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Evaluating the Antibacterial Activities of *Thymus Vulgaris* L. Essential Oil against *Streptococcus Oralis in Vitro* Study

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ABSTRACT

Background: Dental biofilm acting a major factor in the cause of gingivitis and periodontitis. The primary colonizers of dental biofilm are of large magnitude in the sequence steps of biofilm creation. Streptococci make about 60% to 80% of oral bacteria existing in the biofilm which shaped on surfaces of teeth. Chemical anti-biofilm agents were used for dental biofilm control and gingivitis prevention which then prevent periodontitis but it has several side effects which necessitates the need for an anti-biofilm agent that has fewer side effects. Thymus vulgaris L. essential oil has been shown to make bacteriostatic and bactericidal effects toward several dental biofilm bacteria. Nevertheless, the antibacterial effect of it on primary dental biofilm colonizer (Streptococcus oralis) has never been tested which was investigated in this study.

Aim: Evaluating the antibacterial effects of Thymus vulgaris L. essential oil against Streptococcus oralis in vitro.

Materials and Methods: The Streptococcus oralis bacteria were isolated by collection of human supra gingival dental biofilm samples from 10 subjects. Presence of the target microorganisms is confirmed using morphological characteristics, Gram stain, and catalase production test, ability for blood haemolysis, Optochin sensitivity and conventional polymerase chain reaction test. Agar susceptibility test was used to study the sensitivity of Streptococcus oralis to Thymus vulgaris L. essential oil as compared to Chlorhexidine 0.2% as a positive control and 10% Dimethyl sulphoxide as a negative control. The minimal inhibitory concentration was defined by means of two-fold serial microdilution method. The minimum bactericidal concentration was also defined.

Results: The Thymus vulgaris L. essential oil showed higher antibacterial activity against Streptococcus oralis than Chlorhexidine. Bacteria was sensitive to different concentrations of the oil with minimum inhibitory concentration was (0.09%) and the minimum bactericidal concentration was (0.09%) for Streptococcus oralis.

Conclusion: The Thymus vulgaris L. essential oil was effective against Streptococcus oralis with bacteriostatic and bactericidal action, and could be used as a natural active agent to produce oral health care products.

Key words: *Streptococcus oralis, Thymus vulgaris* L. Essential oil, Chlorhexidine, Antibacterial

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INTRODUCTION

Dental biofilm is an overall description for the communal microbial complexity that grows on the tooth surface, fixed in a medium of polymers of salivary and bacterial source [1,2]. Oral bacteria from saliva, which contains over 700 diverse bacterial species, invade teeth. The primary attachment of bacteria to dental surfaces is go ahead by the creation of accommodation layer on the clean dental surfaces primarily made up of salivary glycoproteins, its termed acquired pellicle [3]. Amongst the initial bacterial colonizers of dental biofilm are *Streptococcus oralis (S. oralis)* [4]. Which stick to salivary pellicles on tooth surfaces supplying a film for consequent attachment of the dental biofilm secondary colonizers, therefore, performing a primary function in the development of dental biofilm [5]. Accumulation of dental biofilm has long been recognized to be the etiology of dental decay, gingivitis and periodontitis [6]. Gingivitis is caused by the substances derived from microbial dental biofilm accumulating at or near the gingival sulcus [7]. The inflammation of gingiva which resulted from build-up of bacterial biofilm is regarded as the main jeopardy reason for periodontitis beginning [8]. Mechanical or chemical measures are utilized for the purpose of dental biofilm control. The interdental aids and toothbrushes which considered as mechanical dental biofilm control instruments is more assumed procedure for supra gingival dental biofilm controlling, nevertheless, earlier revisions stated that the toothbrush usage perform only a little removal of dental biofilm and inability to perform cleaning by interproximal instruments every day [9]. And a significant frequency of

