Research Article

Experimental *In vitro* Study to Assess the Antibacterial Activity of Thymus vulgaris Oil on *Streptococcus Sanguinis*

Lubna Fadhil Shallal [1*] , Maha Abdul-Aziz Ahmed[2]
1 Master Student, Department of Periodontology, College of Dentistry, University of Baghdad, Iraq.
2 Professor, Department of Periodontology, College of Dentistry, University of Baghdad, Iraq.
*Correspondence: Lubna.Habib1904@codental.uobaghdad.edu.iq.

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Abstract: Background: The *Streptococcus* genus are the predominant bacteria in the mouth and the *Streptococcus sanguinis* is one of them which performing a primary function for expansion of dental biofilm. Gingival and periodontal disorders are caused by dental biofilm, today, there is a necessity to discover naturally presenting antibacterial compounds from herbs with less side effects as a substitutive to the commonly handled chlorohexidine. Thus, the purpose of this study was to assess the antibacterial activity of thymus vulgaris oil on *Streptococcus sanguinis* bacteria In vitro. Materials and Methods: Human supragingival plaque samples were taken from 10 subjects, then morphological and microscopical examination, biochemical tests, Optochin test, hemolytic ability test and conventional polymerase chain reaction test was applied to assure coincidence of *Streptococcus sanguinis*. The sensitivity of bacteria to Thymus vulgaris oil, the minimum concentration that inhibit the growth and killed the test bacteria were identified a partly as comparable to 0.2% chlorhexidine as a positive control and10 % dimethyl sulphoxide as a negative control. Results: Thymus vulgaris oil presented a significant antibacterial property on *Streptococcus sanguinis* with several grades of inhibition zones. When compared to Chlorohexidine, Thymus vulgaris oil had stronger antibacterial properties. The minimum concentration that inhibited the growth and killed *Streptococcus sanguinis* was (0.09%). Conclusion: Thymus vulgaris oil displayed higher antibacterial action with each concentration on *Streptococcus sanguinis* as compared to Chlorohexidine, therefore; it can be used as a natural substitutive oral health care product to Chlorohexidine.

Keywords: Chlorhexidine, Streptococcus sanguinis, Antibacterial, Thymus vulgaris oil.

Introduction

The *Streptococcus* genus are the main bacteria in the mouth and the *Streptococcus sanguinis* (*S. sanguinis*) is one of the greatest predominant bacteria in the mouth [(1)], which stick to tooth surfaces pellicles supplying a sheet for following union of the late dental biofilm colonizers, thus performing a primary function for expansion of dental biofilm.[(2)] Dental biofilm attaches quickly to tooth surfaces because they do not shed it. This is especially true along gingival edges. If remain unremoved, the microorganisms in dental biofilm multiply and the biofilm environment changes, resulting in increased pathogenicity and the development of a variety of oral disorders, primarily tooth decay and periodontal diseases.[(3)] Mechanical dental biofilm management is the cornerstone of dental disease prevention, but it necessitates patient participation and drive. Brushing and interdental cleaning methods are particularly challenging for old age individuals with physical or mental disabilities, malposed or isolated teeth, orthodontic and bridge appliances, [(4)] thus, chemical dental biofilm management products perform as beneficial adjunctive to accomplish the wanted outcomes.[(5)] The (chlorhexidine) CHX has been handled as an adjuvant

product in conjunction with every day tooth cleaning and flossing program. [4] However, it has several side effects that limit its use.[6, 7] Many herbal formulations have been reported to have antibiofilm efficacy when used as mouth washes and can be used as a substitution to CHX with minimal side effects. [8] Thymus vulgaris oil (TVO) has been considered as antibiofilm, antibacterial, antifungal and antispasmodic activities.[9, 10] Although the TVO's antibacterial efficacy in vitro against a variety of human infections has been well demonstrated, there is limited data concerning the antibacterial effect of TVO on dental biofilm primary colonizers (S. sanguinis). Thus, this study was conducted to assess the antibacterial effect of TVO against S. sanguinis.

Materials and Methods

The Medical Ethical Committee of the University of Baghdad's College of Dentistry accepted the study procedure.

Thymus vulgaris oil

Thymus vulgaris oil (earthroma) brand, fig. (1), 100% pure oil was brought from United States and used in this investigation. The plant parts employed for this oil extraction were leaves and stems, and the place of origin was Spain. The odor intensity was medium, and the extraction process was steam distilled. The liquid was a pale-yellow liquid with a relatively viscous yet clear viscosity.

