Antifungal Effect of Thymus Vulgaris Essential Oil and Chlorhexidine on Candida Albicans Colonies Accumulated on Removable Orthodontic Appliances: A Double- blinded Clinical Trial.

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Abstract

Objectives: To assess the efficacy of *Thymus vulgaris* (*T. vulgaris*) essential oil to decrease the count of *Candida albicans* (*C. albicans*) accumulated on removal orthodontic appliances.

Methods: In this 2 arm parallel controlled clinical trial, Forty-four patients whose removable orthodontic appliances contaminated with *C. albicans* were randomly divided into two groups for use of *T. vulgaris* essential oil and 0.2% Chlorhexidine (CHX) by electronic random sampling. The *C. albicans* colony count was measured at baseline (T0) and at 2 (T1) and 5 (T2) days after the intervention. Microbial samples were collected by a sterile swab from 3 spots on the internal surface of orthodontic appliances. The Friedman test was used for within-group comparisons while the Mann Whitney test was applied to compare the efficacy of *T. vulgaris* essential oil and CHX.

Results: No significant difference was noted in the efficacy of 2% *T. vulgaris* essential oil and 0.2% CHX in decreasing the *C. albicans* colony count on removable orthodontic appliances.

Conclusion: *T. vulgaris* essential oil has favorable antimicrobial activity against *C. albicans*.

Background

Acrylic removable orthodontic appliances are commonly used for myofunctional therapy or simple tooth movements in children and adolescents during the prepubertal or pubertal stage. They can correct some maxillofacial problems by benefitting from the growth potential during this time period. Also, these appliances are used during the maintenance phase after completion of orthodontic treatment to maintain the results and prevent relapse [1]. Long-term daily use of removable orthodontic appliances is imperative to achieve ideal treatment results. However, this pattern of use increases the risk of accumulation of microorganisms on the surface of appliances [2]. Despite the numerous benefits of removable orthodontic appliances, their use can enhance microbial biofilm accumulation on dental surfaces and appliance components such as the clasps, springs, and acrylic base [3]. Evidence shows that use of removable orthodontic appliances can alter the oral microbial flora, increase the salivary count of *Streptococcus mutans*, and enhance the biofilm formation during the active treatment phase [4, 5]. Moreover, the porosities on the internal and external surfaces of the acrylic resin base of such appliances are covered by pathogenic microorganisms mainly *Candida albicans* (*C. albicans*) [6–8].

The acrylic base of removable orthodontic appliances is made of plastic polymers, which contain carbohydrates, and attract several microbial strains such as *C. albicans* that consume such polymeric materials [7, 9]. Moreover, decomposition of acrylic resin can enhance microbial biofilm accumulation [3]. Microbial biofilm contains different microorganisms that strongly adhere to each other and also to the surface. These microorganisms are located on a network of proteins and polysaccharides [10, 11]. Also, use of removable orthodontic appliances increases the count of pathogenic microorganisms such as *Streptococcus mutans*, *Lactobacillus*, and *C. albicans* in unstimulated saliva of patients. Change in oral microbial flora can increase the risk of caries and periodontal disease [12–14].

Chlorhexidine (CHX) is the gold standard for chemical removal of microbial plaque, which is often prescribed as an adjunct to mechanical plaque removal methods. However, long-term use of chemical agents is not recommended [15–18].

Use of herbal essences for their antimicrobial properties has been previously evaluated [19–21]. Several medicinal plants such as thyme, cloves, lavender and tea leaves have been tested for disinfection of the oral cavity [22–27]. Given that medicinal plants are suitable alternatives to chemical agents, application of chemical agents can be limited to acute cases, and herbal products can be recommended for continuous long-term use by orthodontic patients during their treatment course [18].