gingival disease caused by dental biofilm between employers of toothbrush [10]. This is due to the fact that toothbrushes and interdental aids necessitate high personal compliance and skill [11]. Time and motivation also, elderly patients, those with physical or mental limitations and patients with mal posed teeth and orthodontic appliances may have difficulty in brushing and interdental cleaning [12]. However, it was showed that elimination of dental biofilm by mechanical methods does not entirely eliminate all periodontal bacteria from the surface of tooth [13]. These limitations in mechanical dental biofilm removal methods made the adjunctive use of chemotherapeutic agents which are helpful in the reduction of dental biofilm accumulation and prevention of gingivitis and periodontal diseases. The traditional option for chemical biofilm regulation is Chlorhexidine (CHX) mouth rinse, but it has a number of shortcomings, including discoloration of teeth and some restorations, a disagreeable sense of taste, oral mucosa sloughing, and increased production of supra gingival calculus [14-16]. This necessitates need for an anti-biofilm agent that has fewer side effects and can be taken effectively in conjunction with tooth brushing and interdental aids cleaning methods [17]. The rise in illness frequency, infective bacterial resistance to antibiotics and chemical therapies, contagious infections, and budgetary constraints in emerging nations have created a worldwide demand for harmless, active, and cost-effective oral disease preclusion and handling choices. Herbal extracts have gotten a lot of care since they are non-organic and non-artificial, and they had consumed in conventional remedy for a prolonged time [18]. Many herbal formulations have been reported to have anti biofilm efficacy when used as mouth washes and can be used as a substitution to CHX with minimal side effects [19]. Of these herbs, *Thymus Vulgaris* L. (TV). Several investigations have shown that *Thymus Vulgaris* L. Essential Oil (TVEO) has antimicrobial and anti-mycotic effects against pathogens of human body such as *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli) in vitro* [20]. *Klebsiella pneumoniae*, and *Salmonella typhimurium*, [21], *Streptococcus mutans (S. mutans), Streptococcus pyogenes (S. pyogens), Porphyromonas gingivalis (P. gingivalis), Aggregatibacter Action mycetemcomitans (A.a) and Candida albicans (C. albicans)* [22]. The purpose of the present study was to evaluate the antibacterial effects of TVEO against primary dental biofilm colonizer exactly *(Streptococcus oralis).*

MATERIALS AND METHODS

The Medical Ethical Committee of the University of Baghdad's College of Dentistry accepted the study procedure. The current *in vitro* investigation was carried out at Baghdad's Altakadm Learning Center's Laboratory Unit.

Thymus vulgaris oil

Thymus vulgaris oil (earthroma) brand, 100% pure essential oil was purchased from USA used in this study. The country of origin was Spain; the plant part used for this oil extraction was leaves and stems. The extraction method was steam distilled and odour intensity was medium. The colour and consistency were a moderately viscous but clear, pale-yellow liquid.

Subjects and dental biofilm sampling

Human dental biofilm samples were collected at private dental clinic from 10 subjects who had supra gingival dental biofilm regarded as inclusion criteria while the exclusion criteria were subject currently using any mouthwash and taking antibiotic within at least one month before the study. Before gathering the samples, the subjects were knowledgeable about the study and their agreement was gained. To evade communication between tooth surfaces and oral mucosa, cotton rolls were used to separate teeth from buccal/labial mucosa throughout the collection procedure. The collection area was washed with water two times before being dried with air. Clinical isolates from supra gingival dental biofilm were collected from the buccal/labial surfaces of teeth using sterile periodontal Gracey curette. Each scraping was transferred immediately to, and dispersed in 3 ml Brain-Heart Infusion Broth (BHI-B) (TMMEDIA, India). Then, transported to laboratory immediately and incubated aerobically at 37°C for 24 hrs [23].

Isolation of oral *Streptococci* from dental biofilm samples: Dental biofilm samples were cultured on selective medium Mitis Salivarius Agar (MSA) for *Streptococcus spp*. (HIMEDIA, India). After culturing sample by streaking method, the plates were placed inside the incubator and incubated aerobically at 37°C for 24 hrs. From the original MSA plate, *Streptococci* were further sub-cultured to obtain pure bacterial isolate. Using a sterile bacteriological loop, one very small colony was picked and spread on MSA plate. Then, incubated aerobically at 37°C for 24 hours [23].

Identification of *S. oralis* colonies were detected and analysed using morphological characteristics on agar plates, stain method by Gram's stain [26], production of catalase [27], antibiotic sensitivity test [25], hemolytic ability [28], and Polymerase Chain Reaction (PCR) [24].

Polymerase chain reaction

Extraction of deoxyribonucleic acid: The DNA templates were extracted from bacterial growth using the following methodology from the ABIO pure extraction kit (ABIO pure, USA):

One ml of bacterial culture that incubated for 24 hrs was centrifuged for 2 min at 13000 rpm for gathering of pellet cells. One hundred µl of lysozyme was added to the 1.5 ml centrifuge tubes that contained the pellet and 100 µl of nuclease free water also added, and then water bath for 30 minutes at 37°C was used for incubation. For bacterial cell destruction, 20 µL of proteinase K (20 mg/ml) was applied to the tube also, 200 µL of binding buffer also added to the tube, for further lysis, it was incubated in water bath for 30 minutes at 70°C. This was followed by the addition of 200 µl of absolute ethanol to the samples

and the samples were again vortexed for 5 min. to blend the sample. Cautiously, all of the mixtures were then moved to tiny columns, the collecting tube was replaced after being centrifuged for 1 minute at 8000 rpm. The mini column was then filled with 600 µL of prewash buffer, centrifuged for 1 minute at 8000 rpm, the wash buffer was used in the amount of 700 µL at 8000 rpm, centrifuged for 1 minute. The tubes were then filled with 100 µL elution buffer, then incubated for 1 min at room temperature and centrifuged at 5,000 rpm for 5 min. After that, the obtained DNA was stored at –20°C.