Samples collection, isolation and identification of Streptococcus sanguinis

Prior to collecting the samples, the subjects were told about the study and their consent and approval were acquired. Human dental biofilm samples were collected from ten participants met the inclusion criteria which include any subject who had supragingival dental biofilm on their labial/buccal teeth surface, but those who used mouthwash or had taken antibiotics in the month before the study met the exclusion criteria. To avoid contact between tooth surfaces and oral mucosa, cotton rolls were used to isolate teeth from buccal/labial mucosa during the sampling procedure. The collection area was washed with water twice before being dried with air. Using sterile periodontal Gracey curette instruments, clinical isolates from supragingival dental biofilm were obtained from the buccal/labial surfaces of teeth. Each scraping was immediately added to tube containing 3 ml brain-heart infusion broth (BHI-B) (TMMEDIA, India). Then, at once taken to the laboratory and incubated aerobically at 37°c for 24 hrs. [11]

For *Streptococcus* spp. isolation, dental biofilm samples were cultured on the selective medium Mitis Salivarius Agar (MSA) (HIMEDIA, India). [12] After streaking the samples, the plates were placed into the incubator and incubated aerobically for 24 hrs. at 37°c. *Streptococci* were sub cultured from the original MSA plate to yield a pure bacterial isolate so, one very little colony was chosen and spread on MSA plate using a sterile bacteriological loop. After that, the cells were incubated aerobically for 24 hrs. at 37°C. [13]

Morphological appearance on MSA plates [14], Gram 's stain ability [15], catalase producing test [16], antibiotic sensitivity test [14], hemolytic ability [17], and Polymerase chain reaction (PCR) were used to identify and diagnose *S. sanguinis* colonies. [12]

Polymerase chain reaction

1.Deoxyribonucleic acid extraction

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure extraction kit (ABIOpure, USA) as the following:

One ml of overnight culture was centrifuged for 2 minutes at 13000 rpm to extract pellet cells. A100 µl of lysozyme and 100 µl of nuclease-free water were added to the 1.5ml centrifuge tubes containing the pellet and incubated in the water bath for 30 minutes at 37°c. After that, twenty µL of proteinase K (Twenty mg/ml) and two hundred µL of binding buffer were applied to the sample for protein digestion and cell lysis. Following that, two hundred µl of 100% ethanol was applied to the sample's tube. All of the mixtures were then carefully transferred to small columns, centrifuged for 1 minute at 6,000 x g, and the collecting tubes were replaced. The mini column was then filled with 600 µl of prewash buffer, centrifuged for 1 minute at 6,000 x g. Finally, 100 µl of elution buffer was applied to the tubes, which were then incubated for 1 minute at ambient temperature before being centrifuged for 5 minutes at 5,000 rpm. The acquired DNA was then kept at –20°C.

2.Thermal cycling procedure and reaction setup

The following DNA sequences were derived from published source and used in this study, (12), table (1).

Table 1: The PCR primers in this study

Primer name	**Primer sequence**	**Annealing Temp. (°c)**	**Expected Size (bp)**
***S. sanguinis*-F**	**5'GGATAGTGGCTCAGGGCAGCCAGTT3'**	**70**	**313**
***S.sanguinis*-R**	**5'GAACAGTTGCTGGACTTGCTTGTC3'**		

The PCR was carried out in 20 µL of a reaction mixture containing 10 µLfrom GoTaq® G2 Green Master Mix 2X (Promega, USA), 1µL of primer- F 10 pmol/µl (Macrogen, Korea), 1µL of primer – R 10 pmol/µl (Macrogen, Korea) (fig. 1), 2 µL of purified DNA, 6 µL nuclease free water. The final mixture was then transferred into conventional PCR tubes and vortexed for 5 seconds. The DNA amplification was performed by (Thermo Fisher Scientific, USA) (fig 2), using the following thermal profile: 95°c for 5 minutes (initial denaturation), followed by 30 cycles of 95°c during thirty seconds. for denaturation, 70°c during 30 sec. for annealing and extension at 72°C for one minute. Final extension at 72°C for 7 min. and hold at 10°c for ten min. The PCR products were evaluated by electrophoresis gel system with 1.5 % agarose (fig 3), next to ethidium bromide 1 µL (10mg/ml) staining. The ethidium bromide-staining bands in gel were visualized by UV transilluminator.

