermedia, respectively. TV essential oil showed moderate and robust antibiofilm activity against *P. gingivalis* and *P. intermedia*.

Conclusions: TV essential oil showed antibacterial and antibiofilm activity against clinically isolated *P. gingivalis* and *P. intermedia*. Further studies should focus on using TV essential oil as an adjunct to periodontal therapy.

Keywords: *Thymus vulgaris, Essential oil, Porphyromonas gingivalis, Prevotella intermedia, Antibacterial activity, Periodontal disease.*

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Introduction

Periodontal diseases (PD) are multicausal, polymicrobial diseases of the supporting structures of the teeth[1]. Bacterial biofilm is the key etiological factor of PD that can activate immune reactions, inducing an inflammatory response by the host[2]. The human oral cavity harbors over 700 bacterial species. However, a limited number are directly associated with the initiation and progression of PD[3]. A keystone pathogen and red complex member, *P. gingivalis*, is a gram-negative, rod-shaped, anaerobic, non-motile bacterium that colonizes the subgingival sulcus and could be detected in subgingival plaque samples in almost every patient with periodontitis. Pathobiont such as *P. gingivalis*, even though it presents in low abundance in the gingival sulcus, can negatively impact the composition of normal oral flora and encourage the evolution of dysbiotic inflammation-inducing microbiota[4]. *P. intermedia* is one of the orange complex species. It is a gram-negative anaerobic bacterium with a black pigmentation long related to PD, such as periodontitis, puberty-associated gingivitis, and acute necrotizing ulcerative gingivitis[5,6].

Scaling and root planing have been the cornerstone of periodontitis treatment[7]. Instead of focusing on individual periodontal pathogens, this therapy tries to remove the whole biofilm. However, not all patients or sites within the same patient will respond to this mechanical debridement alone[8]. Therefore, the adjunct use of antimicrobial agents might help reduce subgingival biofilm accumulation, especially in inaccessible areas. Furthermore, considering the relatively safe nature of botanical remedies and the drawbacks and issues associated with many chemical and synthetic medications, medicinal plants offer an alternative to these chemical agents[9].

Various herbal products for treating oral and dental diseases have recently regained popularity, and these products are available as mouth rinses, toothpaste, gels, and chips[10]. In addition, natural products such as essential oils (EOs) have increased as antimicrobial agents in medicine and dentistry over the last few decades. Antimicrobial and antioxidant properties of medicinal plants have attracted much interest recently because of their safety and widespread acceptance by consumers. They may protect against infections and prevent carcinomas, cardiovascular, and neurological disorders[11].

TV is a herbaceous plant belonging to the Lamiaceae family that develops in mountainous regions and is used as a drink instead of or even with tea and to add flavor to certain foods. The plant is widely prescribed in traditional medicine to cure many medical disorders, including oral, gastrointestinal, and respiratory ailments[12]. However, given the prevalence of PD, the increase in resistant microorganisms, the negative impacts of some chemotherapeutic agents used in dental practice, and the financial concerns of developing economies, there is a need for innovative, safe, and cost-effective preventive and therapeutic agents. Thus, the need for alternative approaches continues, and natural extracts from plants traditionally used as medicines, such as EOs, are accepted as valuable solutions.

Therefore, the present *in vitro* study aimed to evaluate the antibacterial and antibiofilm profiles of *Thymus vulgaris* essential oil (TVEO) on clinically isolated *P. gingivalis* and *P. intermedia*.

Patients and methods

Plant collection

TV was collected in June 2021 from the Shnrwe range, northeast of Halabja Governorate, 76 km from Sulaimani city, Kurdistan region-Iraq. The collection was carried out in the field involving wild species of the plant under study by trained personnel supervised by the researcher to avoid adulterants or contaminants. A plant taxonomist in the College of the agriculture / University of Sulaimani confirmed the plant. (The scientific committee of the College of Dentistry at the University of Sulaimani accepted the study protocol (Approval number: 500 on 21/9/2021).

Essential oil extraction

Extraction of the EO was performed by hydrodistillation method using the Clevenger apparatus under optimal operating conditions according to the standard protocol[13]. Freshly ground samples were added to sterilized distilled water in a 500 mL round-bottom flask and boiled for 3 hours. The EO was collected, dried under anhydrous sodium sulfate, and stored in a sealed amber vial at 4°C until used.