Reaction setup and thermal cycling protocol

The DNA sequences were referenced from published data and used in this study as follows Table 1 [24].

Table 1: Comparison of sickled RBC in anti-sickling analysis of two methods with pre-treatment (n=49).

Primer name	Primer sequence	Annealing Temp. (°C)	Expected Size (bp)
S. oralis-F	5'TCCCGGTCAGCAAACTCCAGCC3'	66	374
S. oralis-R	5'GCAACCTTTGGATTTGCAAC3'		

The PCR was carried out in 20 µL of a reaction mixture containing 10 µLfrom GoTaq® G2 Green Master Mix 2X (Promega, USA), 1 µL of primer-F 10 pmol/µl (Macrogen, Korea), 1 µL of primer–R 10 pmol/µl (Macrogen, Korea), 2 µL of purified DNA, and 6 µL nuclease free water. The final mixture was then transported into conventional PCR tubes and vortexed for 5 seconds. The DNA amplification was then performed by (Thermo Fisher Scientific, USA), using the following thermal profile: 95°C for 5 min (initial denaturation), followed by 30 cycles of 95°C during 30 sec. for denaturation, 66°C during 30 sec for annealing and extension at 72°C for 1 min. Final extension at 72°C for 7 min. and hold at 10°C for 10 min the 1.5% agarose gel electrophoresis was used for PCR products analysis after staining with ethidium bromide. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system (UV trans illuminator).

Preparing and standardizing inoculum suspension

Preparing and standardizing inoculum suspension were carried out by direct colony suspension as follows [29]. The bacterium stocked in 20% glycerol, which had been frozen at -20°C in BHI-B, were permitted to defrost at ambient temperature, after that, MSA plate was inoculated with *S. oralis* and the plate incubated aerobically at 37°C for 24 hrs. Three to five well isolated colonies were selected from MSA agar plate, the growth was transferred by inoculating loop into a tube containing fresh Mueller Hinton Broth (MHB) (Oxoid, England) medium. The turbidity of the *S. oralis* suspension was standardized to match that of a 0.5 McFarland standard which corresponds to 1×10^8 colony forming unit (CFU/ml) by using Absorbance microplate reader (GloMax, Promega, USA) with bacterial concentration managedto an absorbance of 0.6 and at wave length equal to 600 nano meters (nm) [30].

Antibacterial assessment of the TVEO

For antibacterial assessment of the TVEO, all experiments were done in triplicates to ensure precision and accuracy of the outcomes.

Agar susceptibility test

The antibacterial activities of TVEO in this study was determined by Agar susceptibility test according to [29,31] with some modifications. The Mueller Hinton Agar (MHA) (Mast Group, U.K.) plates were inoculated with prepared suspension of *S. oralis.* The TVEO was diluted with 10% Dimethyl sulphoxide (DMSO) (CDH Co., Ltd. India), [22] to prepare various concentrations of TVEO (100%, 75%, 50%, 25%, 12.5%). Wells (six mm in diameter) were prepared in the plate and filled with 50 µl of various concentrations of TVEO. On the other hand, 0.2% CHX and 10% DMSO were used as the positive and negative controls respectively. Then the plates were incubated at 37°C for about 24 hrs the growth inhibition zones around the wells were measured in millimetres using a ruler (6 mm of each well, was included).

Minimum Inhibitory Concentration (MIC)

According to [31] protocol with some modifications, the MIC of TVEO against test bacteria *(S. oralis)* was carried out by two-fold serial broth micro-dilution method using 96 well cell culture plates (Promega, USA). In a 96 well microtiter plate, one hundred µL of MHB was applied to every one of the twelve wells of the two rows, named well one till well twelve. Then, in well one of each row, (100 µL) of TVEO with a concentration of 25% (which diluted to 12.5% when added to broth) was applied, and a two-fold serial dilution was achieved from well one till well nine. The well ten, which served as a positive control, included 100 µL of the mixture of 0.2% CHX with nutrient broth. Well eleven, on the other hand, included a one hundred µL mixture of DMSO and nutritional broth that served as the negative control. All wells in the 2 row were impregnated with 100 µL of *S. oralis* suspension and incubated overnight at 37°C, with the exception of the first row, which was left blank. After incubation, the loss of turbidity in the wells which mean prevention of bacterial growth was detected by Absorbance microplate reader at wave length equal to 600 Nano Meters (nm). The representative minimum percent of TVEO in the well that displayed no *S. oralis* growth regarded as MIC [22].