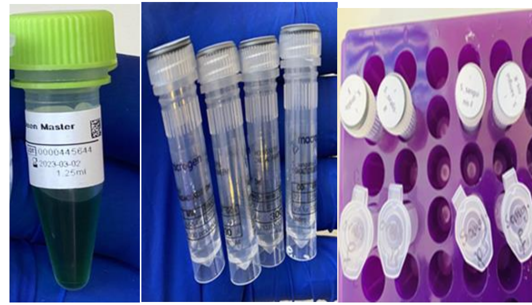

Figure 1: The green master mix and primers

Figure 2: The PCR tubes in 96 well thermal cycler

Figure 3: Gel Electrophoresis System

Preparation of standard bacterial cell suspension

The direct colony suspension method was used to prepare and standardize the inoculum suspension according to Smith and Hussey (2005) technique. [18] The *S.sanguinis* suspension was standardized to match the turbidity of a 0.5 McFarland standard, which equates to 1 X 10^8 colony forming unit (CFU/ml) Using an absorbance microplate reader (GloMax, Promega, USA) with the cell density set to 0.6 and the wave length set to 600 nanometers (nm).[19]

Antibacterial assessment of the TVO

To ensure precision and reproducibility of the results, every investigation aimed antibacterial assessment of the TVO were performed three times at three distinct days.

1.Agar susceptibility experiment

Agar susceptibility protocol for Cavalieri et al. in 2005 and Abdulbaqi et al. in 2016 [18, 20] with some modifications was intended for antibacterial assessment experiment in this study. The Mueller Hinton Agar

(MHA) (Mast Group, U.K.) plates were inoculated with *S.sanguinis* suspension. To generate various concentrations of TVO (100 %, 75 %, 50 %, 25 %, 12.5 %), the TVO was diluted with 10% Dimethyl sulphoxide (DMSO) (CDH Co., Ltd. India) (21). Wells (six mm in diameter) in the plate were made and filled with 50 µL of TVO at previously mentioned concentrations. The positive and negative controls, on the other hand, were 0.2 % CHX and 10 % DMSO, respectively. The plates were then incubated at 37°C for around 24 hrs., after which the inhibition zones of bacterial growth around the wells were measured with a ruler in mm (six mm of every well, was involved).

2.Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) determination

The MIC of TVO on *S. sanguinis* was determined using a two-fold serial broth micro-dilution technique utilizing 96-well cell culture plates (Promega, USA), subsequent to the technique of Abdulbaqi et al. (2016) [20] with some changes. A100 µL of Mueller Hinton Broth (MHB) was added to each of the twelve wells of the two rows, labelled W1-W12. Then, in W1 of each row, (100 µL) of TVO with a concentration of 25 % was added, and a two-fold serial dilution was performed from well one to well nine. The W10, which served as a positive control, included 100 µL of a 0.2 % CHX/nutrient broth mixture. The W11, comprised a 100 µL DMSO/nutritional broth and it was served as the negative control. Each well in the second row were impregnated with 100 µL of *S. sanguinis* suspension and incubated for 24 hrs. at 37°c, with the exception of the first row, which was left blank. After incubation, the loss of turbidity detected using an Absorbance microplate reader at wave length equal to 600 nano meters (nm). The MIC was the concentration of TVO in the well where absence of bacterial growth was noticed (20). The MBC was detected by subculturing a 100µl on MHA plates from MIC tube and one tube before it. The agar plates were incubated overnight at 37°C for 18-24 hours, and the readings were obtained the next day. The concentration which was equal or higher to MIC with no growth of bacteria on MHA after culturing regarded as MBC. [22]

Analytical statistics

The SPSS (Statistical Package for Social Science) version 26 program was used to handle and analyze the data. Descriptive statistics including means and standard deviations (Std) were used. Also, analysis of variance (ANOVA) was utilized to examine the antibacterial effect of data. Tukey's post hoc test was employed to determine whether there was a statistically significant difference between the two concentrations (data not shown). The levels of significance that was used, Non- significant (N.S.) probability value (P – value) > 0.05, Significant (S.) P ≤ 0.05.

Results

The morphological characteristics of *S. sanguinis* on MSA plate appeared as spherical or oval with raised or convex rubbery surfaces, blue in color and adherent to the agar surface (fig. 4). Microscopic examination revealed that cells of *S.sanguinis* colonies showed Gram (+) cocci that were arranged in intermediate or extended chains (fig. 5). On blood agar, the colonies with Alpha hemolytic ability (fig. 6). They were catalase negative (fig. 7) and resistant to Optochin test (fig. 8). The bacteria also identified by conventional PCR. There was a bond between PCR product of suspected bacteria and the primers of *S. sanguinis*. The amplification of *S. sanguinis* primer of bacterial species on 1.5% agarose gel electrophoresis stained with ethidium bromide resulted with 313 bp (fig. 9).