Gas chromatography-mass spectrometry (GC-MS)

The EO sample was analyzed to identify their composition by a GC-MS system composed of an Agilent 7820A GC (Santa Clara, CA, The USA) coupled with a mass spectrometer system.

Subgingival plaque sample collection and growth condition

Samples from subgingival plaques were collected from periodontitis patients who visited the Periodontics Department, College of Dentistry, University of Sulaimani. Written and informed consent from patients

was obtained prior to clinical examination. The samples were collected by isolating 6 mm or deeper periodontal pockets with sterile cotton rolls. A sterilized periodontal curette was used to eliminate supra-gingival plaque or calculus. A sterile paper point (F2 Dia-ProT™) was gently placed into the periodontal pocket until tissue resistance was felt and kept for one minute. After carefully removing the paper points, they were spread over supplemented Columbia agar media. The selective culture media contents per 500 mL were 19.5 grams of Columbia agar base, 2.5µg of hemin (Sigma Aldrich, China), 500µL 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™) within an anaerobic jar (BBL®GasPak system).

Identification, isolation, and confirmation of bacterial strains

Preliminary identification of *P. intermedia* was based on colony morphology, black-pigment production, Gram staining, and anaerobic environment, and finally confirmed by PCR and 16S rRNA Gene Sequencing techniques, and the proved isolated strains were stored in a 25% glycerol (Biochem-France) at -80ºC. *P. gingivalis* was obtained from the microbiology department of the College of Dentistry, University of Sulaimani[14].

DNA extraction

According to the manufacturer's instructions, total bacterial DNAs were extracted from clinical isolate samples using Add Prep Bacterial Genomic DNA Extraction Kit (Add Bio Inc., Korea). First, one mL of an overnight bacterial culture was centrifuged for 30 seconds, followed by the addition of 200µl of lysis solution and 20µl proteinase k solution. After the incubation, 200µl of binding solution and 200µl of absolute ethanol was added and centrifuged. Finally, the genomic DNA was eluted by centrifugation at 13,000 rpm for one minute, and the supernatant was used as a template.

DNA amplification

Specific primer pairs (Macrogen, Korea) targeted at the 16s rRNA gene at 575 base pairs were used to confirm the presence of P. intermedia(15). Specific primers were as follows: Forward: 5'-TTT GTT GGG GAG TAA AGC GGG- 3' Reverse: 5'- TCA ACA TCT CTG TAT CCT GCG T - 3'.

PCR Product preparation and protocol

The PCR preparation was carried out in a total volume of 20µl containing 2µl of reverse primer (10µM), 2µl of forward primer (10µM), 10µl of 2X concentrated Taq polymerase (Add Start Taq Master), 1µl of nuclease-free water and 5µl of DNA template. The PCR reaction involves three steps in a digital automated DNA thermocycler apparatus (VeritiTM 96 well). The DNA sample was first denatured for 10 minutes at 95ºC, followed by 35 cycles of amplification (denaturation of the DNA template for 30 seconds at 95ºC, annealing the specific primers for 30 seconds at 65ºC, and then an extension of primers at 72ºC for 30 seconds). The final extension was performed for 5 minutes at 72ºC for one cycle[16]. The PCR product was electrophoresed at 80 V on a 2% agarose gel for 35 minutes. Three µl of ethidium bromide were used to stain the gel. A 100bp plus DNA ladder was used as a molecular weight marker. Gel purification was performed using the gene JET™ Gel extraction kit (Fermentas, UK). Additionally, the standard sequencing for the PCR product was performed by ©Macrogen, Inc., South Korea.

Antibacterial activity

The disc diffusion assay was used to evaluate the sensitivity of the clinically isolated periodontal pathogens to the tested EO[17]. A swab of the bacteria 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., UK), enriched with hemin and vitamin K1 under an aseptic environment, sterile empty paper discs 6mm in diameter (CHMLAB, Spain) were impregnated with 30 µL of different concentrations (5%, 10%, and 20%) of TVEO and placed on the agar plates. Chlorhexidine (CHX) 0.12% mouthwash was used as a positive control. The Petri plates were left for 15 minutes at room temperature to allow the diffusion of the oils, and then they have incubated anaerobically at 37˚C for 24 hrs.

