Original Article

Antimicrobial Effect of *Zataria Multiflora* Extract in Comparison with Chlorhexidine Mouthwash on Experimentally Contaminated Orthodontic Elastomeric Ligatures

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Abstract

Objectives: Long-term use of orthodontic appliances and fixation ligatures creates a favorable environment for the accumulation of oral normal microflora and increases the risk of enamel demineralization and periodontal disease.

The aim of this study was to compare the antimicrobial effects of Zataria Multiflora extract and 0.2% chlorhexidine (CHX) mouthwash on experimentally contaminated orthodontic elastomeric ligatures.

Materials and Methods: In this lab trial study, Iranian and foreign-made elastomeric ligatures were experimentally contaminated in *Streptococcus mutans*, *Enterococcus faecalis* and *Candida albicans* suspensions. Ligatures were then decontaminated using 0.2% CHX as the control, 0.5 mg/ml *Zataria multiflora* extract mouthwashes as the test and phosphate buffered saline (PBS) as the negative control for one hour. Antimicrobial properties of both solutions were evaluated by comparing the mean viable bacterial cell count on both rings after decontamination, using SPSS version 15 software.

Results: The mean viable bacterial cell count on Iranian ligatures was greater than that on foreign-made ligatures before disinfection (P=0.001), however this difference for *C. albicans* was not statistically significant (P=0.061). Chlorhexidine mouthwash completely eliminated all tested microorganisms attached to both elastomeric rings, but Zataria extract was only capable of completely eliminating *C. albicans* from both ligatures. Statistically significant differences were found in viable bacterial counts on both ligatures before and after disinfection with Zataria extract (P=0.0001).

Conclusion: Zataria multiflora extract has antimicrobial properties and can be used for disinfection of elastomeric ligatures. In vivo studies are required to evaluate the efficacy of the incorporation of this herbal extract in mouthwashes for orthodontic patients.

Key Words: *Zataria multiflora*, *Streptococcus mutans*, *Candida albicans*, *Enterococcus faecalis*, Chlorhexidine

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INTRODUCTION

Use of fixed orthodontic appliances often results in increasing levels of periodontal pathogens in supragingival and subgingival biofilms, causing gingival inflammation during orthodontic treatment [1, 2]. During fixed orthodontic treatment, plaque accumulates around the brackets and causes demineraliza-

tion of the adjacent enamel, which is clinically important [3]. Malocclusion is known as the 3rd most common oral health problem. A higher demand for orthodontic treatment has been observed in the past decades in both children and adults. Orthodontic therapy is very popular in Iran as well and an epidemiological study on Iranian schoolchildren showed that about 36.1% of them needed orthodontic treatment [4].

Orthodontic procedures were meant to be non-invasive; but orthodontic therapy using fixed and removable buccal appliances causes iatrogenic side effects like periodontal disease [5]. Human oral microflora is highly diverse and may be changed by several endogenous and exogenous factors [6]. Literature shows that presence of fixed or removable orthodontic appliances in the oral cavity provides retentive spaces for accumulation of microbial plaque and initiation of caries, which in turn compromise oral health and result in subsequent periodontal infection [7-11]. Isolation of aerobic and anaerobic bacteria from blood samples of patients with orthodontic appliances highlighted the role of dental biofilm accumulated on these appliances in this respect [12].

Three methods are conventionally used for the attachment of wire to brackets: ligation with steel wires, elastomeric ligatures, and self-ligating brackets.

All these methods have their own advantages and disadvantages; but literature suggests that elastomeric ligatures enhance the retention of dental biofilm [13].

Candida albicans, known as the most prevalent fungus in oral normal flora, is frequently isolated from dental plaque and carious lesions of children with early childhood caries [14]. Streptococcus mutans also appears to be the most commonly involved acidogenic microorganism in development of caries [15]. Enterococcus faecalis is an enteric facultative Gram-positive bacterium that grows independently in the root canal system causing endodontic infections [16].

