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Antimicrobial Effects of *Zataria multiflora* Extract Mouthrinse on Orthodontic Elastomeric Ligatures- an In Vitro Study

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Abstract

Objectives: The aim of this study was to evaluate and compare antibacterial and antifungal effects of different concentrations of *Zataria multiflora* extract mouthrinse.

Methods: In this lab trial study, 128 elastomeric ligatures were divided into 2 groups of 64. Before disinfection eight ligatures of each group were randomly selected to evaluate the microbial load. The remaining ligatures in each group (n = 58) were divided into 7 subgroups of 8. All samples in group 1 (G1) contaminated with *Streptococcus mutans* (*S. mutans*) and in group 2 (G2) contaminated with *Candida albicans* (*C. albicans*). Then, *Zataria multiflora* extract in 1%, 0.1%, 0.01%, 0.001%, 0.0001% concentrations as decontamination agent were used in 5 subgroups of each group. Positive control consisted of penicillin in G1 and nystatin in G2. For the negative control in both groups phosphate buffered saline was used. The average number of adhered viable bacterial cell after performing the disinfection protocols were calculated and compared. The collected data was statistically analyzed by Kruskal-wallis, Wilcoxon and Mann-Whitney test using SPSS software version 18 at a significant level of 0.05.

Results: Results showed that penicillin, nystatin, *Zataria multiflora* extract in 1% and 0.1% concentrations completely eliminated *C. albicans* and *S. mutans* on elastomeric ligatures. Statistically significant differences were found between the number of adhered *S. mutans* and *C. albicans* before and after performing the all five concentrations of disinfection solutions (P = 0.01). Also higher concentrations of *Zataria multiflora* showed more antibacterial effectse in both groups.

Conclusions: *Zataria multiflora* extract mouthrinse showed antibacterial and antifungal characteristics and could be useful for patients under orthodontic treatment.

Keywords: Disinfection, Orthodontics, Ligatures, *Zataria multiflora*

1. Background

Poor oral hygiene and inappropriate removal of dental plaque during orthodontic treatment usually result in gingival inflammation and white spot lesions (1, 2). Prolonged period of treatment and retentive spaces of orthodontic appliances are the main reasons of plaque accumulation and gingival inflammation during orthodontic treatment (1). It has been shown that using a fixed orthodontic appliance can change oral microbial flora which often leads to an increase in the levels of subgingival and supragingival pathogens in dental plaque (3). Several studies have reported an increase in *Streptococcus mutans* (*S. mutans*) levels in saliva and dental plaque of patients with orthodontic appliances (4-6).

High acid tolerance microorganisms, *S. mutans* and lactobacilli, and their interaction with sugar cause a decrease of PH level in plaque which can lead to initiation of caries lesions (7). *Candida albicans* (*C. albicans*) are frequently seen in the oral cavity of almost half of the healthy population, and may be associated with orthodontic appliance contamination and pathologies (8). Also, it can be isolated from plaque in immune-compromised patients who seek orthodontic treatment (9). Therefore more cautious approaches, like using mild herbal mouthwashes, are required when providing fixed orthodontic treatments for immunocompromised children regarding the increased possibility of candidal infection (10).

Plaque control interventions, mainly done by mechan-

ical removal methods (tooth brushing, flossing) can be enhanced by incorporating chemical or herbal mouthrinse into oral hygiene regimens (11). Simoes Moraes et al. (12) stated that high concentrations of chlorhexidine (CHX) decreased plaque formations of *S. mutans* around metal brackets. Sari and Birinci (11) also reported applying CHX for one week can significantly decrease colonization of microorganisms however it had no effects on lactobacillus. Despite good antimicrobial effects of CHX, discoloration of teeth, soft tissue irritations and also undesirable taste can lead to incompliant patients (13, 14).

Recently, using herbal mouthrinses like garlic extraction and percica has been increased. Also, *Zataria multiflora* (ZM) which is called Avishan Shirazi in Iran, is a newly introduced herbal solution which studies on its antimicrobial effects in denistry is scarce. ZM belong to the Laminaceae family of plants and its main components are carvacrol (50.7%), thymol (13.38%) and P-cymene (8.27%) (15). ZM has a favorable taste and smell which makes its acceptance great by patients.

2. Objectives

The objective of this study was to evaluate and compare antibacterial and antifungal effects of different concentrations of *Zataria multiflora* extract mouthrinse.

3. Methods

This in vitro experimental study was conducted on 128 polyurethane orthodontic elastomeric ligatures (Ortho Technology, USA).To imitate the tensile force and simulating the adherence effects of ligatures during orthodontic treatment, they were placed on metal brackets (Dentaurum Co, Germany) for a week (16, 17). Then they were removed from brackets and were incorporated into the experiment.