Determination of the minimum inhibitory and minimum bactericidal concentrations

The broth macro dilution method was used to determine the minimum inhibitory concentration (MIC) of the tested EO[18]. The stock solution was prepared by dissolving TVEO in an aqueous solution containing 10% dimethyl sulfoxide (DMSO) and 0.05% polysorbate (Tween) 80 (Biochem-France). CHX mouth rinse 0.12 % was used as a positive control and Muller Hinton broth (MHB)(Oxoid-UK) as a negative control

to allow bacterial growth. Two-fold serial dilutions of the EO from 100 - 0.20 µL/mL were made in 10 sterile glass test tubes containing 900µL of MHB and 100µL of bacterial inoculum containing 1.5×10^8 CFU/mL (McFarland standard no.5) to yield a final volume of 1 mL per each tube. The tubes were secured with cotton plugs and incubated for 24 hours at 37˚C under anaerobic conditions in an anaerobic jar and observed for bacterial growth. The MIC of an EO was defined as the lowest concentration that inhibited the visible growth of the bacteria. After incubation, the tubes were observed for bacterial growth. Usually indicated by turbidity or a pellet of microorganisms in the bottom of the tubes[19].

The minimum bactericidal concentration (MBC) was determined by spreading a loopful sample from each test tube containing different concentrations of the EO on the supplemented MHA Petri plate. The MBC was described as the lowest concentration resulting in no bacterial growth[20].

Antibiofilm activity

A qualitative tube method was used to screen the effect of TVEO in preventing biofilm formation by clinical strains[21]. First, the content of the incubated transparent glass test tubes used to determine the MIC of the EO that contained different oil concentrations was carefully discarded. Next, the tubes were washed with a sterile phosphate buffer saline (pH=7.3) from (Biochem, France) to remove the planktonic bacteria and left in an inverted position for 45 minutes to dry completely. Later on, each tube was stained with one mL of 1% crystal violet and incubated at room temperature for 15 minutes. Then, the excess dye was removed by washing the tubes with sterile distilled water. The biofilm formation was judged qualitatively 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 (+), moderately adherent (++) or strongly adherent (+++)[21]. All assays were performed in duplicate.

Statistical analysis

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

Results

Bacterial strains

The previously isolated *P. gingivalis* strain used in this study was obtained from the Microbiology Department, College of Dentistry, University of Sulaimani[14]. In addition, the *P. intermedia* strain was isolated and confirmed from subgingival plaque samples obtained from patients with periodontitis. The bacteria identification was initially performed by observing colony morphology, color, growth environment, and gram staining. The colonies on the plates after 48 hours were small, round, opaque, and convex, with black pigmentation developed after one week. The bacterial identification was finally confirmed by PCR technique and DNA sequencing, indicating the presence of *P. intermedia*, as shown in Figure 1.

Chemical composition of TVEO

Based on three successive hydro distillation extractions, The yield of TVEO was 1.3%. The GC-MS analysis of the EO identified 27 compounds. The main compound was thymol (42.34%), whereas γ-Terpinene (22.77%), p-Cymene (12.68%), α-Terpene (5.06%), β-Myrcene (2.97%), α-Thujene (2.355), and β-Bisabolene (2.33%) were observed in considerable amount as presented in Table 1.

The Antibacterial Activity of TVEO

The result of the antibacterial activity of TVEO against clinically isolated periodontal pathogens by agar disc diffusion assay is displayed in Table 2 and Figure 2. Inhibition zones above 6 mm (including the disc diameter) were taken as positive results in this test. The clinical strains were sensitive to TVEO at 5%, 10%, and 20% concentrations used in the present study and produced inhibition zones ranging from 9.55±0.30 mm to 15.85±0.30 mm for *P. gingivalis* and from 10.55±0.25 mm to 16.85±0.40 mm for *P. intermedia*. Agar disk diffusion test on 10% dimethyl sulfoxide and 0.05% Tween-80 showed no inhibitory effect on clinical strains used in this study. CHX produced mean inhibition zones of 14.09±0.24 mm, and 15.17±0.23mm against clinically isolated *P. gingivalis* and *P. intermedia*, respectively.