Thymus (thyme) is an important medicinal herb that belongs to the Lamiaceae family. Thymus, Ziziphora, and Zataria are the three main genera of the Lamiaceae family. The thymus genus has 14 species in Iran, and Thymus vulgaris (T. vulgaris) is one of the most important species [33]. Thymol and carvacrol are the main constituents of T. vulgaris essence. Also, it contains tannin, flavonoids, and saponins [34]. The T. vulgaris essential oil has several pharmaceutical and therapeutic applications [25]. It also has high chance of acceptance by patients due to its favorable taste and odor, insignificant toxicity and side effects, and low cost [35].

Essential oil is the alcoholic extract with a volatile oil, which can be extracted by different techniques such as the cold-pressed extraction method, distillation, extraction by solvent, distillation under vacuum using a microwave, maceration, or extraction with absorbent oils obtained from herbal products [36]. Distillation is an important method of essential oil extraction, which is based on separating the components or substances in two phases [37]. Evidence shows that Zataria multiflora (Shirazi thyme) has suitable antimicrobial and antifungal properties for irrigation of root canals in endodontic treatment in vitro, compared with chemical irrigating solutions [38, 39]. Also, Zataria multiflora essential oil is effective for disinfection of acrylic plates and orthodontic elastics in vitro [40, 41]. Favorable antifungal activity of T. vulgaris essential oil, compared with CHX, has also been reported for disinfection of removable orthodontic appliances in vitro [42].

Specific objectives and hypotheses

This study aimed to compare the effects of T. vulgaris essential oil and CHX on C. albicans accumulated on removable orthodontic appliances. The null hypothesis was that there was no difference in Antifungal efficacy of T. vulgaris essential oil and CHX.

Materials And Methods

Trial design and any changes after trial commencement

This double-blind, single-center, parallel clinical trial with 1:1 ratio was approved by the ethics committee of Islamic Azad University, School of dentistry, Shiraz Branch (Reference: IR.IAU.SHIRAZ.REC.1397.004)

and registered in the Iranian Registry of Clinical Trials (IRCT20180923041092N1). There were no changes to the trial after its commencement.

Participants, eligibility criteria and settings:

Patients with removable orthodontic appliances with a midline expansion screw whose orthodontic treatment had commenced 6 to 8 months earlier in the Orthodontics Department of School of Dentistry of Islamic Azad University, Shiraz Branch were enrolled in this study. The medical and dental history of patients and their oral health, dental status, type of malocclusion, dental arrangement, and oral hygiene were evaluated before the study. Of 83 candidates for participation in the study, those with large diastema or periodontal problem, patients using antimicrobial mouthwashes, those who reported antibiotic intake in the past 3 months, and patients with very poor oral hygiene were excluded. According to the inclusion and exclusion criteria, 55 eligible patients were selected. The participants were briefed about the study, and written informed consent was obtained from them.

Primary sampling to detect patients with *C. albicans*:

One main inclusion criterion for the study was presence of *C. albicans* on removable orthodontic appliances of patients. Thus, a primary sampling was performed for assessment of the presence/absence of *C. albicans* on removable orthodontic appliances. For this purpose, a sterile swab dipped in sterile distilled water was used to collect samples from three spots on the internal surface of the removable appliance that was in contact with the oral mucosa. The three spots were (I) at the center of the appliance, (II) close to the gingival margin of teeth #4 and #5, or D and E in the right side, and (III) close to the gingival margin of teeth #4 and #5, or D and E in the left side. The collected sample was streak cultured on a specific culture medium for *C. albicans*. The plates were coded and sent to a microbiology lab for the microbial culture. Each participant was allocated a code. Of 55 patients, 44 were positive for *C. albicans* and were enrolled in the study.

Microbial culture:

The Sabouraud dexterosus agar containing chloramphenicol is a specific culture medium for *C. albicans* [26]. Thus, the collected microbial sample was streak cultured on this culture medium, and the plate was then incubated for 24–48 h. The proliferated colonies of *C. albicans* were observed on the culture medium after this time period.

Staining:

Lactophenol cotton blue dye, which is a strong dye for diagnostic staining of slides, was used for identification of *C. albicans* colonies, and their differentiation from other microorganisms.