Minimum bactericidal concentration (MBC)

The MBC defined as the lowest concentration of an antimicrobial agent required to kill 99.9% of the bacterial population [32]. It was detected by sub culturing 100 µl from the contents of the tube that showing no turbidity (MIC) and one tube before the tube that showing no turbidity (MIC) and then spread by cotton swab on MHA plates. The incubation of MHA plates was at 37°C overnight for 18-24 hrs. And in the next day, readings were taken. The plate that was showed no growth of bacteria on agar plate after incubation was considered as MBC [33].

Statistical analysis

Data processing and analysis were carried out by using SPSS (Statistical Package for Social Science) version 26 program. Descriptive statistics including means and Standard Deviations (S.D.) were used. The data of antibacterial effect was evaluated by Analysis of Variance (ANOVA) and Tukey's post hoc test was used to analyse any statistically significant difference between each two concentrations.

The levels of significance that was used: Non-Significant (NS) probability value (P-value)>0.05, Significant (S.) P ≤ 0.05.

RESULTS

The morphological determination of *S. oralis* colonies on MSA plates seemed as spherical or ovoid in shape with raised or convex smooth surfaces, blue in colour, concentric circle structure inside colonies of *S. oralis* and non-adherent to the agar surface. Microscopic examination showed that the cells of *S. oralis* had Gram (+) ability, organized in long or short chain and round or oval in shape. The catalase production for *S. oralis* was negative, also on blood agar the hemolysis ability was Alpha and the Optochin sensitivity was resistant. The PCR showed that the *S. oralis* bacteria identified by conventional PCR. There was a bond between PCR product of suspected bacteria and the primers of *S. oralis.* The amplification of *S. oralis* primers were seen on 1.5% electrophoresis agarose gel system stained with Ethidium Bromide resulted in 374 bp PCR products.

Agar susceptibility of *S. oralis*: As the conc. of the TVEO increased, the diameter of the inhibitory zones becomes larger. Each conc. started from 12.5%, 25%, 50%, 75%, 100% was effective against *S. oralis* bacteria. The summary of mean values in (mm) and standard deviation (S.D.) values of the inhibition zones of TVEO against *S. oralis* are presented in Table 2.

Table 2: The statistical analysis of S. oralis inhibition zones by different conc. of TVEO, CHX and DMSO.

Agent	Conc.	No.	Mean in (mm)	± S. D.	ANOVA test
CHX	0.002	9	25.9	0.5	*F=8626.0 *P=0.000 S. *d. f.=6
DMSO	0.1	9	0	0	
TVEO	0.125	9	30.76	0.79	
	0.25	9	38.34	0.69	
	0.5	9	41.68	0.56	
	0.75	9	50.83	0.73	
	1	9	56.25	0.5	

*P=Probability, *d.f.=Degree of freedom, *F=freedom

All the concentrations of TVEO displayed higher mean values of the inhibition zones than CHX, conversely, the 10% DMSO presented no inhibition zone on *S. oralis* bacteria. The 100% of TVEO presented an extreme mean value of inhibition zone which was 56.25 mm. One-way ANOVA test revealed significant difference in comparison to different conc. of TVEO with CHX and

By using Tukey's post hoc test for comparison between every pair of dissimilar conc. for TVEO and between each conc. of TVEO with CHX and DMSO, the results obtained were demonstrated in Table 3 and revealed significant differences.

Table 3: Comparisons of mean values of S. oralis inhibition zones between each pair of different concentration for TVEO and between each concentration of TVEO with CHX and DMSO by Tukey's post hoc test.

Agents Conc.		Mean difference	P-value	*Desc.
DMSO 10%	CHX 0.2%	-25.9	0	S.
	TVEO 12.5%	-30.76	0	S.
	TVEO 25%	-38.34	0	S.
	TVEO 50%	-41.68	0	S.

	TVEO 75%	-50.83	0	S.
	TVEO 100%	-56.25	0	S.
CHX. 0.2%	DMSO 10%	25.9	0	S.
	TVEO 12.5%	-4.86	0	S.
	TVEO 25%	-12.44	0	S.
	TVEO 50%	-15.78	0	S.
	TVEO 75%	-24.93	0	S.
	TVEO 100%	-30.35	0	S.
TVEO 12.5%	DMSO 10%	30.76	0	S.
	CHX. 0.2%	4.86	0	S.
	TVEO 25%	-7.57	0	S.
	TVEO 50%	-10.92	0	S.
	TVEO 75%	-20.06	0	S.
	TVEO 100%	-25.48	0	S.
DMSO 10%	38.34	0	S.	
TVEO 25%	CHX. 0.2%	12.44	0	S.
TVEO 12.5%	7.57	0	S.	
TVEO 50%	-3.34	0	S.	
TVEO 75%	-12.48	0	S.	
	TVEO 100%	-17.91	0	S.
DMSO 10%	41.68	0	S.	
TVEO 50%	CHX. 0.2%	15.78	0	S.
TVEO 12.5%	10.92	0	S.	
TVEO 25%	3.34	0	S.	
TVEO 75%	-9.14	0	S.	
TVEO 100%	-14.56	0	S.	
TVEO 75%	DMSO 10%	50.83	0	S.
	CHX. 0.2%	24.93	0	S.
	TVEO 12.5%	20.06	0	S.
	TVEO 25%	12.48	0	S.
	TVEO 50%	9.14	0	S.
	TVEO 100%	-5.42	0	S.
TVEO 100%	DMSO 10%	56.25	0	S.
	CHX. 0.2%	30.35	0	S.
	TVEO 12.5%	25.48	0	S.
	TVEO 25%	17.91	0	S.
	TVEO 50%	14.56	0	S.
	TVEO 75%	5.42	0	S.
	*Desc.=Description			