Forsberg et al. showed that elastomeric rings resulted in accumulation of a greater number of microorganisms in dental plaque around brackets than steel ligatures. Using elastomeric rings in orthodontic therapy results in significant accumulation of microorganisms such as *S. mutans, C. albicans* and a few others in dental plaque around brackets causing enamel demineralization [17].

Failure in maintaining proper oral hygiene by orthodontic patients may result in oral conditions like tooth decay and periodontal disease. Therefore, cariogenic microorganisms in dental biofilm must be eliminated as much as possible during orthodontic treatment [18]. Normally, elastomeric rings are cut by scissors and placed in the mouth without prior disinfection or sterilization. This act has been questioned by the recently suggested biohazard measures, as they may become contaminated during processing and manipulation by dental assistants or orthodontists before final insertion. The rough surface and the absorption properties of elastomeric rings further contribute to the formation of bacterial plaque on their surfaces, resulting in accumulation of higher number of microorganisms on tooth surfaces [17, 18].

In order to control dental plaque formation, different chemical mouthwashes are used, which may cause allergic reactions and mucosal irritation [19].

Several chemical mouthwashes are available in the market; CHX is known to be highly effective for prevention of dental plaque formation and elimination of pathogenic microorganisms like *S. mutans* and *C. albicans*. In most studies on mouthwashes, CHX is used as the gold standard or positive control for evaluation of the efficacy of other products [20] and is routinely prescribed for use by orthodontic patients [21]. However, CHX has several side effects such as undesirable tooth discoloration, unpleasant taste and causing dryness and burning sensation in the mouth, leading to patient dissatisfaction [22, 23].

Fig 1. Left: Washing of contaminated ligatures; Right: *S. mutans* colonies isolated from ligatures

Recently, use of herbal mouthwashes such as persica and garlic extract has increased by orthodontic patients. Persica was reported to be ineffective [24] and garlic extract enhanced biofilm formation by *S. mutans* on orthodontic wires [25]. *Streptococcus mutans*, which is the major pathogen causing enamel demineralization around orthodontic brackets, has not been fully investigated in terms of its susceptibility to different mouthrinses [17].

Zataria multiflora is an herbal plant belonging to the Laminaceae family. It grows in Iran, Pakistan and Afghanistan and is known as Avishan Shirazi in Iran. The main components of the essential oil of this plant include phenolic compounds such as carvacrol, thymol and eugenol [26]. Using herbal disinfectants is safer than chemical compounds such as CHX. To the best of our knowledge, literature is scarce on the antimicrobial activity of Zataria multiflora extract and its application for disinfection of elastomeric ligatures. However, some previous reports have demonstrated the antimicrobial efficacy of *Z. multiflora* extract against yeasts and Gram-positive and Gram-negative clinical isolates of *S. aureus*, especially methicillin-resistant *Staphylococcus aureus (MRSA)* [26, 27]. Therefore, this study aimed to evaluate and compare the antimicrobial activity of Zataria multiflora extract and CHX against microbial colonization on experimentally conta

minated Iranian and foreign-made elastomeric ligature modules.

However several side effects such as undesirable tooth discoloration, unpleasant taste, dryness and burning sensation in the mouth, are reported which discouraged patients to use this mouthwash [22, 23].

Recently, the use of herbal mouthwashes such as persica and garlic extract by orthodontic patients is increasing.

Persica was reported non-effective [24] and garlic extract showed to increase biofilm formation by *S. mutans* to orthodontic wire [25]. *Streptococcus mutans*, which is the major pathogen for demineralization on the enamel surrounding orthodontic brackets, has not been fully investigated [17].

Zataria multiflora is an herbal plant belonging to the Laminaceae family that geographically grows in Iran, Pakistan and Afghanistan known as Avishan Shirazi (in Iran). The main components of the essential oil of this plant are phenolic compounds such as carvacrol, thymol and eugenol [26].

Using herbal disinfectants is more safe than chemical components such as chlorhexidine. To our best knowledge, there aren't observed any comprehensive and comparative study on comparison of antimicrobial activity of Zataria multifelora essence solution and Chlorhexidine for disinfection of elastomeric ligatures in literature.