Organisms: *Streptococcus mutans* (Strain PTCC1 1683) and *Candida albicans* (PTCC 5027) were obtained from Iranian Research Organization for Science and Technology (IROST). Further *S. mutans* seeded on Mitis Salivarius agar (Merck, Germany) containing 1% potassium tellurite and *C. albicans* on Sabouraud dextrose agar (Oxoid, UK) plates by standard protocols to provide fresh colonies for making microbial suspension. The density of the bacterial suspension was adjusted with sterile phosphate buffered saline (PBS) to that of 0.5 McFarland standard concentration and used for the experiments.

3.1. Experimental Groups

128 Elastomeric ligatures were randomly divided into 2 groups:

G1: 64 elastomeric ligatures were immersed in 1×10 7 CFU/mL *S. mutans* suspension on a reciprocal shaker (100 rpm) at 37°C for 2 hours.

G2: 64 elastomeric ligatures were immersed in 1×10^6 CFU/mL *C. albicans* suspension as above mentioned protocol.

3.2. Assessment of the Adhered Microbial Cell Before Disinfection

Sixteen ligatures (8 of each group) were randomly selected to count the adhered viable microbial and fungal colonies before using *Zataria multiflora* extract mouthrinse. Each ligature was separately transferred to a sterile micro tube containing 1 ml of sterile distilled water and washed 3 times with phosphate buffered saline, Ph = 7. Next, micro tubes were sonicated at 45 KH (Elma, Germany) for 10 minutes to dislodge the adhered viable cells. 100 μL of final sonicated distilled water was spread on blood agar plates as a growth medium for *S. mutans* and Sabouraud dextrose agar plates for *C. albicans*. Plates were incubation for 48 hours in 37°C and 30°C (18). Then bacterial and fungal colonies were counted separately on each plate and the average number of adhered colony forming units (CFUs) was calculated based on similar studies (19).

3.3. Disinfection Materials

Five mm ZM extract (1m g/mL) was obtained from Barij Essence Pharmaceutical Co, Kashan, Iran. ZM was diluted by distilled water and used in 5 concentrations of 1, 0.1, 0.01, 0.001, 0.0001 mg/ mL.

Penicilline V potassium, 250 mg (Farabi pharmaceutical Co, Iran) in form of oral suspension was used as positive control for *S. mutans* in G1 nystatine 100,000 (Emad pharmaceutical Co, Iran units in form of oral suspension was used as positive control for *C. albicans* in G2 phosphate buffered saline was used as negative control in both groups.

3.4. Disinfection Protocol

In each study group, the 56 remained contaminated elastomeric ligatures were divided into 7 subgroups (N = 8). Five subgroups were placed in sterile Falcon tubes containing 5 mL of different concentrations of *Zataria multiflora* extract including 1%, 0.1%, 0.01%, 0.001%, 0.0001% and the other 2 subgroups were placed in penicillin and Nystatin falcon tubes. They were incubated on a shaker (100

rpm) at 37°C for 1 minute. Thereafter each ligature was transferred to a sterile micro tube containing 5 mL of sterile distilled water, washed for 3 times and then sonicated as method explained earlier (20).

At the end, 100 μL of each washed distilled water samples was spread on blood agar plates and Sabouraud Dextrose for *S. mutans* and *C. albicans* respectively as method explained earlier. The average number of adhered CFUs after performing the disinfection protocol by each mouthrinse was calculated (Figure 1) by an automatic colony counter (colony counter Funke-Gerber GMBH, Nr. 2774, Berlin/Munchen).

The collected data was statistically analyzed by Kruskal-wallis, Wilcoxon and Mann-Whitney test using SPSS software version 18 at a significant level of 0.05.

4. Results

Mean values and standard deviation (SD) of CFUs in G1 and G2 before and after disinfecting protocol are presented in Table 1.

Results showed that penicillin, nystatin, *Zataria multiflora* extract in 1% and 0.1% concentrations completely eliminated *C. albicans* and *S. mutans* on elastomeric ligatures.

As shown in Table 1, the maximum CFU of attached viable cells both in G1 and G2 were belonged to the contaminated elastomeric ligatures before applying disinfection solutions fallowed by negative controls. The minimum CFUs both in G1 and G2 were seen in elastomeric ligatures disinfected by 1% of *Zataria multiflora* extract mouthrinse and positive controls.

As it shown in Tables 2 and 3, there was a statistically significant difference between CFUs of elastomeric ligatures before and after performing the all five concentrations of disinfection mouthrinse in both groups (P value = 0.01). In G1 and G2, there was a statistically significant difference between high concentrations of ZM (1%, 0.1%, and 0.01%) and attenuated concentration of 0.0001% in regard to bacterial elimination (Figure 1). In G2, ZM of 1% and 0. 1% was statically more effective than 0.001% (Figure 2). There were no significant differences among the other subgroups (P value > 0.05).