Figure 4: The morphology of *S. sanguinis* colonies on MSA plate

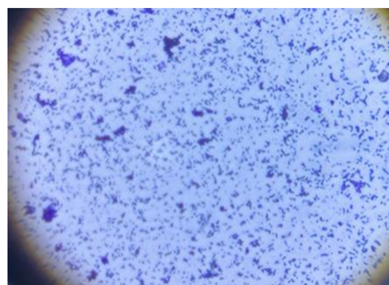

Figure 5: Microscopic appearance of *S.sanguinis*

Figure 6: Alpha hemolytic result of *S.sanguinis* on blood agar

Figure 7: Negative result of catalase test for *S.sanguinis*

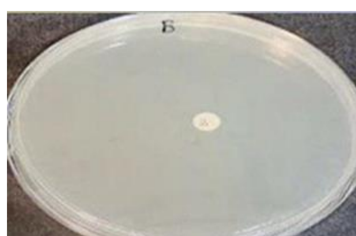

Figure 8: Optochin sensitivity test of *S.sanguinis*

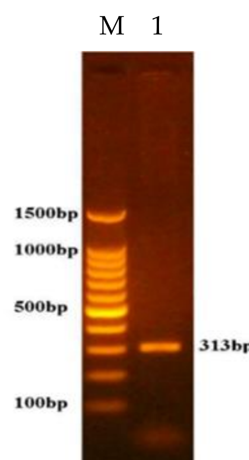

Figure 9: Amplification of *S. sanguinis* primer. M: 100bp ladder marker, Lanes resemble 313 bp PCR products

Agar susceptibility experiment showed that as the conc. of the TVO increased, the diameter of the inhibitory zones grew larger. All concentrations were effective against *S.sanguinis* bacteria, starting at 12.5 %, 25 %, 50 %, 75 %, and 100 %.

The 10 % DMSO revealed no inhibition zone on *S.sanguinis* bacteria, while the TVO at 12.5 %, 25 %, 50 %, 75%, and 100 % displayed higher mean values of the inhibition zones than CHX. The maximum mean value of inhibition zone revealed by 100 % TVO was 60.90 mm., the results of the one-way ANOVA test demonstrated significant difference between the varied concentrations of TVO with CHX and DMSO (table 2,3).

The findings of the comparison for the mean values of inhibition zones between every couple of dissimilar conc. for TVO and between each conc. of TVO with CHX and DMSO by Tukey's post hoc test which were significantly differences (data not shown).

Table 2: The statistical analysis of *S.sanguinis* inhibition zones by different conc. of TVO, CHX and DMSO

Agents Conc.	No.	Mean in(mm)	± S. D.	ANOVA test
CHX 0.2%	9	29.11	0.78	*F=8085.0
DMSO 10%	9	0.00	0.00	*P=0.000 S. *d. f.=6
TVEO 12.5%	9	38.14	0.65	

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

Table 3: The statistical analysis of *S.sanguinis* inhibition zones by different conc. of TVO, CHX and DMSO

Agents conc.	No.	Mean in(mm)	± S. D.	ANOVA test
TVEO 25%	9	41.61	0.63	F=8085.0
TVEO 50%	9	44.78	0.58	P=0.000 S.
TVEO 75%	9	51.37	0.81	d. f.=6
TVEO 100%	9	60.90	0.71	

In this study, experiments showed that the MIC and MBC for TVO against *S.sanguinis* was at 0.09% (0.9 μl/ml).

Discussion

Herbal rudiments were acquiring awareness as both protective plaque formation methods and as assessment remedies. The TVO exhibited antibacterial against microorganisms associated with formation of periodontal diseases [21], but there is restricted data concerning the anti-bacterial effect of TVO on primary dental biofilm colonizer (*S.sanguinis*). Therefore, this study conducted.

The TVO used in this study was from the company (earthroma), gas chromatography mass spectrometry (GC/MS) analysis revealed 17 compounds were identified. Oxygenated monoterpenes were the major portion of this TVO. 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%). According to the composition of the oil, it is obvious that the TVO used in this study belong to thymol chemotype. The composition of TVO was almost similar to that used in previous study

which mentioned that thymol also being the major constituent, Thymol (43.19%), p- γ-Terpinene (6.365%), Cymene (28.55%) and Carvacrol (3.14%), (23). By way of earlier stated the distinction of the chemical structure of the EOs may be because of numerous reasons for example the inherited features, topographical source of plant material and collecting period. [24]

In this study, agar wells susceptibility experiment presented well anti-bacterial effect on *S.sanguinis* bacteria. The diameters of the inhibition zones were raised as the conc. of the TVO raised. A study conducted by Schött et al. (2017) [25] who observed obvious anti-bacterial effect of TVO against *Streptococcus mutans* (*S. mutans*). Also, a study done by De Oliveira et al. (2021) [26] found that the antibacterial activity of TVO on *S.mutans*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus sobrinus*, *Lactobacillus acidophilus* and *Actinomyces naeslundii* was effective. The TVO used in this study 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 had higher antimicrobial activity than CHX.