However there are reports those *Z. multifelora* essential oils has significant antimicrobial activity against yeast, gram positive, gram negative, clinical isolates of *S. aureus*, especially *MRSA* [26, 27]. Therefore, the main aim in this study was to evaluate and compare the antimicrobial property of Zataria multifelora essence and Chlorhexidine on reduction of microbial colonization on experimentally contaminated Iranian and foreign elastomeric ligature module.

MATERIALS AND METHODS

1. Oral microbial growth and *Zataria multiflora* extract:

Streptococcus mutans (Strain PTCC[1] 1683), *Enterococcus faecalis* (PTCC 1394), and *Candida albicans* (PTCC 5027) were obtained from Iranian Research Organization for Science and Technology (IROST). In order to have fresh microbial cultures, *S. mutans* was seeded on Mitis Salivarius agar (Merck, Germany) containing 1% potassium tellurite, *E. faecalis* was cultured on blood agar (Merck, Germany) and *C. albicans* on Sabouraud dextrose agar (Oxoid, UK) plates. Fresh colonies of Bacterial species and *Candida* were inoculated in brain heart infusion (BHI) broth and incubated for 4–6 hours, to the point when growth was considered to be in the logarithmic phase. The density of the bacterial suspension was adjusted with sterile PBS to that of 0.5 McFarland standard concentration and used for the experiments.

The 0.5mg/ml *Zataria multiflora* extract solution (Barij Essence Pharmaceutical Co, Kashan, Iran) was used as mouthwash for evaluation of its antimicrobial effect on tested microorganisms in comparison with 0.02% CHX (Shar Daroo, Iran).

2. Microbial attachment to orthodontic ligatures:

To evaluate the effect of *Zataria* extract on microbial biofilm formation, we used sterile Iranian conventional orthodontic elastomeric polyurethane ligatures (M.I.B Co, Iran) and foreign-made orthodontic elastomeric ligatures with the same shape and composition (Ortho Technology, USA). These ligatures are manufactured by injection moulding.

Several rapid and easy methods have been developed for detection of bacteria among those, viable microbial cell counting (28) was used in this study. For viable cell counting, 31 Iranian and 31 Ortho Technology ligatures were immersed in *S. mutans*, *E. faecalis* and C. *albicans* suspensions on a reciprocal shaker (100rpm) at 37ºC for 2 hours. Ten rings of each group were randomly selected to determine the adhered viable microbial cells before using any mouthwash and each one was separately transferred to a sterile microtube containing 1 mL of sterile PBS and washed 3 times with PBS and sonicated at 45 KH (Elma, Germany) for 5 minutes.

Finally, 10 µl of sonicated PBS was spread on Mitis Salivarius agar (*S. mutans*), blood agar (*E. faecalis*) and Sabouraud dextrose agar (*C. albicans*) plates. After incubation for 2–3 days, bacterial and fungal colonies were counted on each plate, and the number of colony-forming units (CFUs) was calculated as a percentage of biofilm before performing the disinfection protocol on 21 remaining contaminated rings.

3. Evaluation of antimicrobial activity of *Zataria multiflora* extract:

The broth macro-dilution test evaluated by CLSI (Clinical and Laboratory Standards Institute) was used to compare the antimicrobial activity of *Z. multiflora* and 0.2 CHX as follows:

Three remaining 21 contaminated Iranian and Ortho Technology orthodontic ligatures were divided into 3 sterile Falcon tubes (n=7) containing 2ml of *Zataria multiflora* extract (as the test), 0.2% CHX (as the positive control) and sterile PBS (as the negative control) and incubated on a shaker (100 rpm) for 1 hour. Each ligature was then separately transferred to a sterile microtube containing 1ml of sterile PBS, washed for 3 times and then sonicated as explained earlier.

Finally, 10 µl of washed PBS was spread on specific agar plates as explained earlier. The relative number of CFUs was calculated and the mean number of viable microbial cells remained on 7 rings decontaminated by each solution after performing the disinfection protocol was calculated and analyzed using Wilcoxon, Kruskal-Wallis and the Mann-Whitney tests by SPSS version15 software. $P < 0.05$ was considered statistically significant.

