

Comparison of the Antibacterial Effect of Zataria Multiflora Essence with Chlorhexidine Mouthwash against Streptococcus Mutans and Lactobacillus Acidophilus

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Abstract

Introduction and Aim: Dental caries is the most common and chronic disease of the oral cavity worldwide. *Streptococcus mutans* and *Lactobacilli* are the most important etiological agent of dental caries. The aim of the present study was to compare the antimicrobial effect of Zataria multiflora essence with chlorhexidine (CHX) on the growth of *streptococcus mutans* (*S. mutans*) and *lactobacillus acidophilus* (*L. acidophilus*).

Material and Method: In this *in vitro* study, the standard strains of *S. mutans* (PTCC = 1683) and *L. acidophilus* (PTCC: 1643) were obtained as lyophilized ampoules. Zataria multiflora essence was extracted using the Clevenger apparatus. Serial dilutions of 0.2% CHX and Zataria multiflora essence were prepared. The minimum inhibitory concentration (MIC) values of CHX and essence of Zataria multiflora were determined using the modified Epsilometer test. Data were analyzed using the Mann-Whitney test and SPSS version 18. A p-value less than 0.05 was considered statistically significant.

Results: The MIC levels of 0.2%CHX and Zataria multiflora for *S. mutans* and *L. acidophilus* were (0.00125,0.01) and (0.005,0.04) mg/ml, respectively. The antibacterial activity of CHX (0.2%) was more than Zataria multiflora essence. The antibacterial activity of 0.2% CHX and Zataria multiflora against *S. mutans* was more than *L. acidophilus*.

Conclusion: Chlorhexidine is more active and effective against *S. mutans* and *L. acidophilus* strains compared with Zataria multiflora essential oil.

Keywords: Antibacterial, Chlorhexidine, Lactobacillus Acidophilus, Streptococcus Mutans, Zataria Multiflora.

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INTRODUCTION

Dental caries is one of the most common and costly diseases in the world and is considered a major problem for health service providers. [1] *S. mutans* and *Lactobacilli* are the most important etiological agent of dental caries. [1,2] The biofilm and microbial community (dental plaque) on the teeth surface is the first stage of dental caries. The microbial plaques are mainly composed of indigenous microbial flora and cariogenic bacteria. Dental caries is progressed by

specific types of acid-producing bacteria [3]. Several species (approximately 25 species) of oral *streptococci* reside in the oral cavity. The most prevalent caries-associated microorganisms in the oral cavity are *Streptococcus sobrinus* (*S. sobrinus*) and *Streptococcus mutans* (*S. mutans*) [4]. However, other *streptococcal* species, including *Streptococcus sanguinis* and *Streptococcus salivarius* are less harmful and considered as part of the normal human oral microflora [3]. *Lactobacilli* are more prevalent in the advanced areas of carious lesions, and these

bacteria may be associated with dentinal caries [5].

Various antimicrobial agents have been introduced to target oral bacteria. Mouthwashes with active ingredients are the most important antimicrobial agent developed to tackle oral bacteria [6]. There are currently many chemical mouthwashes designed to control dental plaque formation. There are also side effects for chemical mouthwashes including allergic reactions and mucosal irritation [7].

Chlorhexidine with a broad-spectrum antimicrobial activity is one of the most commonly used chemical agents for the elimination of oral pathogenic microorganisms and the prevention of dental plaque formation. In clinical studies, chlorhexidine is considered as the positive control and the gold standard in plaque and biofilm prevention [8]. There are potential side effects associated with the use of chlorhexidine in the oral cavity including tooth discoloration, taste disturbance, burning sensation in the oral mucosa, allergies, dry mouth, and harmful systemic effects following the ingestion of CHX [9].

Chemical drugs in addition to their therapeutic effects may trigger adverse drug reactions and unexpected side effects due to their chemical constituents. Medicinal plants and plant-based herbal products have been developed and manufactured due to the limitation of chemical drugs including drug interactions and side effects, drug resistance, and rising prescription drug costs [2].

The Persian traditional medicines (Iranian traditional medicines) are one of the most popular and ancient forms of herbal medicines. Iranian traditional medicines have a long historical tradition and have been used for the treatment of various diseases such as oral diseases [2].

Zataria multiflora Boiss (Shirazi thyme) is a dicotyledonous plant that belongs to the Lamiaceae family and typically grows in the warm and mountainous areas of Iran. *Zataria multiflora Boiss* is mainly characterized by its medicinal and cosmetic values [10]. This plant is rich in phenolic content and has been used as an anti-infectious agent in Iranian traditional medicine [11].

The essence of *Zataria multiflora Boiss* contains numerous chemical compounds with antibacterial properties including terpene compounds (thymol and carvacrol) [12].