5. Discussion

During orthodontic treatment, presence of fixed appliances makes many retentive spaces for microbial plaque and also makes tooth brushing more difficult. Therefore insufficient removal of attached plaque can increase the activity of pathogens in biofilms. Further, acidogenic activities of pathogens could lead to enamel decalcification and white spot lesions particularly around the brackets on buccal surface of teeth (21, 22). Previous studies reported that during orthodontic treatment bacterial colonization on elastomeric ligatures are higher than stainless steel ligatures (23, 24). In the present study elastomeric ligatures were used because they are more commonly used by orthodontists.

Applying different antimicrobial agents during orthodontic treatment have been evaluated in previous studies. Bretas et al. (25) reported that 0.4% Stannous fluoride gel could only make small changes in *S. mutans* colony count adhered to elastomeric rings and steel ligatures in orthodontic patients. Salehi et al. (26), showed that persica and matrica mouthrinsescan significantly reduced biofilm formation of *S. mutans* around brackets in orthodontic patients without any side effects such as discoloration in CHX use. ZM as antifungal and anti bacterial agent has been studied a lot in recent years. Sedigh-Shams et al. (27) in 2016 showed that ZM could be used as an irrigate for dental root canals contaminated with *C. albicans* with similar effects to the popular one of NaOCl. In this study ZM extract mouthrinse was used. It is an herbal plant and usually grows in Iran, Pakistan and Afghanistan. According to Eftekhar et al. (28) carvacrol and thymol are the main components of ZM which produce antimicrobial effects against fungus, *E. coli* and *S. aureus* Zomorodian supported the idea of using ZM in mouthwashes and denture cleansers, since they show high efficacy in inhibiting microbial strains, even in the plantitic form (29). Furthermore, anti-inflammatory activity and pleasant odor and flavor of ZM are additional advantages to their antimicrobial activities to be used as a mouth rinse and other oral hygienic products (30).

Results of the current study showed that there were significant differences in fungal and bacterial colony counts after using ZM extract on the surface of elastomeric ligatures. Although higher concentrations of ZM showed better antimicrobial and antifungal effects, the least concentration of ZM (0.0001%), also, represented significant antimicrobial effects compared to negative control. Sedigh-Shams et al. (27) reported that the minimum fungicidal concentration of ZM was 1 mg/mL. Based on this study, a solution of 1% or 0.1% of ZM could be as effective as penicillin/nystatine (completely eliminating the *S. mutans* and *C. albicans*). Jafari et al. (31) also reported that higher concentrations of ZM were more comparable to nystatine in regard to elimination of *C. albicans* of acrylic plates. The reason of testing very small percentages of ZM in this study was to obtain the minimum effective dosage of ZM since no

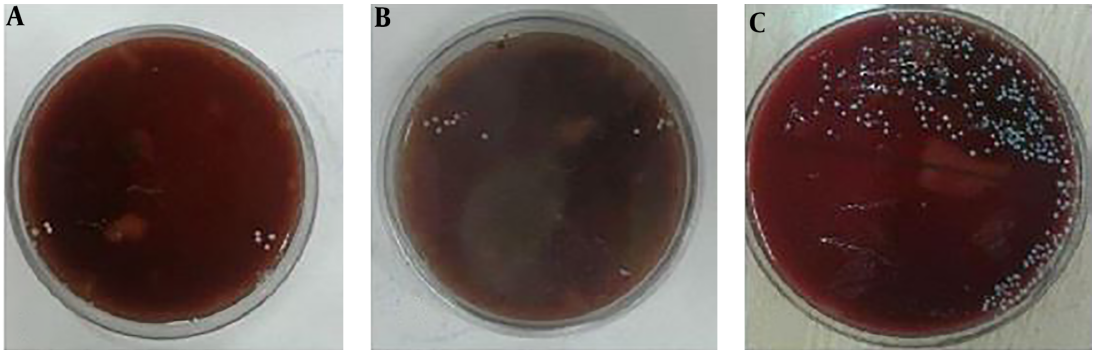

Figure 1. *S. mutans* colonies isolated from ligatures disinfected; A, with 0.1% ZM; B, with 0.01% ZM; and C, phosphate buffer saline (negative control).

Table 1. Descriptive Statistics for CFUs of Study Samples Before and After Disinfection[a]

Study Groups	Before Disinfectants	After Disinfection with ZM					Positive Control	Negative Control
		1%	0.1%	0.001%	0.01%	0.0001%		
G1	699.62 ± 33.34	0 ± 0	0 ± 0	0.62 ± 0.49	0.25 ± 0.25	1.62 ± 0.53	0 ± 0	322 ± 26.36
G2	257.5 ± 39.95	0 ± 0	0 ± 0	0.12 ± 1.04	0.12 ± 0.12	7.6 ± 3.00	0 ± 0	122 ± 17.20

[a]Values are expressed as mean ± SD.