RESULTS

Mean and standard deviation (SD) of viable microorganisms attached to Iranian and Ortho-technology ligatures before and after disinfecting protocol are shown in tables 1 and 2. As are shown in these tables, a higher density attached viable cells for both tested bacteria on Iranian is present in comparison with Ortho-technology ligatures before disinfection (P=0.001), but a statistically significant difference (P=0.061) for attached viable *C. albicans* on both ligatures before entering to disinfection protocol.

Chlorhexidine mouth wash, which used as gold standard in the present study, completely cleaned both ligatures.

Zataria essence solutions also completely cleaned *C. albicans* from both Iranian and a Ortho-technology ligature as there weren't any viable *Candida* cells after decontamination with *Zataria*.

There were seen statistically significant differences between both bacterial viable cell counts on both rings before and after disinfection with *Zataria* essence (P=0.0001). The mean and standard deviation (SD) of viable microorganisms attached to Iranian and Ortho-Technology ligatures before and after applying the disinfection protocol are shown in Tables 1 and 2.

As shown, viable bacterial cells attached to Iranian ligatures had a higher density than those attached to Ortho Technology ligatures before disinfection (P=0.001), but attachment of viable *C. albicans* colony count was not significantly different (P=0.061) before the disinfection protocol.

Chlorhexidine mouthwash, which was used as the gold standard in the current study, completely disinfected both ligatures. *Zataria* extract solution completely eliminated the *C. albicans* colonies from both Iranian and Ortho Technology ligatures as there was no viable *C. albicans* colony after disinfection with *Zataria* extract. Statistically significant differences were seen in the viable bacterial cell counts on both rings before and after disinfection with *Zataria* extract (P=0.0001). However PBS, used as the negative control in the current study, decreased the mean viable microbial count but not significantly (Table 3).

DISCUSSION

Greater emphasis has been placed on oral hygiene measures during orthodontic treatment in the recent years. Immunocompromised patients and those with systemic conditions receiving orthodontic treatment require additional care because of the risk of bacteremia and infection [29].

Results of the current study showed that Iranian elastomeric ligatures are more susceptible for attachments of all tested microorganisms in comparison with Ortho Technology ligatures. The retention of microbial plaque to three different elastic ligatures was determined by Condo *et al.* in an *in viv*o study. They reported no statistically significant difference among the elastic ligatures; while a higher microbial colonization was seen on Iranian polyurethane ligatures than on polyurethane foreign-made ligatures in the current study [30].

Arun *et al.* compared the S. *mutans* and *lactobacilli* counts on stainless steel and elastomeric ligature rings using the same method applied in the current study [31]. They reported significantly higher colonization of both bacteria on elastomeric rings than on stainless steel rings. Also, they reported higher colonization of *S. mutans* than *lactobacilli* on elastomeric ligatures.

Table 1. Mean (SD) of attached microorganisms on Iranian ligature rings before and after disinfection

Microorganisms	**Before**	**After**		
Disinfectants	**-**	**Zataria**	**Chlorhex.**	**PBS**
S. mutans	835.6 (83.9)	48.9 (8.04)	0 (0)	620 (25.8)
E. faecalis	696.3 (28.3)	22.2 (4.7)	0 (0)	502 (21.7)
C. *albicans*	103.9 (15.64)	0 (0)	0 (0)	24.4 (5.9)

Table 2. Mean (SD) of attached microorganisms on Ortho-technology ligature ring before and after disinfection

Microorganisms	**Before**	**After**		
Disinfectants	**-**	**Zataria**	**Chlorhex.**	**PBS**
S. mutans	595.5 (49.3)	10.9 (2.2)	0 (0)	302.5 (32.6)
E. faecalis	447.5 (36.7)	11.9 (2.3)	0 (0)	330 (29.45)
C. *albicans*	87.5 (11.34)	0 (0)	0 (0)	45.25 (4.64)

Table 3. P value for comparison of mean viable microbial cells isolated from Iranian and Ortho-technology ligature rings before and after disinfection

Microorganisms	**Zataria**	**Chlorhex.**	**PBS**
P value (Iranian)			
C. *albicans*	<0.001	<0.001	0.057
E. faecalis	0.001	0.001	0.063
S. mutans	0.001	<0.001	0.061
P value (Foreign)			
C. *albicans*	<0.001	<0.001	0.053
E. faecalis	0.001	<0.001	0.054
S. mutans	0.001	0.001	0.052

(Wilcoxon)

In the current study, a higher colonization of *S. mutans* was seen in comparison with *E. faecalis* and *C. albicans*, similar to Arun's study.