The inhibition zones of TVEO 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 TVEO at 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, TVEO at 20% was more potent than CHX and produced more expansive inhibition zones on both clinical strains ($p < 0.05$). Finally, TVEO at all concentrations and CHX were more active on *P. intermedia*, producing wider bacterial inhibition zones than *P. gingivalis.*

MIC and MBC of TVEO against tested bacteria

The MIC values of TVEO against *P. gingivalis and P. intermedia* clinical strains measured by the broth macro dilution method were 1.56 µL/mL and 0.78µL/mL, respectively. The MBCs of TVEO that eradicated the clinical strains, as shown in (Figure 3- A, B, C, and D) were 3.125 µL/mL and 1.56µL/mL for *P. gingivalis* and *P. intermedia,* respectively (Table 3).

Antibiofilm activity of TVEO

The results obtained by the test-tube method to measure the effect of TVEO on the adherence and biofilm-forming potential of the tested clinical strains are presented in Table 4. The violet color intensity on the tube's walls compared to the negative control tube was used to evaluate the antibiofilm-forming ability of the oil against the clinical strains. The results showed that the TVEO effect did not reveal a clear pattern, varying from a nearly complete absence of biofilm at the highest concentration and strong biofilm formation at the lowest concentration. TVEO showed a moderate anti-biofilm effect against *P. gingivalis* and a strong effect on *P. intermedia* at MIC concentrations. According to the results, CHX 0.12% mouthwash prevented visible biofilm formation by *P. intermedia* and *P. gingivalis* clinical strains. At the same time, the negative control test tube that contained bacterial inoculum and MHB without CHX displayed strong biofilm formation by *P. gingivalis* and *P. intermedia*, as shown in Figure 3(E and F).