The MIC for TVEO that inhibited *S. oralis* growth was at 0.09% (0.9 µl/ml). The MBC for TVEO that killed *S. oralis* was at 0.09% (0.9 µl/ml).

DISCUSSION

Investigators are always looking for innovative ways to incorporate beneficial formulae into dental products. Essential Oils (EOs) have a long history of application in a variety of fields, including dentistry. Hence, EOs appears to be viable elements for new oral maintenance products and dental ingredients utilized by both dentists and patients [34].

In dental care and oral hygiene control, EOs are handled as an ingredients of mouth rinses (*i.e.*, Cool Mint and Listerine Antiseptic), impermanent filing constituents (eugenol-filling materials, *i.e.,* zinc oxide-eugenol base material), sterile solutions and toothpastes [35,36]. The TVEO's ability to suppress a variety of infective and/or spoilage microorganisms in *in vitro* settings have earlier been demonstrated [37-39]. Evidence suggested that toothpaste containing TVEO show important antimicrobial and anti-biofilm activities against microorganisms associated with formation of periodontal diseases [40]. Up to now, there is no information on the antibacterial activity of TVEO on primary dental biofilm colonizer *(S. oralis).* Thus, the goal of this investigation was to see if TVEO has any antibacterial properties against *S. oralis.*

The *Streptococcus* spp. *(S. oralis)* isolated from supra gingival dental biofilm samples in this study was identified by morphological characteristics on agar plates, Gram's stain, production of catalase, antibiotic sensitivity test, haemolytic ability and conventional PCR

system were used as a confirmation tests, the PCR technique has been shown to be fast to perform, dependable and are suitable for identification of cultured streptococcus spp. The CHX used as a positive control to compare with TVEO. The CHX is the most frequently used molecule for chemical dental biofilm control. However, its limitations, such as adverse effects of the long-term use [41], development of bacterial resistance [42], and tooth staining, [43] motivate the search for new alternatives that could be incorporated to the mouthwashes.

Chemical analysis of TVEO was performed and 17 compounds were identified as it was reported by Manufacture Company (earthroma). Oxygenated monoterpenes were the major portion of this TVEO. The major constituent was thymol (49.11%), followed by p-cymene (19.33%), γ-terpinene (7.87%), Linalool (5.03%), Carvacrol (3.54%), β-caryophyllene (3.22%), α-pinene (2.04%) and Borneol (1.95%). It is obvious that the oil from TV used in this study belong to thymol chemotype.

Previous studies mentioned also that thymol being the major constituent in which the concentration were thymol (38.1%) along with γ-terpinene (5.2%), p-cymene (29.1%), β-Caryophyllene (3.1%), linalool (3.7%) and Carvacrol (2.3%), (44-46). Other study stated that the chemo type of TVEO was linalool where it is taken from France (linalool, 76.2%, linalyl acetate, 14.3%), while the chemical type of TVEO which was taken from Jablanicki, Serbia was of geraniol type (geraniol, 59.8%, geranyl acetate 16.7%), [47]. Inherent issues such as kind, cultivar, and clone, as well as ecological factors such as topographical source, weather environments, earth, active and technological issues, agriculture techniques, kinds of gathering procedures, fresh material packing forms, and dispensation machineries, all affect the structures of essential oils [48]. Generally, the phenolic compound's carvacrol and thymol seemed to be linked to many antimicrobial activities of the TVEO [49].

While the exact mechanism of action of these compounds are not obviously known, it is widely assumed that the hydroxyl group on these two compounds intermingles with the cytoplasmic membrane, altering its permeability, and affecting the lipid organization and firmness of its bilayer, causing an increase of proton passive flux crosswise the membrane, causing destruction of cytoplasmic membrane and outflow of cellular elements [49-52].