Table 2. The Result of Multi Comparison Test[a, b]

G1 *S. mutans*	Before Disinfection	ZM Concentrations				
		1	0.1	0.01	0.001	0.0001
ZM concentrations						
1	S[b]	-	NS[d]	NS	NS	S
0.1	S	-	-	NS	NS	S
0.01	S	-	-	-	NS	S
0.001	S	-	-		-	NS

[a]Showing significance or insignificancy of differences between CFUs adhered to the elastomeric ligature contaminated with *S. mutans* before and after disinfection.
[b]Wilcoxon test.
[c]Significant.
[d]Not significant.

Table 3. The Result of Multi Comparison Test

G2 *C. albicans*	Before Disinfection	ZM Concentrations				
		1	0.1	0.01	0.001	0.0001
ZM concentrations						
1	S[c]	-	NS[d]	NS	S	S
0.1	S	-	-	NS	S	S
0.01	S	-	-	-	NS	S
0.001	S	-	-		-	NS

[a]Showing significance or insignificancy of differences between CFUs adhered to the elastomeric ligature contaminated with *C. albicans* before and after disinfection.
[b]Wilcoxon test.
[c]Significant.
[d]Not significant

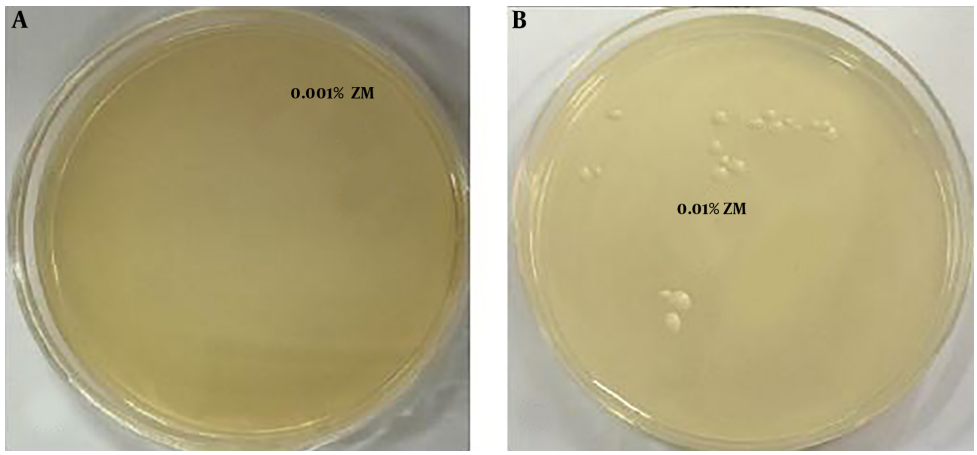

Figure 2. *C. albicans* colonies isolated from ligatures disinfected in; A, 0.001% ZM; and B, 0.01% ZM.

study has been done so far on adverse effects and citotoxicity of ZM.

Amanlou et al. (32), reported that in patients with denture ostomatities, a gel of ZM could efficiently reduce surface erythema and colony counts of *C. albicans* in saliva more than miconazole. However in comparison to miconazole, ZM was less effective in reduction of the colony count of *C. albicans* on the denture surface (32). Aghili et al. (20) investigated antibacterial effect of ZM extract in comparison to CHX mouthrinse, ZM could efficiently decrease colony counts on elastomeric ligatures. Result of this study showed that incorporating all five concentration of ZM could efficiently reduce CFUs and therefore it could be used as a base of mouthrinse. However further studies on a larger variety of ZM concentrations in oral environment is recommended to overcome the limitations of this study.

5.1. Conclusions

Zahra Moradi extract mouthrinse in 5 concentrations (1%, 0.1%, 0.01%, 0.001%, and 0.0001%) were effective in reduction of colony counts of *S. mutans* and *C. albicans* adhered to elastomeric orthodontic ligatures.

Higher concentrations of ZM (1% and 0.1%) were completely eliminated the CFUs of *S. mutans* and *C. albicans* same as positive control (penicillin and nystatin).

Footnotes

Authors' Contribution: Hossein Aghily designed the study. Ahmad Mosadegh has done the microbiological exams Alireza Akrami performed the infection and disinfection of samples Mohammad Reza Hakimimeiboodi and Zahra Moradi analyzed the data Zahra Ebrahimi Nik drafted the manuscript.

Conflict of Interests: Zahra Ebrahimi Nik as the corresponding author, declare that this study was done without any conflict of interest.

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