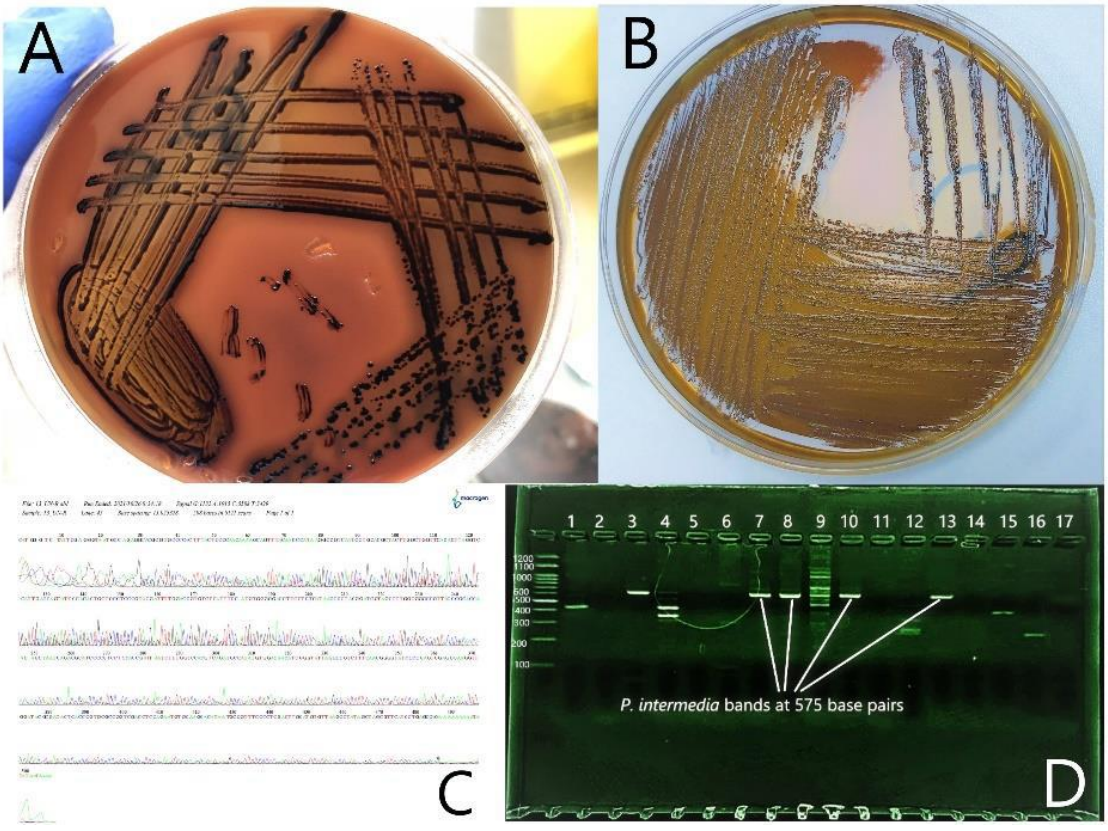

Figure 1: Black pigmented colonies of **A**: *P. gingivalis* and **B**: *P. intermedia* on blood agar after ten days of anaerobic incubation. **C:** 16S rRNA gene sequence graph of the purified colonies of *P. intermedia* performed in Macrogen - South Korea. **D:** PCR products in agarose gel electrophoresis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

Table 1: Results of GC-MS analysis of TVEO.

Peak	Area%	CAS#	M_W	M_F	Compound name
1	2.33	2867-05-2	136.23	$C_{10}H_{16}$	α-Thujene
2	0.14	80-56-8	136.23	$C_{10}H_{16}$	α-Pinene
3	0.85	127-91-3	136.23	$C_{10}H_{16}$	β-Pinene
4	2.35	123-35-3	136.23	$C_{10}H_{16}$	β-Myrcene
5	0.69	99-83-2	136.23	$C_{10}H_{16}$	α-Phellandrene
6	5.06	99-86-5	136.23	$C_{10}H_{16}$	α-Terpene
7	12.68	99-87-6	134.22	$C_{10}H_{14}$	p-Cymene
8	22.77	99-85-4	136.23	$C_{10}H_{16}$	γ-Terpinene
9	0.24	586-62-9	136.23	$C_{10}H_{16}$	Terpinolene
10	0.13	7299-40-3	154.25	$C_{10}H_{18}O$	trans-β-Terpineol
11	1.21	562-74-3	154.25	$C_{10}H_{18}O$	Terpinen-4-ol
12	0.40	98-55-5	154.25	$C_{10}H_{18}O$	α-Terpineol
13	0.84	104-46-1	148.2	$C_{10}H_{12}O$	Anethole
14	42.34	89-83-8	150.22	$C_{10}H_{14}O$	Thymol
15	0.87	380-28-5	192.25	$C_{12}H_{16}O_2$	Carvacrol acetate
16	0.20	2395-97-3	238.28	$C_{16}H_{14}O_2$	9,10-Dimethoxyanthracene
17	1.32	87-44-5	204.35	$C_{15}H_{24}$	Caryophyllene
18	0.13	17699-05-7	204.35	$C_{15}H_{24}$	α-Bergamotene
19	0.41	475-20-7	204.35	$C_{15}H_{24}$	Longifolene
20	0.16	6753-98-6	204.35	$C_{15}H_{24}$	α-Caryophyllene
21	0.29	21747-46-6	204.35	$C_{15}H_{24}$	Virdiflorene
22	2.97	495-61-4	204.35	$C_{15}H_{24}$	β-Bisabolene
23	0.31	17627-44-0	204.35	$C_{15}H_{24}$	α-Bisabolene
24	0.26	6750-60-3	220.35	$C_{15}H_{24}O$	Spathulenol
25	0.32	1139-30-6	220.35	$C_{15}H_{24}O$	Caryophyllene oxide
26	0.49	112-39-0	270.45	$C_{17}H_{34}O_2$	Palmitic acid, methyl ester
27	0.14	112-61-8	298.5	$C_{19}H_{38}O_2$	Methyl stearate

Table 2: Mean and standard deviations of inhibition zones and p- values of CHX and TVEO at different concentrations on clinical strains used in the study.