In this experiment, agar wells diffusion assay showed obvious anti-bacterial effect on *S. oralis*, as the concentration of TVEO progress, the widths of the inhibition zones increased. A study conducted by Fani and Kohanteb [22] who observed effective anti-bacterial effect of TVEO by agar disk diffusion assay against *(S. pyogenes, S. mutans, P. gingivalis, and A.a.)* isolated from oral cavity. Another study done by Tardugno [53] found that the antibacterial activity of TVEO on Lactobacillus species and *S. mutans* taken from the oral cavity was effective.

Significant differences were found in comparison between each pair of different conc. for TVEO and between each conc. of TVEO with CHX and DMSO. Hence, the TVEO of 12.5%, 25%, 50%, 75% and 100% concentrations showed larger inhibition zones than 0.2% CHX and statistically all these concentrations presented significant differences, which suggests that they have higher antimicrobial activity than CHX.

The MIC of TVEO needed to inhibit *S. oralis* growth in broth media was 0.09% concentration (0.9 µl/ml).

Monika [44] study the MIC of TVEO on ordinary strains of bacteria and one hundred twenty clinical isolates which were isolated from patients with infections of the oral cavity, gut cavity, respiratory and genitourinary tracts, skin, and from the clinic atmosphere, hence, it was ranged from 0.25-0.75 µl/ml for *S. aureus* and the MIC for *Enterococcus* species strains ranged from 0.5-1 µl/ml.

Another study showed that the MIC for TVEO against *Enterococcus faecalis* isolated from infected root canals was 1 µl/ml [54].

On the contrary, very weak inhibitory activities of TVEO with very high MIC value on oral *Streptococci* were as well described by [55]. The TVEO analysis by Gas Chromatography/Mass Spectrometry (GC/MS) had done by these researchers show no carvacrol and occurrence of few quantities of thymol (0.24%). These 2 phenolic compounds showed main job for the inhibition of bacterial growth; thus, these results may be a description for the little (high value of MIC) or no inhibitory effects of TVEO on oral Streptococci as mentioned by the researcher. Higher values of MIC (62.5 mg/mL) for TVEO on pathogenic bacteria of periodontal tissues are reported by some investigators [56]. These discrepancies in MIC standards stated by diverse researchers from several areas are primarily belong to the fact that TVEO chemical structure and dynamic constituents' concentrations (thymol, γ-terpinene, carvacrol, P-cymene, Linalool) are significantly specified by the plant genetic constitution and effect of ecological issues counting topographical environments, kind of soil, temperature, term of gathering cutting of plant, and more important, the oil drawing technique [49,55,57,58].

The TVEO in this study presented a bactericidal activity on Streptococcus spp. The MBC of the oil that kill *S. oralis* was 0.09% (0.9 µl/ml) concentration Previous studies revealed that thyme had bactericidal effect against Staphylococcus aureus, Bacillus cereus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumoniae, and Bacillus subtilis [59,60].

CONCLUSION

The *Thymus vulgaris* L. essential oil was more effective against *S. oralis* than CHX. With bacteriostatic and bactericidal action, and might be handle as a natural substitutive effective product to CHX to produce oral health care products.