Microorganisms absorb cationic chromatophores due to their slight negative charge, which reveals the morphology of microorganisms under a microscope. For staining, two drops of distilled water were applied at the center of a sterile slide and one drop of lactophenol cotton blue dye was added to distilled water. Next, some colonies were added and dissolved in the dye using a sterile loop such that a

homogenous suspension was obtained. Next, the slide was inspected under a microscope at x4, x10, x40 and finally x100 magnification to identify and study the microorganisms.

Intervention:

After finding the patients with orthodontic appliances contaminated with *C. albicans*, patients were randomly divided into two groups of 2% *T. vulgaris* essential oil and 0.2% CHX control group.

Intervention group:

In the intervention group, the patients received *T. vulgaris* essential oil spray. In order to prepare the *T. vulgaris* essential oil, some research was conducted to find *T. vulgaris* with higher concentrations of thymol and carvacrol. Thus, *T. vulgaris* was obtained from Aliabad Tang village in Beyza city of Fars Province, Iran, and *T. vulgaris* essential oil was extracted by distillation. Analysis of the essence was performed by gas chromatography/mass spectrometry (GC-MS; Agilent Technologies-5975C- MS,7890A-GC) to measure the concentrations of thymol and carvacrol. A clinical trial aiming to determine the minimum inhibitory concentration (MIC) of *T. vulgaris* essential oil against *C. albicans* reported that the MIC of *T. vulgaris* essential oil against *C. albicans* was 1.56% (equal to 15.6 $\mu\text{L/mL}$).⁴² Thus, in this study, 2% concentration of *T. vulgaris* essential oil was formulated after taking into account the possibility of procedural errors and in order to increase the effect size.

In the formulation of *T. vulgaris* essential oil, distilled water was used as the aqueous phase, *T. vulgaris* essential oil was used as the organic phase, and Span 80 and Tween 80 were used as surfactants. In order to prepare 2% formulation, 2 wt% of a mixture of Tween 80 and Span 80 along with the essence were added to 96 wt% of distilled water. Regarding the order of mixing, first Tween 80, Span 80, distilled water and essence were separately weighed. Next, distilled water was added to Tween 80, and the essence was added to Span 80. Eventually, the mixture of essence and Span 80 was added to the mixture of water and Tween 80. The final solution was mixed for 1–3 min, and sonication was performed for 15 min at room temperature in order for the components to form a homogenous mixture. After preparing 2% *T. vulgaris* essential oil, it was packaged in the form of 50 mL sprays, which were filled with the solution using a funnel. The sprays were also coded. The patients received comprehensive instructions on how to use the sprays and also regarding daily hygienic measures. Also, comprehensive instructions for use were provided on the labels of the sprays. The patients were requested to spray the internal surface of their orthodontic appliance from 15 cm distance every night after washing their appliance and then rinse it again 15 min after spraying.

Control group:

The control group received 0.2% CHX Najo in the form of spray with the same packaging and labeling as those administered among the test group. Similarly, the control subjects were provided with thorough

instructions on how to use the spray and perform daily hygienic measures. Also, comprehensive instructions for use were provided on the labels of the sprays. The sprays were then coded.

Outcomes (primary and secondary) and any changes after trial commencement

The second microbial sampling was performed in both groups at baseline prior to the commencement of clinical trial (T0), and at 2 (T1) and 5 days (T2) after using the sprays. A sterile swab was used for sampling from three spots of the appliance as explained earlier for the first sampling. The person preparing and providing the sprays to the participants and those responsible for culture and diagnosis of the slides were all blinded to the group allocation of samples. There were no changes to the trial after its commencement

Sample size calculation:

A total of 40 participants were required to achieve 85% power (instituted by G power, version 3.0.1; Franz Faul universitat, Kiel, Germany) and detect significant differences considering the effect size of 0.47 ($P < 0.05$). Considering the possible dropouts, the sample size was increased to 44.