Oil %	Inhibition zones (*P. gingivalis*)			Inhibition zones (*P. intermedia*)		
	TVEO	CHX 0.12%	*p*-value	TVEO	CHX 0.12%	*p*-value
5%	9.55±0.30	14.10±0.15	0.001	10.55±0.25	15.15±0.05	0.001
10%	10.33±0.25	14.20±0.25	0.000	12.50±0.40	15.05±0.15	0.003
20%	15.85±0.30	13.98±0.33	0.02	16.85±0.40	15.30±0.36	0.028

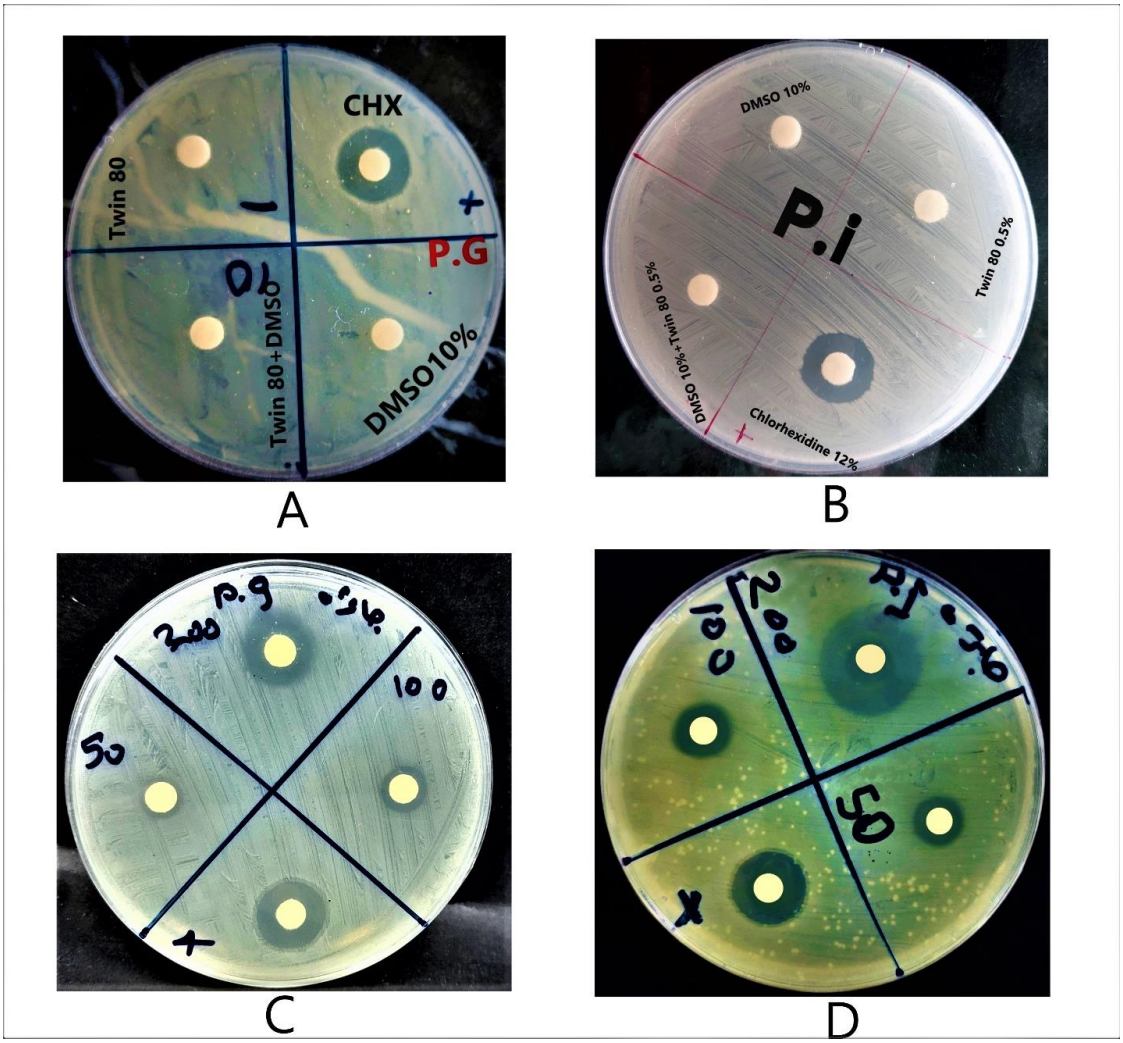

Figure 2: Disc diffusion assay revealed no inhibitory effect of solvents used to prepare TVEO stock solution on tested bacteria (A: *P. gingivalis* and B: *P. intermedia*). Inhibition zones of different concentrations of TVEO on clinical strains (C: *P. gingivalis* and D: *P. intermedia*).