The aim of the present study was to compare the antimicrobial effect of *Zataria multiflora* essence with CHX on the growth of *S. mutans* and *L. acidophilus*.

MATERIALS AND METHODS

Ethical considerations

The present *in vitro* study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Ethics ID: IR. AJUMS.REC. 1398.845).

Extraction of *Zataria multiflora* Boiss essence

At the outset of the study, a weight of 1kg of *Zataria multiflora Boiss* (Shirazi thyme) was obtained from the authentic grocery store in Ahvaz, Southwest of Iran. The quality of Shirazi thyme was confirmed by the AJUMS microbiology laboratory. The *Zataria multiflora* essence was extracted using steam distillation and Clevenger apparatus (Schott DURAN, Germany). In the extraction process, 500 g of thyme was dried and powdered using an electric mill grinder. The plant powder was poured into a two-liter Zhvzhh balloon (volumetric balloon) and 700 ml water was added, accordingly. The balloon was then attached to the Clevenger apparatus and placed on the heater. The mixture of plant material and water were brought to boil and condensed by indirect cooling with water. Finally, the condensed mixture was filtered and the essence was separated from the water. The essence was kept in a dark bottle.

Chlorhexidine 0.2% was prepared from Shahre Daru Pharmaceutical Company (Tehran, Iran).

Culture Media and Strains of Microorganisms

In this *in vitro* study, the standard strains of *S. mutans* (PTCC = 1683) and *L. acidophilus* (PTCC: 1643) were obtained as lyophilized ampoules from the Fungi Culture Collection of Pasteur Institute (Tehran, Iran).

Minimum Inhibitory Concentration Measurement

The minimum inhibitory concentration (MIC) values of CHX and essence of *Zataria multiflora* on plates of solid growth medium were determined using the modified Epsilometer test (E-test). The modified E-test is a simulated version of the standard E-test that determines the susceptibility of an organism [13]. The bacterial suspension was prepared and adjusted to the tube turbidity equivalent to 0.5 McFarland standard (1.5×10^8 CFU/ml). The bacterial suspension was cultured on the Mueller-Hinton medium using the disk diffusion method. Different concentrations of *Zataria multiflora* essence were prepared in absolute ethanol using the serial dilutions method (0.16, 0.08, 0.04, 0.02, 0.01, 0.0005, 0.0025, 0.00125 mg/ml). Serial dilutions of 0.2% chlorhexidine were also prepared using sterile distilled water (0.02, 0.01, 0.005, 0.0025, 0.00125, 0.000625, 0.0003125, 0.00015625 mg/ml).

Sterile blank paper disks (6 mm, Padtan Teb Inc., Tehran, Iran) were impregnated with 10 μ l of various concentrations of the *Zataria multiflora* essence and 0.2% CHX and placed in the oven at 37°C for 2 hours to dry completely. Each disk was then placed on the agar plates and incubated at 37°C for 48h to allow microorganisms to grow on the medium. The discs were placed on the Müller-Hinton medium consecutively and at intervals from higher to lower concentrations. A total of eight dilutions (eight

concentrations) were prepared for each study substance. All experiments were repeated three times.

In this study, tetracycline disk was used as a positive control for *L. acidophilus* and amoxicillin disk was used as a positive control for *S. mutans*. Blank disks (filter paper without antimicrobial material) were used as a negative control for both types of microorganisms. The MIC values were read as the antimicrobial concentrations at the points where dense colonial growth intersected the disks. The test was performed in triplicate for each culture. The MIC values were determined at the particular threshold level where dense colonial growth passes through the disc [14]. An equivalent concentration was prepared for each disc.

Data analysis

The non-parametric Mann-Whitney test was used for the comparison of the studied groups. Data were analyzed using SPSS (SPSS Inc., Chicago, IL, USA) version 18. P-value less than 0.05 (≤ 0.05) was considered statistically significant.

RESULTS

In the present study, the antibacterial activity of chlorhexidine (%0.2) and *Zataria multiflora* essence against strains of *S. mutans* and *L. acidophilus* were evaluated. The MIC values (mg/mL) of chlorhexidine (%0.2) and *Zataria multiflora* extract were measured.

The MIC values of CHX (%0.2) against strains of *S. mutans* and *L. acidophilus* are presented in Table 1. The MIC values of CHX (0.2%) against strains of *S. mutans* and *L. acidophilus* using modified E-test were 0.00125 (mg/mL) and 0.005 (mg/mL), respectively (Table 1). The MIC values of *Zataria multiflora* essence against strains of *S. mutans* and *L. acidophilus* are presented in Table 2. The MIC values of *Zataria multiflora* against strains of *S. mutans* and *L. acidophilus* using modified E-test were 0.01(mg/mL) and 0.04 (mg/mL), respectively (Table 2).

