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ORIGINAL

# Study the antimicrobial effects of *Zataria multiflora*-based mouthwash on the microbial community of dental plaques isolated from children: A candidate of novel plant-based mouthwash

*Estudio de los efectos antimicrobianos del enjuague bucal a base de Zataria multiflora sobre la comunidad microbiana de placas dentales aisladas de niños: Un candidato a enjuague bucal novedoso a base de plantas*

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## Abstract

***Background:*** *Zataria multiflora* is a medicinal plant with high antimicrobial effects. It is mainly used as an oral specie in food. The present survey was aimed to assess the antimicrobial effects of *Z. multiflora*-based mouthwash on the microbial community of dental plaques.

***Methods:*** Two-hundred dental plaque samples were collected from individuals. Culture technique was used to assess their microbial contamination. *Z. multiflora* was collected and use in a base of mouthwash in 1% concentration. Antimicrobial effects of *Z. multiflora*-based mouthwash was examined against isolated bacteria and compared to antibiotic agents using the disk diffusion. Minimum inhibitory concentration of *Z. multiflora*-based mouthwash was also studied against isolated bacteria.

***Results:*** *Streptococcus mutans* (19%), *Enterobacter cloacea* (17.50%), and *Staphylococcus aureus* (15%) were the most commonly identified bacteria amongst the dental plaque samples. *H. pylori* (8%) had the lowest prevalence. The mean ranges of the diameter of the growth inhibition zones were 5.71±0.92 (*S. aureus* against tetracycline) to 15.68±0.55 (*S. mutans* against azithromycin) mm. *Z. multiflora* mouthwash (1%) harbored the highest antimicrobial effects against *S. mujtans* (15.33±0.81 mm), and *S. aureus* (12.01±1.10 mm), while showed the lowest against *E. coli* (8.38±0.46 mm) and *E. cloacea* (10.52±0.84 mm). The lowest MIC levels were obtained for *S. mutans* (2 mg/ml). The highest MIC level was found for *E. cloacea* (8 mg/ml). The MIC levels of *Z. multiflora* mouthwash against *E. coli* and *H. pylori* bacteria were higher than examined concentrations.

***Conclusion:*** *Z. multiflora*-based mouthwash may be a useful herbal-based mouthwash against bacteria in dental plaque samples.

*Key words:* *Zataria multiflora*, mouthwash, antimicrobial effects, dental plaques.

## *Resumen*

***Antecedentes:*** *Zataria multiflora* es una planta medicinal con altos efectos antimicrobianos. Se utiliza principalmente como especie oral en la alimentación. El presente estudio tenía como objetivo evaluar los efectos antimicrobianos de un enjuague bucal a base de *Z. multiflora* sobre la comunidad microbiana de las placas dentales.

***Métodos:*** Se recogieron doscientas muestras de placa dental de individuos. Se utilizó una técnica de cultivo para evaluar su contaminación microbiana. Se recogió *Z. multiflora* y se utilizó en una base de enjuague bucal en una concentración del 1%. Se examinaron los efectos antimicrobianos del enjuague bucal a base de *Z. multiflora* contra las bacterias aisladas y se compararon con los agentes antibióticos mediante la difusión en disco. También se estudió la concentración mínima inhibitoria del enjuague bucal a base de *Z. multiflora* frente a bacterias aisladas.

***Resultados:*** *Streptococcus mutans* (19%), *Enterobacter cloacea* (17,50%) y *Staphylococcus aureus* (15%) fueron las bacterias más comúnmente identificadas entre las muestras de placa dental. *H. pylori* (8%) tuvo la menor prevalencia. Los rangos medios del diámetro de las zonas de inhibición del crecimiento fueron de 5,71±0,92 (*S. aureus* frente a la tetraciclina) a 15,68±0,55 (*S. mutans* frente a la azitromicina) mm. El enjuague bucal de *Z. multiflora* (1%) albergó los mayores efectos antimicrobianos contra *S. mujtans* (15,33±0,81 mm), y *S. aureus* (12,01±1,10 mm), mientras que mostró los más bajos contra *E. coli* (8,38±0,46 mm) y *E. cloacea* (10,52±0,84 mm). Los niveles de MIC más bajos se obtuvieron para *S. mutans* (2 mg/ml). El nivel de CIM más alto se encontró para *E. cloacea* (8 mg/ml). Los niveles de CIM de *Z. multiflora* contra las bacterias *E. coli* y *H. pylori* fueron superiores a las concentraciones examinadas.