Interim analyses and stopping guidelines

Not applicable.

Randomization:

Before the trial commencement, randomization process, allocation concealment, and implementation were performed by a researcher who was not involved in the study. Random Allocation Software program was used for simple electronically-generated randomization.

Randomization ensured patients' allocation to both groups with 1:1 ratio. Allocation concealment was ensured by using numbered, sealed, opaque envelopes prepared before the trial commencement. The envelopes were sequentially opened only after the envelope had been irreversibly assigned to a participant

Blinding:

Double-blinding was ensured in this study. Only the chemist who cooperated with us was aware of the distribution of disinfectants. She assigned a number to each bottle that only she was aware of.

Statistical analysis:

Data were analyzed using SPSS version 22. Distribution of data was evaluated using the Kolmogorov-Smirnov test. Since data were not normally distributed, non-parametric tests were applied. The Friedman test was used to assess the efficacy of *T. vulgaris* essential oil and CHX within each group. The Mann Whitney test was applied to compare the efficacy of *T. vulgaris* essential oil and CHX.

Results

Participant flow

Of 83 patients, 28 were excluded since they did not meet the eligibility criteria. Of the remaining 55, 44 had removable orthodontic appliances contaminated with *C. albicans* and were enrolled in the study. Recruitment began in April 2019 and was completed in August 2019. Two participants were later excluded since their orthodontic appliance broke and they did not cooperate well after the trial commencement; The CONSORT diagram is shown in Fig. 1.

Baseline data

The baseline characteristics for age, sex and the time point at which patients were recruited were similar in both groups (Table 1).

	Thymus vulgaris	Chlorhexidine
Age	10.4±1.9	9.9±1.1
Sex		
Boy	11 (52.38%)	13 (61.90%)
Girl	10 (47.62%)	8 (38.10%)

Table 1

Basic characteristics for patients in each groups

Numbers analyzed for each outcome, estimation and precision, subgroup analysis

For data analysis, the colony count was classified into five groups of rare, few, moderate, many and numerous (Table 2). The Friedman test revealed that continuous use of *T. vulgaris* essential oil or CHX significantly decreased the colony count ($P < 0.05$, Table 3). The Mann Whitney test showed that this reduction was not significantly different between the two groups of *T. vulgaris* essential oil and CHX (Table 3). Figure 2 shows the change in the mean rank of colony count (contamination rate) in the two groups. Although the mean difference in the first and third samplings was the same and equal to 0.1, the 2% *T. vulgaris* essential oil graph was completely descending, while the 0.2% CHX graph reached a plateau from the second phase on.

Classification	Number of colonies
Rare	Number of colonies $\leq$ 10
Few	10<Number of colonies $\leq$ 30
Moderate	30<Number of colonies $\leq$ 60
Many	60<Number of colonies $\leq$ 100
Numerous	Number of colonies>100

Table 2
Classification of colony count data

Table 3

Within-group and between-group comparisons of the mean rank of colony count

Groups	Number	Mean Rank			P value ^a
		T0	T1	T2	
Thymus vulgaris	21	2.50	1.87	1.63	0.002
Chlorhexidine	21	2.60	1.67	1.73	0.001
P value ^b		0.505	0.809	0.501	
T0: first day of intervention; T1: Two days after intervention; T2: Five days after intervention.					
^a : Friedman test; p < 0.05					
^b : Mann Whitney test; p < 0.05					

Figure 3 shows the relative frequency distribution of different levels of colony count in the two groups at different time points. The relative frequency of rare class was 6.7% in both groups at baseline while the frequency of rare class was 53.3% in the 2% T. vulgaris essential oil and 26.7% in the CHX group in the final sampling, which indicated higher efficacy of T. vulgaris essential oil than CHX. The relative frequency of numerous class was 80% in T. vulgaris essential oil group at baseline, which decreased to 40% in the final sampling. The remaining 40% was distributed among different classes, with rare class having a higher frequency. This rate was 66.7% at baseline in the CHX group, which decreased to 33.3% in the final sampling. The remaining percentage mainly belonged to the few class, which indicated the superiority of T. vulgaris essential oil to CHX.