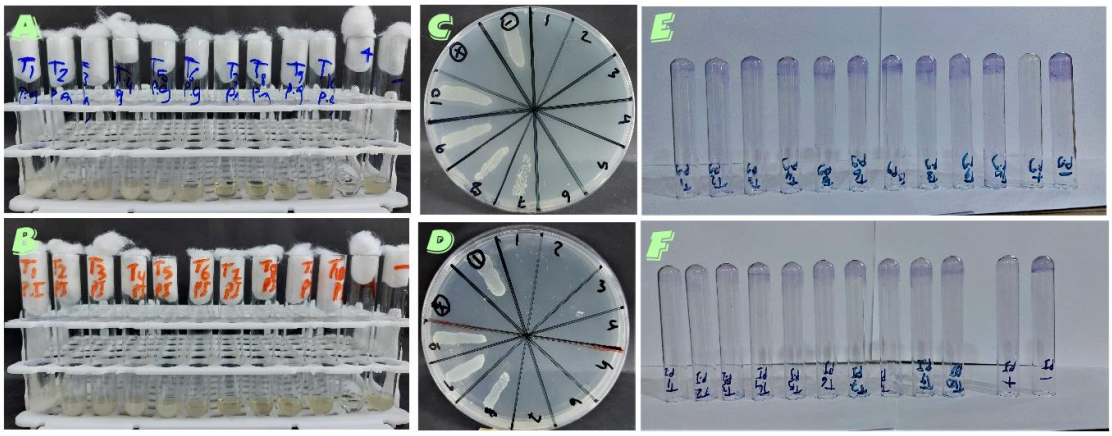

Figure 3: A and B: Broth macro dilution method to determine MIC values of TVEO on clinically isolated *P. gingivalis* and *P.intermedia*, respectively. C: and D: Agar culture method to confirm MBCs of TVEO against *P.gingivalis* and *P. intermedia*, respectively. E and F: Qualitative antibiofilm assay using tube method to assess the anti-biofilm activity of two-fold dilution series of TVEO against clinically isolated *P. gingivalis* and *P. intermedia*, respectively.

Table 3: The MIC and MBC values of TVEO on clinically isolated *P. gingivalis* and *P. intermedia* by broth dilution assay.

Bacteria	TVEO	
	MIC	MBC
P. gingivalis	1.56 µL/mL	3.125 µL/mL
P. intermedia	0.78 µL/mL	1.56 µL/mL

Table 4: Qualitative biofilm formation was judged by observing a visible film lining the walls of the tubes.

Bacteria	TVEO	Positive control (CHX 12%)	Negative control (MHB)
P. gingivalis	++	0	+++
P. intermedia	+	0	+++

Discussion

Periodontal disease is multifactorial, with periodontal pathogens playing a central role in the disease process. Over the years, the primary treatment of PD has remained constant: the removal of plaque biofilm and calculus from supra and subgingival surfaces through scaling and root planning[7]. However, all patients might not respond to mechanical debridement only[8]. One of the reasons for this is that some periodontal pathogens invade the gingival tissues and are thus spared from mechanical debridement that provides the source for recolonizing the periodontal pocket and resurgence of the disease[22]. In addition, previous decades have seen an indiscriminate use of commercial antimicrobials, albeit prolonged use of these antimicrobials 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[23].

The present *in vitro* study tested the antibacterial and antibiofilm efficacy of TVEO against clinically isolated *P. gingivalis* and *P. intermedia*. The results indicated that the TVEO has comparable antibacterial activity against both clinical strains, as measured by agar disk diffusion and broth macro dilution methods. Besides, TVEO exhibited considerable antibiofilm activity against both clinical strains used in this study as measured by the qualitative tube method. The diameter of the inhibition zones may measure the antibacterial efficacy of plant extracts or specific components. However, it is necessary to address that the diffusion of lipophilic extracts such as EOs from a filter paper disc into an agar medium is insufficient. It may provide negative results or smaller inhibition zones[24].