***Conclusión:*** El enjuague bucal a base de *Z. multiflora* puede ser un útil colutorio a base de plantas contra las bacterias presentes en las muestras de placa dental.

*Palabras clave:* *Zataria multiflora*, enjuague bucal, efectos antimicrobianos, placas dentales.

## Introduction

Dental plaque is the name given to the aggregations of bacteria and their products which accumulate on the tooth surface[1]. When plaque accumulates on the crowns of teeth the natural, smooth, shiny appearance of the enamel is lost and a dull, matt effect is produced[2]. As it builds up, masses of plaque become more readily visible to the naked eye[3]. Additionally, dental plaque bacteria can be a dangerous sources of infections for other parts of the body, such as gastrointestinal tract, head, neck, and nasopharynx[4].

In direct smears, the early plaque is dominated by cocci and rods, most of which are Gram-positive. In the mature plaque (after about 7 days) the percentage of cocci in the plaque decreases rapidly and filaments and rods constitute about 50% of organisms in plaque[5]. Studies revealed that *Streptococcus mutans*, *Enterobacter cloacea, Staphylococcus aureus, Klebsiella pneumonia, Helicobacter pylori,* and *Escherichia coli*, are the most important and frequent bacterial species isolated from the oral cavity and dental plaque samples, globally[6,7].

Several antimicrobial choices are available for the infections of the oral cavity. However, bacterial isolates of the dental plaque samples harbored severe resistance toward commonly used antimicrobial agents, particularly aminogylocides, tetracyclines, penicillins, cephalosporins, and quinolones[8]. Thus, studying the profile and pattern of antibiotic resistance amongst bacterial isolates of dental plaques as novel reservoirs of bacteria seems essential.

*Zataria multiflora* (*Z. multiflora*) is a 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[9]. This plant is known as potential antimicrobial agents and mainly used as a spice in different foodstuffs[10]. Edible nature of this plant make it possible to use it in several types of oral drugs, particularly herbal mouthwash[11].

This study was aimed to assess the antimicrobial effects of *Z. multiflora*-based mouthwash on the microbial community of dental plaques as a novel candidate of plant-based mouthwash in vitro condition.

## Materials and methods

### Samples and inclusion criteria

A total of 200 children referred to the dentistry clinics for routine check-ups were assessed in this survey. All children with dental plaque samples were included in this survey. Dental plaque presence is the prominent inclusion factor. All children who had received antimicrobial options or antibacterial mouthwashes three months before the experiment were excluded from the research. Dental plaque sample was taken from the gingival crevice at the most profound pocket reading and removed from the clinical site using a sterile universal curette. The curette tip was inserted into the depths of the crevice/pocket, moved coronally while in contact with the tooth surface to remove both sub and supragingival plaque.

### Preparation of plant materials

*Z. multiflora* was purchased from traditional groceries. A total of 3500 grams of *Z. multiflora* was used to create an ethanol extract. It was cleaned, dried at room temperature for 24 hours, and processed in a blender until its texture became smooth. To obtain the extract from the processed *Z. multiflora*, 2000 ml of a 96% ethanol solution was used, and this process was repeated thrice. The initial extract was filtered and evaporated at temperatures ranging from 50 to 60 °C to obtain a 100% pure *Z. multiflora* extract. The pure extract was weighed, stored in a sealed glass container, and subsequently placed in a desiccator before being used as mouthwash.

### Mouthwash formulation

As much as 100 ml of mouthwash was produced for each formulation with *Z. multiflora* extract as the active substance. The formulations of *Z. multiflora* mouthwash according to **table I**. Propylene glycol was included in the *Z. multiflora* extract and placed in a glass beaker. It was then raised to 60 °C, stirred with a magnetic stirrer at 300 rpm and Tween 80, and sorbitol and aquadest were added. Benzoic acid and sodium benzoate were dissolved in aquadest and added to the solution and stirred with a magnetic stirrer until homogeneous. Subsequently, 100 ml of the sorbitol and aquadest ad was stirred until the solution became clear, and Oleum menthae piperitae was added.

**Table I:** Formulation of Z. multiflora mouthwash.

| Components | Frequency (%) |
|---|---|
| *Z. multiflora* ethanolic extract | 1 |
| Propylene glycol | 25 |
| Tween 80 | 5 |
| Oleum menthe piperitae | 0.25 |
| Benzoate acid | 0.1 |
| Sodium benzoate | 1 |
| Sorbitol 70% | 15 |
| Aquadest | 100 |

### Quality assessment of Z. multiflora mouthwash

Organoleptic, acidity, stability, weight mass, viscosity, irritation, and contact time of *Z. multiflora* mouthwash were assessed using the method described previously[12].