REFERENCES

1. Marsh PD, Lewis MA, Williams D, et al. Oral microbiology,5th ed.Churchill-Livingston: Elsevier 2009.
2. Dumitrescu AL, Kawamura M. Etiology of periodontal disease: dental plaque and calculus. InEtiology and pathogenesis of periodontal disease 2010.
3. Larsen T, Fiehn NE. Dental biofilm infections–an update. Apmis 2017; 125:376-384.
4. Samaranayake L. Essential microbiology for dentistry-E-Book. Elsevier Health Sciences 2018.
5. Bathla S. Periodontics revisited. JP Medical Ltd 2011.
6. Nair AA, Malaiappan S. The comparison of the antiplaque effect of Aloe Vera, chlorhexidine and placebo mouth washes on gingivitis patients. J Pharm Sci Res 2016; 8:1295.
7. Murakami S, Mealey BL, Mariotti A, et al. Dental plaque–induced gingival conditions. J Clin Periodontol 2018; 45:17-27.
8. Slot DE, Echeverría JJ, Lindhe J. Mechanical supragingival plaque control. J Periodontal Implant Sci 2015; 2:677-703.
9. Ismail AI, Szpunar SM. The prevalence of total tooth loss, dental caries, and periodontal disease among Mexican Americans, Cuban Americans, and Puerto Ricans: findings from HHANES 1982-1984. Am J Public Health 1990; 80:66-70.
10. Lang NP, Lindhe J, editors. Clinical periodontology and implant dentistry. 2: John Wiley and Sons 2015.
11. Santos A. Evidence-based control of plaque and gingivitis. J Clin Periodontol 2003; 30:13-16.
12. Adriaens PA, Adriaens LM. Effects of nonsurgical periodontal therapy on hard and soft tissues. J Periodontol 2000. 2004; 36:121-145.
13. Sajadi FS, Moradi M, Pardakhty A, et al. Effect of fluoride, chlorhexidine and fluoride-chlorhexidine mouthwashes on salivary Streptococcus mutans count and the prevalence of oral side effects. J Dent Res Dent Clin Dent Prospects 2015; 9:49.
14. Haydari M, Bardakci AG, Koldsland OC, et al. Comparing the effect of 0.06%-, 0.12% and 0.2% Chlorhexidine on plaque, bleeding and side effects in an experimental gingivitis model: a parallel group, double masked randomized clinical trial. BMC Oral Health 2017; 17:1-8.
15. James P, Worthington HV, Parnell C, et al. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. Cochrane Database of Systematic Reviews. 2017.
16. Pradeep AR, Suke DK, Martande SS, et al. Triphala, a new herbal mouthwash for the treatment of gingivitis: A randomized controlled clinical trial. J Periodontol 2016; 87:1352-1359.
17. Poureslami H. The effects of plant extracts on dental plaque and caries. Contemporary approach to dental caries 2012; 20:395-402.
18. Mehta S, Pesapathy S, Joseph M, et al. Comparative evaluation of a herbal mouthwash (Freshol) with chlorhexidine on plaque accumulation, gingival inflammation, and salivary *Streptococcus* mutans growth. J Int Soc Prev Community Dent 2013; 3:25.
19. Lemos MF, Lemos MF, Pacheco HP, et al. Seasonal variation affects the composition and antibacterial and antioxidant activities of *Thymus vulgaris*. Ind Crops Prod 2017; 95:543-548.
20. Boruga O, Jianu C, Misca C, et al. *Thymus vulgaris* essential oil: chemical composition and antimicrobial activity. J Med Life 2014; 7:56.
21. Fani M, Kohanteb J. *In vitro* antimicrobial activity of Thymus vulgaris essential oil against major oral pathogens. Evid Based Complementary Altern Med 2017; 22:660-666.
22. Obaid ZM, AMA-A. Anti-Bacterial Effects of Commiphora Myrrha and Ziziphus Spina-Christ Leaves Extracts Against *Streptococcus Mitis* (Primary Colonizer of Dental Plaque) *In vitro* Study. J Res Med Dent Sci 2020; 8:57-64.
23. Hoshino T, Kawaguchi M, Shimizu N, et al. PCR detection and identification of oral streptococci in saliva samples using gtf genes. Diagn Microbiol Infect Dis 2004; 48:195-199.
24. England PH. Identification of *Streptococcus* species, Enterococcus species and morphologically similar organisms. UK Stand Microbiol Investig 2014; 4:1-36.
25. Smith AC, Hussey MA. Gram stain protocols. American Society for Microbiology 2005; 1:14.
26. Reiner K. Catalase test protocol. American society for microbiology 2010; 1-6.
27. Buxton R. Blood agar plates and hemolysis protocols. Am Society Microbiol 2005; 1-9.
28. Cavalieri S, Harbeck R, McCarter Y, et al. Manual of antimicrobial susceptibility testing. American Society for Microbiology. PAHO: Washington, DC, USA. 2005
29. Razak FA, Othman RY, Abd Rahim ZH. The effect of Piper betle and Psidium guajava extracts on the cell-surface hydrophobicity of selected early settlers of dental plaque. J Oral Sci 2006; 48:71-75.
30. Abdulbaqi HR, Himratul-Aznita WH, Baharuddin NA. Anti-plaque effect of a synergistic combination of green tea and *Salvadora persica L*. against primary colonizers of dental plaque. Arch Oral Biol 2016; 70:117-124.
31. Parvekar P, Palaskar J, Metgud S, et al. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of silver nanoparticles against *Staphylococcus aureus.* Biomater investig dent 2020; 7:105-109.
32. Thosar N, Basak S, Bahadure RN, et al. Antimicrobial efficacy of five essential oils against oral pathogens: An *in vitro* study. Eur J Den. 2013; 7:071-077.