Results of GC-MS:

The interpretation of GC-MS results revealed that the essential oil used in this study contained 43.7% thymol. Table 4 lists the 14 main constituents of this essential oil.

Table 4
Main constituents of *T. vulgaris*
essential oil.

Compound	%
Alpha- Phellandrene	1.30
Alpha-Pinene	5.65
Camphene	1.88
Beta-Pinene	0.42
Beta-Myrecene	1.35
Alpha-Terpinene	0.76
Cymene	16.96
Gamma-Terpinene	4.52
Cis-Sabinenehydrate	1.46
Camphor	1.16
Borneol	4.33
Alpha-fenchyl acetate	0.94
Thymol methyl ether	1.77
Thymol	43.7

Harms

No harm was observed or reported from the participants in the study.

Discussion

Orthodontic appliances can impair the balance of microorganisms in the oral environment and may even lead to disease [10, 11]. The acrylic base of removable orthodontic appliances is made of polymethyl methacrylate-based auto-polymerizing resin while the wires are made of stainless steel. The pathogenic microorganisms can lodge in resin porosities on the external and internal surfaces of acrylic components. Also, degradation of acrylic resin can cause bacterial biofilm accumulation [13].

The mechanical plaque removal methods such as tooth brushing are suitable for elimination of the accumulated biofilm. Nonetheless, mechanical methods alone are not highly effective in many individuals due to poor motivation or hand skills [43]. Thus, chemical plaque removal agents are always considered along with the mechanical methods. Many chemical and herbal products are used for elimination or reduction of microorganisms [44]. CHX is the gold standard for chemical removal of the

microbial plaque. Nonetheless, CHX is a synthetic chemical agent, which can have many side effects following long-term application [18]. Thus, long-term use of chemical agents is not recommended [15]. In the recent years, the demand for alternative plaque removal methods has greatly increased. Herbal products are gaining increasing popularity due to their organic nature and fewer side effects [27]. The results of this clinical trial showed that *T. vulgaris* essential oil and CHX effectively decreased the *C. albicans* colonies accumulated on orthodontic appliances. In this study, the *T. vulgaris* plant was collected early in April, and the aerial part of the plant was used to ensure its high quality.

T. vulgaris is a medicinal plant with antimicrobial properties [45]. It has favorable taste and odor and low cytotoxicity [35]. Nonetheless, the *in vivo* effect of *T. vulgaris* essential oil on *C. albicans* isolated from removable orthodontic appliances has not been previously evaluated. According to a study by Kaviani et al, the MIC of *T. vulgaris* against *C. albicans* isolated from the surface of acrylic appliances was 1.56% (15.6 $\mu\text{L/mL}$) [42]. Thosar et al. reported the MIC of *T. vulgaris* essential oil to be 1.6% (16 $\mu\text{L/mL}$), with the difference that they used standard-strain *C. albicans* *in vitro* [24]. Moreover, Fani et al. showed that the MIC of *T. vulgaris* essential oil was 1.63% (16.3 $\mu\text{L/mL}$) [46]. In this clinical trial, 2% *T. vulgaris* essential oil was formulated to compensate for the possible procedural errors.

Although immersion of orthodontic appliances in disinfecting solutions for a certain period of time is the conventional method for disinfection of these appliances at home [47], immersions can lead to water sorption and subsequent adverse changes in the acrylic resin structure. In this study, the spraying technique was used instead of immersion to prevent possible changes in acrylic resin structure. Also, spraying is cost-effective and easier for patients [13, 48].

The results of this clinical trial revealed that 2% *T. vulgaris* essential oil and 0.2% CHX had suitable antifungal effects on orthodontic appliances contaminated with *C. albicans*. Nonetheless, the reduction in colony count was not significantly different between the two groups.