There was a significant difference between the antibacterial activity of chlorhexidine (0.2%) and *Zataria multiflora* essence against strains of *S. mutans* and *L. acidophilus* ($p=0.025$) (Table3). The antibacterial activity of chlorhexidine (0.2%) was more than *Zataria multiflora* essence.

Zataria multiflora essence showed an inhibitory effect on the growth of *S. mutans* (MIC = 0.01) and *L. acidophilus* (MIC = 0.04). The inhibitory effect CHX (0.2%) was more than *Zataria multiflora* essence. The antibacterial activity of CHX (0.2%) and *Zataria multiflora* against *S. mutans* was better than *L. acidophilus* (Figure 1,2).

DISCUSSION

Streptococcus mutans is the main etiological factor of dental caries. Therefore, any approach to dental caries prevention

and management should target the reduction or elimination of *S. mutans* biofilms [15].

The role of *S. mutans* in the initiation of dental caries and *lactobacilli* in caries progression has long been confirmed in previous studies. Therefore, the quantity of these two bacteria in the oral cavity is considered a strong indicator of dental caries [1, 16].

The present *in vitro* study, examined the antibacterial activity of CHX (0.2%) and *Zataria multiflora* against *S. mutans* and *L. acidophilus* bacteria.

The results of the study showed that *Zataria multiflora* essence had inhibitory effects on the growth of *S. mutans* (MIC = 0.01) and *L. acidophilus* (MIC = 0.04). However, the antibacterial activity of chlorhexidine (0.2%) was more than *Zataria multiflora* essence.

The present study also showed that *S. mutans* was more sensitive to chlorhexidine (0.2%) and *Zataria multiflora* than *L. acidophilus*.

Chlorhexidine with a chlorine phenylbiguanide property has a broad-spectrum usage in dentistry including inhibition of smooth surface caries, dentures disinfectant, and reducing the microbial plaque [17].

Chlorhexidine can adhere to the surfaces charged with bacteria and has broad activity against gram-positive and gram-negative bacteria. CHX has the potential to increase the permeability of the cell wall and/or coagulation of the cytoplasm of bacterial cells. The cationic nature of CHX inhibits the growth of gram-positive facultative anaerobic bacterium like *S.mutans*. Additionally, chlorhexidine remains to the tooth surface even after salivary clearance [15].

Nowadays, there has been a growing interest in herbal medicine research and analysis to reduce the side effects of chemical drugs. *Zataria multiflora Boiss* is a valuable herbal medicine from the *Lamiaceae* family which is used traditionally for medicinal purposes [18]. In addition to its medicinal properties, *Zataria multiflora Boiss* is a popular aromatic flavoring spice that is cost-effective and easily accessible [19].

Limited studies investigated the antibacterial activity of *Zataria multiflora* essence or extract against *S. mutans* [20-23].

Aghili *et al.*[7], 2015 studied the antimicrobial activity of *Zataria multiflora* extract and 0.2% CHX mouthwash on experimentally contaminated orthodontic elastomeric ligatures and concluded that *Zataria multiflora* extract has sufficient antimicrobial activity and can be used as a disinfectant solution especially for disinfection of the elastomeric ligatures which was consistent with the results of the present study [7].

Yaghooti Khorasani *et al.* [24], 2012 evaluated the maximum inhibitory concentrations of two common mouthwashes including Thymol-based (ORION Ö) and CHX

against *S. mutans* and *Streptococcus sanguis* and concluded that both mouthwashes had a significant effect against bacterial growth which was in line with the results of the present study .

Jafari Nadoushan *et al.*[6], 2013 in an *in vitro* study examined the antibacterial activity of the *Zataria multiflora* essence and chlorhexidine on orthodontic elastic rings contaminated with *S. mutans* using broth macrodilution method and concluded that *Zataria multiflora* essence can be helpful for disinfection of orthodontic appliances which was in agreement with the results of the present study .

Haghighati *et al.*[25], 2003 evaluated the antimicrobial activity of ten herbal extracts including *Thymus vulgaris* and chlorhexidine on *Actinobacillus actinomycetemcomitans*, *S. mutans*, and *Candida albicans* and concluded that *Thymus vulgaris* had effective antibacterial activity against oral pathogens especially *Candida albicans* which was consistent with the results of the present study [25].

Zomorodian *et al.*[3], 2015 investigated the antimicrobial activity of essential oils of seven Iranian aromatic plants against oral pathogens and concluded that *Satureja khuzestanica*, *Zataria multiflora*, and *Satureja bachtiarica* exhibited the highest antimicrobial activity against oral pathogens which was consistent with the results of the present study [3].