The antibacterial effect of TVO associated with the phenolic compound's thymol and carvacrol. [27] The TVO and thymol can rupture cell membrane and interposed deoxyribonucleic acid to influence the normal function of cells [28]. The thymol which is stated to dissolve Gram-negative bacteria's outer membrane and make their cytoplasmic membrane extra leaky. [29] Carvacrol has ability to interrupt the folding and coding of proteins [30], as well as prevent the structuring of bacterial protein (flagellin) required for movement of bacteria. [31]

The MIC of TVO needed to inhibit *S.sanguinis* growth in broth media was 0.09 % conc. (0.9 µl/ml). Also, the CHX 0. 2 % used in this experiment as a positive control showed bacteriostatic effect against *S.sanguinis*.

The outcome of MIC in this study was in acceptance with previous reports which found a bacteriostatic effect of TVO against oral pathogenic bacteria *Lactobacillus* species and *S. mutans*, in addition, other author founded a bacteriostatic activity for the TVO on *Staphylococcus aureus* (*S. aureus*) and *Enterococcus* species. [32, 33]

A previous research was conducted to assess the activity of TVEO on *S.sanguinis* bacteria and the result reveled antibacterial activity of TVEO on this bacteria with MIC was 160 µg/ml.[34]

Conversely, high value of MIC or no inhibitory activity of TVO on some periodontal and *Streptococcal* species were reported. [35, 36] The fact that TVO chemical composition and active constituent concentrations are greatly influenced by environmental factors such as temperature, physical situations, and term of gathering are mainly cause the differences in MIC values presented by diverse researchers from different areas. [37, 38]

In this study, the TVO presented killing activity on *S. sanguinis*. The MBC of the oil that kill S. sanguinis was 0.09 % (0.9 µl/ml) conc., other investigations who stated that TVO had killing activity on *Streptococcus salivarius*, *S. mutans*, *Streptococcus pyogenes*, *S. aureus* and *Enterococcus faecalis*. [34, 39]

Conclusion

The Thymus vulgaris oil was more effective as antibacterial agent than 0.2% CHX against *S. sanguinis*, and could be utilized as a natural substitutive energetic product to chlorhexidine as periodontal health maintenance agent.

Conflict of interest: None.

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العنوان:دراسة مختبريه لتقييم فعالية التضاد البكتيري لزيت الزعتر البري على المكورات المسبحية نوع سانكوينس.

الباحثون: لبنى فاضل شلال ,مها عبد العزيز احمد.

المستخلص:

الخلفية:المكورات المسبحيه هي من اهم البكتريا الشائعه في الفم وان مكورات البكتريا المسبحيه نوع سانكوينس هي واحدة منها حيث تلعب دورا اساسيا في تكوين طبقة البلاك السنية.يعد سبب امراض والتهاب اللثة هو مادة البلاك السنية حيث ان هناك حاجة لاكتشاف مضادات بكتيرية طبيعية من الاعشاب مع تاثير جانبي اقل من الكلورهكسيدين .لذلك كان الغرض هو دراسة تاثير زيت الزعتر البري مختبريا على مكورات البكتريا المسبحيه نوع سانكوينس .

المواد والطرق العمل :اخذ عينات من مادة البلاك السنيه من ١٠ مشاركين ومن ثم فحص البكتريا بواسطة الشكل والتحليلات البايوكميائية وكذلك تفاعل البوليمراز المتسلسل حيث تم تعين الحساسيه والحد الادنى لمنع وقتل البكتريا بوسطة زيت الزعتر البري مقارنة بالكلورهيكسيدين.

النتائج: اظهرت النتائج ان لزيت الزعتر البري تاثير فعال على البكتريا حيث كان الحد الادنى لمنع وقتل البكتريا ٠.٠٩٪.

الاستنتاج:كان زيت الزعتر البري تاثير فعال وهو اعلى تاثيرا من الكلورهيكسيدين وبالتالي يمكن استخدامه كمنتج بديل للكلورهكسيدين من اجل انتاج منتجات العنايه بالفم.