### Isolation of bacteria from the dental plaque samples

The dental plaque sample from each child was cultured into a sterile tube containing 5% sheep blood agar, chocolate agar and a selective medium and transported to the microbiology laboratory. All media were incubated at 37°C and 42 °C for 24 to 48 h. after Gram staining and

microscopy, different biochemical tests were performed to identify bacterial strains. The basic biochemical tests used to identify bacterial strains includes Starch Test, Simon Citrate, Oxidase, Catalase, Voges Proskauer, Urease, Indole, Methyl Red and Coagulase Test. Analytical Profile Index (API 20E) (BioMeriouxVitek, Inc., MO, USA) system was used to identify bacteria[13].

### Antibacterial effects of mouthwash against isolated bacteria

The simple disk diffusion method was used to assess the antimicrobial effects of synthesized mouthwash. For this purpose, isolated bacteria were cultured on Muller Hinton agar media. A total of 1000 µl of 1% *Z. multiflora* mouthwash were poured into the blank disk and located at the surface of each media. For comparison, penicillin (10 µg/disk), tetracycline (30 µg/disk), azithromycin (15 µg/disk), gentamicin (10 µg/disk), and ampicillin (10 µg/disk) (Oxoid, UK) antibiotic disks were accompanies. All guidelines were performed according to the Clinical and laboratory standard institute (CLSI)[14-16]. The Minimum Inhibitory Concentration (MIC) of synthetic *Z. multiflora* mouthwash was also assessed. For this purpose, 1, 2, 4, and 8 mg/ml concentration of mouthwash were prepared and the MIC value was determined using the previously described method[17].

### Statistical analysis

Collected data were transferred to the Microsoft Office Excel software and arranged well. Then, they were statistically analyzed using the SPSS software and chi-square and analysis of variance tests (ANOVA). $P<0.05$ was consider z significant level[18-22].

## Results

**Table II** shows the distribution of bacteria strains isolated from dental plaque samples. As shown, *S. mutans* (19%), *E. cloacea* (17.50%), and *S. aureus* (15%) were the most commonly identified bacteria amongst the dental plaque samples. *H. pylori* (8%) had the lowest prevalence amongst the examined dental plaque samples. Statistically significant difference was obtained between the distribution of different bacteria ($P<0.05$).

**Table III** shows the dimeter of the growth inhibition zone of bacteria against synthetic mouthwash compare to antimicrobial agents. The mean ranges of the diameter of the growth inhibition zones were 5.71±0.92 (*S. aureus* against tetracycline) to 15.68±0.55 (S. mutans against azithromycin) mm. *Z. multiflora* mouthwash (1%) harbored the highest antimicrobial effects against *S. mujtans* (15.33±0.81 mm), and *S. aureus* (12.01±1.10 mm), while showed the lowest against *E. coli* (8.38±0.46 mm) and *E. cloacea* (10.52±0.84 mm). Statistically significant differences were obtained between the diameter of the growth inhibition zone of bacteria treated with different antimicrobial agents ($P<0.05$).

**Table IV** shows the MIC values of *Z. multiflora* mouthwash against different isolated bacteria. Findings showed that the lowest MIC levels were obtained for *S. mutans* (2 mg/ml). The highest MIC level was found for *E. cloacea* (8 mg/ml). The MIC levels of *Z. multiflora* mouthwash against *E. coli* and *H. pylori* bacteria were higher than examined concentrations (non detected).

**Table II:** Distribution of bacteria strains isolated from dental plaque samples.

| Samples | N. collected | Distribution of bacteria (%) | | | | |
|---|---|---|---|---|---|---|
| | | *S. aureus* | *S. mutans* | *E. cloacea* | *E. coli* | *H. pylori* |
| Dental plaques | 200 | 30 (15) | 38 (19) | 35 (17.50) | 20 (10) | 16 (8) |

**Table III:** Dimeter of the growth inhibition zone of bacteria against synthetic mouthwash compare to antimicrobial agents.