TV is an important medicinal plant that belongs to the Lamiaceae family; The antimicrobial activity of EOs depends on their chemical constituents. The antimicrobial activity of TVEO is related to the phenolic compounds (Thymol and γ-Terpinene)[25]. TVEO was reported to show antimicrobial activity against oral pathogens due to the high thymol and p-cymene. The phenolic compound thymol, the main component of TVEO, was reported to disintegrate the outer membrane of Gram-negative bacteria and make the bacterial cytoplasmic membrane more permeable to adenosine triphosphate[26]. Another constituent of TVEO, carvacrol, exhibits antimicrobial potential against *Streptococcus mutans* and *Candida albicans*[27,28]. Carvacrol emulsion might also be a promising alternative to sodium hypochlorite in the irrigation of tooth root canal systems and in eradicating intracanal bacteria[29]. Studies also proved the potent antimicrobial activity of thymol against *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, which are essential in the initiation and progression of PD[30]. In another *in vitro* study, TVEO exhibited higher or equal antimicrobial properties against oral pathogens than CHX[31].

In the present study, significant discrepancies in the mean diameter of the inhibition zone among different thyme EO concentrations were noted. Therefore, it can be claimed that the impact of the oil rises with increasing concentration. Comparing the inhibition zone of TVEO to that of a positive control group revealed that bacterial growth in the presence of CHX was less than that of

thyme EO, particularly at 5 %and 10 %; however, this is less relevant than the herb's safety. Nevertheless, the growth of clinical strains was reduced, and a more prominent bacterial inhibition was seen at higher concentrations (20%). In addition, the oil was more effective against *P. intermedia* than *P. gingivalis*, providing a foundation for future research.

Data on the antibacterial activity of TVEO on clinically isolated *P. gingivalis* and *P. intermedia* are very limited in the literature. In our study, *P gingivalis* and *P. intermedia* clinical isolates were sensitive to TVEO with a mean MIC of 1.56 µL/mL and 0.78 µL/mL, respectively. Other investigators reported a 6.25 mg/mL MIC value of thyme EO on *P. gingivalis*[32]. Furthermore, *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* clinical isolates were sensitive to TVEO with 32 µg/mL as MIC[30]. However, a higher MIC value of 62.5 mg/mL of TVEO on *A. Actinomycetemcomitance* and *P. gingivalis* was reported by other researchers[33].

The discrepancies in MIC values reported by different studies from various regions are mainly attributed to the fact that the chemical composition and active ingredients concentrations 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 procedure[34,35]. In addition, other factors might influence the MIC values, including differences in the antibacterial assay methodologies, such as the growth medium contents, the incubation conditions, and the emulsifying agents or solvents used that may modify the antibacterial properties of the EOs. Furthermore, variations of the cell wall structure among different strains of the same bacterial species might also affect MIC results[36].

Biofilm-induced PD comprises a wide range of inflammatory conditions that affect the supporting structure of the tooth, which may result in tooth loss and contribute to systemic inflammation. Therefore, strategies that can disrupt any stage of biofilm formation are considered potentially valuable in controlling biofilm-related infections[37]. The present study evaluated the power of TVEO to prevent biofilm formation by *P. gingivalis* and *P. intermedia*. The simple qualitative tube method results showed that TVEO successfully suppressed biofilm formation in a concentration-dependent manner. Analysis of the results regarding *P. gingivalis* showed that TVEO moderately inhibited biofilm formation. At the same time, it showed a potent anti-biofilm effect toward *P. intermedia*. Generally, the biofilm formation intensity was inversely proportional to the concentration of the EO, as it decreased with increased concentration and vice versa. In accordance with the current study, several studies reported the antibiofilm potential of TVEO against various fungal and bacterial strains[38-40].

Study limitations

The present *in vitro* study had some limitations as it was conducted using 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. In addition, this study examined a limited number of clinically isolated periodontal pathogens.

Conclusions

The present *in vitro* study showed evidence for the antimicrobial and antibiofilm activity of TVEO against clinically isolated *P. gingivalis and P. intermedia*. More research is recommended to identify and isolate the active components of TV extracts in different areas and seasons and comprehend their mechanism of action on biofilm formations. Furthermore, *in vivo* studies examining the effect of TVEO are highly recommended.

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