Misra et al. reported a significantly greater number of both aerobic and anaerobic microorganisms on teeth ligated with elastomeric rings than with steel ligatures [32].

Forseberg et al. also reported higher colonization of *S. mutans* on elastomeric rings than on stainless steel ligatures. They also reported a significant increase in the salivary level of *S. mutans* and *lactobacilli* after the insertion of fixed orthodontic appliances with elastomeric ligatures [18]. However, two types of elastomeric ligatures were compared in the current study; our findings showed a higher colonization of *S. mutans*, which is in agreement with the earlier findings by Forseberg et al.

Turkkahraman et al. [33] also reported higher colonization of microorganisms on teeth ligated with elastomeric rings than steel ligatures; however, this difference was not statistically significant. One limitation of the current study may be the underestimation of bacterial colonization due to the use of non-elastic rings in the current in vitro study. Using ligatures around brackets in orthodontic patients may result in greater microbial colonization.

There are several studies evaluating the effect of different mouthwashes and dentifrices containing various antimicrobial agents on salivary *S. mutans* and dental plaque of patients with fixed orthodontic appliances with controversial results [3, 24, 25, 32].

Magnusson et al. showed that dentifrices with antimicrobial agents only resulted in small or no change in *S. mutans* count in the saliva and dental plaque of orthodontic patients [33]. Zataria multiflora extract has Carvacrol, thymol and linalool in its composition, which have antimicrobial activity [26]. This is an herbal, affordable extract and can be easily accepted by orthodontic patients for disinfection purposes [34].

Several reports are available about the antimicrobial activity of *Zataria multiflora* extract against clinical isolates of *S. aureus*, especially MRSA, *E. coli*, and yeasts [26, 27, 35].

This study also showed significant antimicrobial activity of Zataria extract on *C. albicans*, *S. mutans* and *E. faecalis*.

Antibacterial activity of Zataria solution on *S. mutans* in comparison with CHX in the current study was also confirmed by Yaghotti et al. They compared the efficacy of a thymol-based mouthwash (with constituents similar to those of Zataria) with that of CHX against *S. mutans* and *S. sanguinis* [36].

Amanlou et al. compared miconazole gel with *Zataria multiflora* Boiss. Gel for treatment of denture stomatitis and showed a significantly greater reduction in surface erythema of the palate than miconazole gel [23].

Disinfection of elastomeric rings using chemical disinfectants caused a decrease in their tensile strength. Disinfection of elastomeric ligatures in 5% glutaraldehyde solution is recommended, but literature shows that repeated disinfection of elastomeric ligatures can damage these ligatures [37, 38].

Evangelista et al. also reported a decrease in tensile strength of elastomeric ligatures, disinfected with phenol and glutaraldehyde [39].

Comparison of these results to those of other studies was impossible because there are no published studies assessing bacterial contamination of conventional elastomeric rings and efficacy of herbal disinfection.

Our findings suggest that further investigations are required to provide detailed information about the commercially available conventional and modified elastomeric rings and the efficacy of herbal mouthwashes available in the market.

CONCLUSION

1- Colonization of *S. mutans* and *E. faecalis* on Iranian and Ortho Technology ligatures was significantly different.
2- Colonization of *C. albicans* on the two ligatures was not significantly different.

3- *Zataria multiflora* extract showed antimicrobial activity for disinfection of elastomeric ligatures.
4- Maximum antimicrobial activity of *Zataria multiflora* extract was seen against C. albicans.

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