| Tested antimicrobial agents | Diameter of the growth inhibition zone of bacteria (mm) | | | | |
|---|---|---|---|---|---|
| | *S. aureus* | *S. mutans* | *E. cloacea* | *E. coli* | *H. pylori* |
| *Z. multiflora* mouthwash (1%) | 12.01±1.10 [a*] | 15.33±0.81 [a] | 10.52±0.84 [a] | 8.38±0.46 [b] | 10.57±0.61 [a] |
| Penicillin | 8.63±0.34 [b] | 9.66±0.23 [c] | 10.14±0.22 [a] | 8.15±0.51 [b] | 10.33±0.35 [a] |
| Tetracycline | 5.71±0.92 [c] | 6.61±0.28 [d] | 8.17±0.37 [b] | 8.81±0.36 [b] | 8.66±0.25 [a] |
| Azithromycin | 13.24±0.95 [a] | 15.68±0.55 [a] | 11.43±0.52 [a] | 10.93±0.44 [a] | 9.90±0.60 [a] |
| Gentamicin | 6.03±0.32 [c] | 11.82±0.39 [b] | 11.97±0.93 [a] | 8.55±0.81 [b] | 9.93±0.65 [a] |
| Ampicillin | 7.15±0.41 [c] | 10.17±0.16 [c] | 10.08±0.09 [a] | 9.72±0.74 ab | 9.71±0.69 [a] |

*Dissimilar small letters in each column show significant statistical differences ($P<0.05$).

**Table IV:** MIC values of *Z. multiflora* mouthwash against different isolated bacteria.

| Treatment | MIC (mg/ml) | | | | |
|---|---|---|---|---|---|
| | *S. aureus* | *S. mutans* | *E. cloacea* | *E. coli* | *H. pylori* |
| *Z. multiflora* mouthwash | 4 | 2 | 8 | ND* | ND |

*Non detected.

## Discussion

Infections may cause several life-threating diseases globally[23-38]. In this regard, medical plants and traditional medicine act as healing sciences[39]. In this study, *Z. multiflora*-based mouthwash was used as an antimicrobial agent on bacteria isolated from dental plaque samples. Total distribution of *S. aureus, S. mutans, E. cloacea, E. coli,* and *H. pylori* amongst the examined dental plaque samples was 15%, 19%, 17.50%, 10%, and 8%, respectively.

An Indian survey[40] revealed that Streptococcus spp. was the most commonly detected bacteria (51.00%), followed by E. coli (19.00%) and Veillonella spp. (19.00%). A research on United Kingdome[41] showed that *Tannerella forsythensis* (65.00%), *Porphyromonas gingivalis* (49.00%), and *Actinobacillus actinomycetemcomitans* (55.00%) were the most commonly detected pathogens isolated from dental plaque samples. Similarly in Korea[42], *Streptococcus, Corynebacterium, Neisseria*, and *Fusobacterium* were the most commonly detected bacterial strains in dental plaque samples. Brazilian survey[43] showed that the predominant species in all 600 samples included *Corynebacterium diphtheriae, Enterococcus faecalis, S. aureus, Acinetobacter baumannii, P. aeruginosa*, and *E. coli* were predominant bacterial pathogens in the dental plaque samples of children with chronic periodontitis.

Findings of the current research revealed that *Z. multiflora*-based mouthwash had the highest antimicrobial effects on *S. mutans* and *S. aureus*. The main reason for the lower antimicrobial effects of the mouthwash against other bacteria is that they were Gram-negative and have Lipopolysaccharide (LPS) in their cell walls which inhibit the penetration of essential oils and herbal extracts. Aghili et al. (2015)[44] reported that the *Z. multiflora* extract had the higher antimicrobial effects on experimentally contaminated orthodontic elastomeric ligatures compared to chlorhexidine mouthwash. Milho et al. (2021)[45] stated that the essential oils of Cymbopogon citratus (DC.) Stapf and Lippia alba (Mill.) seem to be the most promising in fighting microbial biofilm in *S. mutans*, given their high capacity to reduce biofilm at low concentrations. Significant effects of herbal mouthwash against pathogenic bacteria recovered from the dental plaques have similarly been reported by Tusi et al. (2020) (Iran)[46] and Pedrazzi et al. (2015) (Brazil)[47].

## Conclusion

According to the high antimicrobial effects of the *Z. multiflora*-based mouthwash compared to antimicrobial agents even in low concentrations, its application as a novel and herbal-based mouthwash particularly against Gram-positive bacteria responsible for dental plaque formation has been recommended. However, some additional surveys should perform to assess other effects of *Z. multiflora*-based mouthwash on the oral cavity.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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