In the previous studies, the antibacterial activity of medicinal plants was evaluated using the broth macrodilution method, disk plate method, and well plate [3, 6, 13, 14, 25]. However, in the present study, the antibacterial activity and the MIC value of the studied agents were measured using the modified E-test.

The present study, also evaluated the effect of *Zataria multiflora* against *L. acidophilus*. The antibacterial activity of *Zataria multiflora* against *L. acidophilus* bacterium has not been examined in the previous studies. Therefore, no similar study concerning the effect of *Zataria multiflora* against *L. acidophilus* was found to be compared with the present study.

Many studies have shown the antibacterial property of carvacrol and thymol against both Gram-positive and Gram-negative bacteria [26, 27]. The antibacterial activity of *Zataria multiflora* is directly associated with the phenolic compound of plant extract including carvacrol, thymol, linalool, and p-cymene [28]. The mechanism of action of phenolic compounds includes cytoplasmic disorganization, control of biological electron flow, and coagulation of the cell content. The mechanism of action of herbal essences against bacteria could be explained due to the inactivation of different molecules of the bacteria and the multidimensional properties of herbal essences which target bacterial colonization. Hence, natural ingredients and medicinal herbs have become popular compared to chemical antimicrobial and synthetic agents [18].

CONCLUSION

There was a significant difference between chlorhexidine antibacterial activity and *Zataria multiflora* essence against *S. mutans* and *L. acidophilus* strains. The antibacterial activity of CHX (0.2%) was more than *Zataria multiflora* essence. The antibacterial activity of CHX (0.2%) and *Zataria multiflora* against *S. mutans* was better than *L. acidophilus*.

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AUTHORS' CONTRIBUTION

Hossein Malekzadeh: Methodology, Data curation, Formal analysis, Writing - original draft. Mansour Amin: Methodology, Data curation, Formal analysis, Writing - original draft. Fatemeh Babadi: Methodology, Data curation, Formal analysis, Writing - original draft. Yasin Karimizadeh: Methodology, Writing - original draft.

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ABBREVIATION

Z. multiflora: *Zataria multiflora*

CHX: Chlorohexidine

MIC: Minimum Inhibitory Concentration

S. mutans: *Streptococcus mutans*

L. acidophilus: *Lactobacillus acidophilus*

E-test: Epsilometer test

AJUMS: Ahvaz Jundishapur University of Medical Sciences

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Table 1: The MIC values of chlorhexidine (%0.2) against strains of S. mutans and L. acidophilus

Bacterium strain	Number of tests	Minimum inhibitory concentration [MIC] of chlorhexidine [0.2 %] mg/ml							
		0.002	0.001	0.0005	0.00025	0.000125	0.0000625	0.00003125	0.000015625
<i>Streptococcus mutans</i> (PTC C 1683)	1	-	-	-	-	+	+	+	+
	2	-	-	-	-	+	+	+	+
	3	-	-	-	-	+	+	+	+
<i>Lactobacillus acidophilus</i> (PTC C 1643)	1	-	-	+	+	+	+	+	+
	2	-	-	+	+	+	+	+	+
	3	-	-	+	+	+	+	+	+

[+]= Bacterium growths detected, [-]=Bacterium growths undetected

Table 2: The MIC values of Zataria multiflora essence against strains of *S. mutans* and *L. acidophilus*

Bacterium strain	Number of tests	Minimum inhibitory concentration [MIC] of <i>Zataria multiflora</i> essence [0.2 %] mg/ml							
		0.16	0.08	0.04	0.02	0.01	0.005	0.0025	0.00125
		Disk 1	Disk 2	Disk 3	Disk 4	Disk 5	Disk 6	Disk 7	Disk 8
<i>Streptococcus mutans</i> (PTCC 1683)	1	-	-	-	-	+	+	+	+
	2	-	-	-	-	+	+	+	+
	3	-	-	-	-	+	+	+	+
<i>Lactobacillus acidophilus</i> (PTCC 1643)	1	-	-	+	+	+	+	+	+
	2	-	-	+	+	+	+	+	+
	3	-	-	+	+	+	+	+	+

[+]= Bacterium growths detected, [-]=Bacterium growths undetected

Table 3: Comparison of the MIC values of CHX and Zataria multiflora essence against strains of *S. mutans* and *L. acidophilus*

Bacterium	MIC of chlorhexidine (0.2 %) mg/ml	MIC of <i>Zataria multiflora</i> mg/ml	Test statistics*	p-value
<i>Streptococcus mutans</i>	0.00125	0.01	-2.236	0.025
<i>Lactobacillus acidophilus</i>	0.005	0.04	-2.236	0.025
Test statistics*	-2.236	-2.236	-	-
p-value	0.025	0.025	-	-

*Mann-W