The antimicrobial properties of medicinal plants mainly depend on the presence and concentration of phenolic compounds, saponins, and flavonoids in their structure. These compounds can adversely affect the plasma membrane of microorganisms or inhibit their structural cell membrane enzymes and exert their antimicrobial effects as such [49].

Thymol and carvacrol are among the main constituents of *T. vulgaris* essential oil, and the antifungal properties of this plant can be attributed to these ingredients. Carvacrol is an iso-thymol that increases the activity of ATPase and inhibits the activity of the enzyme responsible for non-specific permeability of the cell membrane of microorganisms such as bacteria, and consequently increases their sensitivity to external factors entering the cell [50]. Thus, difference in the MIC of *T. vulgaris* essential oil can be attributed to the variations in the percentage of its constituents.

Assessment of the mean rank-time graph revealed that continuous use of *T. vulgaris* essential oil during the study period caused a continuous reduction in *C. albicans* colony count. However, CHX caused a reduction in colony count to some extent and then reached a plateau. Also, assessment of the relative

frequency of *C. albicans* colonies revealed that *T. vulgaris* essential oil was more effective than CHX for reduction of the relative frequency of *C. albicans* colonies.

Generalizability

The patients recruited for this study were 10 to 12 years old, this shows the age range for removable orthodontics appliance and allowing the results to be generalized across this age range.

Limitation:

Short intervention period: to evaluate the effect of prolonged use of *T. vulgaris* essential oil on oral mucosa and teeth, enlarging treatment duration is necessary. Further studies on the antimicrobial efficacy of *T. vulgaris* essential oil against other microorganisms accumulated on orthodontic appliances and dentures are required prior to extensive clinical use of this essential oil as an alternative to CHX.

Conclusion:

Although this study found no significant difference in antifungal efficacy of 2% *T. vulgaris* essential oil and 0.2% CHX against *C. albicans* isolated from removable orthodontic appliances, 2% *T. vulgaris* essential oil can be suggested as an alternative to CHX for elimination of fungal contamination of orthodontic appliances, considering its lower cytotoxicity, fewer side effects, and higher patient acceptance, compared with CHX.

Abbreviations

T. vulgaris: *Thymus vulgaris*; *C. albicans*: *Candida albicans*; CHX: Chlorhexidine; GC-MS: Gas Chromatography/Mass Spectrometry; MIC: Minimum Inhibitory Concentration;

Declarations

Ethics approval and consent to participate:

This clinical trial was approved by the ethics committee of Islamic Azad University, School of dentistry, Shiraz Branch (Reference:IR.IAU.SHIRAZ.REC.1397.004) and registered in the Iranian Registry of Clinical Trials (Trial registration:IRCT20180923041092N1. Registered 23 November 2019 - Retrospectively registered, <https://www.irct.ir/user/trial/34180/view>).

Availability of data and material:

The datasets used and/or analyzed during the present study are available from the corresponding author.

Competing interests:

The authors declare that they have no competing interests.

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No funding was provided for this study.

Authors' contributions:

NN and TB contributed in the design of the study, wrote the first draft of the manuscript, revised the manuscript, participated in data interpretation, and gained permission from the Medical Ethics Committee, Shiraz branch, Islamic Azad university, Iran. A KK participated in acquisition of data and revision of the manuscript. A KK and RS made contributions to prepare the extract of *Thymus vulgaris* and mouthwashes. provided critical comments. S N Participated in microbial evaluation and data interpretation. All authors read and approved the final manuscript

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Figures

Figure 1

CONSORT flow diagram

Figure 2

Change in the mean rank of colony count in the two group s T0: first day of intervention; T1: Two days after intervention; T2: Five days after intervention

Figure 3

Relative frequency of different levels of *C. albicans* colony count at different time points. T0: First day of intervention; T1: Two days after intervention; T2: Five days after intervention.

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