33. Lapinska B, Szram A, Zarzycka B, et al. An *in vitro* study on the antimicrobial properties of essential oil modified resin composite against oral pathogens. Materials 2020; 13:4383.
34. Dagli N, Dagli R, Mahmoud RS, et al. Essential oils, their therapeutic properties, and implication in dentistry: A review. J Int Soc Prev Community Dent 2015; 5:335.
35. Haas AN, Wagner TP, Muniz FW, et al. Essential oils-containing mouthwashes for gingivitis and plaque: Meta-analyses and meta-regression. J dent 2016; 55:7-15.
36. Bajpai VK, Baek KH, Kang SC. Control of Salmonella in foods by using essential oils: A review. Int Food Res J 2012; 45:722-734.
37. Nezhadali A, Nabavi M, Rajabian M, et al. Chemical variation of leaf essential oil at different stages of plant growth and *in vitro* antibacterial activity of *Thymus vulgaris* Lamiaceae, from Iran. Beni-Suef Univ J Basic Appl Sci 2014; 3:87-92.
38. Kohiyama CY, Ribeiro MM, Mossini SA, et al. Antifungal properties and inhibitory effects upon aflatoxin production of *Thymus vulgaris L*. by Aspergillus flavus Link. Food Chem 2015; 173:1006-1010.
39. de Oliveira Carvalho I, Purgato GA, Píccolo MS, et al. *In vitro* anticariogenic and antibiofilm activities of toothpastes formulated with essential oils. Arch Oral Biol 2020; 117:104834.
40. Bernardi A, Teixeira CS. The properties of chlorhexidine and undesired effects of its use in endodontics. Quintessence int 2015; 46.
41. Kaspar JR, Godwin MJ, Velsko IM, et al. Spontaneously arising *Streptococcus mutans* variants with reduced susceptibility to chlorhexidine display genetic defects and diminished fitness. Antimicrob Agents Chemother 2019; 63:00161-19.
42. Van Strydonck DA, Slot DE, Van der Velden U, et al. Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: a systematic review. J Clin Periodontol 2012; 39:1042-1055.
43. Sienkiewicz M, Łysakowska M, Denys P, et al. The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. Microb Drug Resist 2012; 18:137-148.
44. Grzesiak B, Głowacka A, Krukowski H, et al. The *in vitro* efficacy of essential oils and antifungal drugs against *Prototheca zopfii*. Mycopathologia 2016; 181:609-615.
45. Sienkiewicz M, Łysakowska M, Kowalczyk E, et al. The ability of selected plant essential oils to enhance the action of recommended antibiotics against pathogenic wound bacteria. Burns 2017; 43:310-317.
46. Satyal P, Murray BL, McFeeters RL, et al. Essential oil characterization of *Thymus vulgaris* from various geographical locations. Foods 2016; 5:70.
47. Russo M, Suraci F, Postorino S, et al. Essential oil chemical composition and antifungal effects on Sclerotium cepivorum of Thymus capitatus wild populations from Calabria, southern Italy. Revista Brasileira de Farmacognosia 2013; 23:239-48.
48. Rota MC, Herrera A, Martínez RM, et al. Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. Food control 2008; 19:681-687.
49. Shapiro S, Guggenheim B. The action of thymol on oral bacteria. Oral microbiol immunol 1995; 10:241-246.
50. Dorman HD, Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J Appl Microbiol 2000; 88:308-316.
51. Xu J, Zhou F, Ji BP, et al. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli.* Lett Appl Microbiol 2008; 47:174-179.
52. Tardugno R, Pellati F, Iseppi R, et al. Phytochemical composition and *in vitro* screening of the antimicrobial activity of essential oils on oral pathogenic bacteria. Nat Prod Res 2018; 32:544-551.
53. Al-Badr RJ, Al-Huwaizi HF. Effect of Tea Tree, Thymus Vulgaris and Nigella Sativa Oils on The Elimination of Enterococcus Faecalis (*In Vitro* Study). J Baghdad Coll Dent 2017; 29:55-62.
54. Imelouane B, Amhamdi H, Wathelet JP, et al. Chemical composition and antimicrobial activity of essential oil of thyme (Thymus vulgaris) from Eastern Morocco. Int J Agric Biol 2009; 11:205-208.
55. Rodriguez-Garcia A, Galan-Wong LJ, Arevalo-Niño K. Development and *in vitro evaluation of biopolymers as a delivery system against periodontopathogen microorganisms*. Acta Odontol Latinoam 2010; 23:158-163.
56. Hernndeza T, Canalesa M, Durana A, et al. Variation in the hexanic extract composition of Lippia graveolens in an arid zone from Mexico: environmental influence or true chemotypes. Plant Sci J 2009; 3.
57. Soković MD, Vukojevic J, Marin PD, et al. Chemical composition of essential oilsof thymus and mentha speciesand their antifungal activities. Molecules. 2009; 14:238-249.
58. Mohsenipour Z, Hassanshahian M. The inhibitory effect of Thymus vulgaris extracts on the planktonic form and biofilm structures of six human pathogenic bacteria. Avicenna J Phytomedicine 2015; 5:309.

59. Hamelian M, Varmira K, Veisi H. Green synthesis and characterizations of gold nanoparticles using Thyme and survey cytotoxic effect, antibacterial and antioxidant potential. J Photochem Photobiol B 2018; 184:71-79.

60. Kot B, Wierzchowska K, Piechota M, et al. Antimicrobial activity of five essential oils from lamiaceae against multidrug-resistant Staphylococcus aureus. Nat Prod Res 2019; 33:3587-3591.