Dental Research Journal

Original Article

Antibacterial effects of aqueous and alcoholic extracts of *Zataria multiflora* in comparison with chlorhexidine mouthwash on some pathogenic oral streptococci: An *in vitro* study

Parnian Baradaran Noveiri[1], Rayehehossadat Rezvaninejad[2], Ali Azarm[3], Raziyehsadat Rezvaninejad[4]

[1]Student Research Committee, Faculty of Dentistry, Hormozgan University of Medical Sciences, Bandar Abbas, [3]Student Research Committee, Rafsanjan University of Medical Sciences, Rafsanjan, [4]Assistant Professor, Department of Oral Medicine, School of Dentistry, Kerman University of Medical Sciences, Kerman, Iran, [2]MSc Student, Faculty of Dentistry, McGill University, Montreal, QC, Canada

ABSTRACT

Background: Increasing antibiotic resistance to pathogenic microorganisms (Streptococci) has led scientists around the world to turn to medicinal plants. In this study, the effects of aqueous and alcoholic extracts of *Zataria multiflora* on the *in vitro* growth of *Streptococcus mutans* and *Streptococcus sanguis* have been considered and compared with 0.2% chlorhexidine mouthwash.

Materials and Methods: In this *in vitro* study, the inhibitory growth zone was accessed by the disc diffusion method after 48 h of incubation at 37 C. To find out the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of treatments, colony counts of cultured bacteria on nutrient agar have been considered at serial dilution at 1/2-1/1024 dilution rates. An independent *t*-test was used to compare the antibacterial effects of extracts while the level of significance of was considered to be 5% ($P < 0.05$).

Results: The inhibitory growth zones of aqueous and alcoholic extracts on *S. mutans* were 26.8 mm and 35.8 mm, respectively, whereas growth zones for *S. sanguis* were considered as 25.8 mm and 33.2 mm, sequentially. Comparisons showed better effects of alcohol compared to aqueous extract ($P > 0.05$). The MIC and MBC assessments showed the same results ($P > 0.05$). In all comparisons, the effects of 0.2% chlorhexidine mouthwash were significantly better than both *Z. multiflora* aqueous and alcoholic extracts ($P > 0.05$).

Conclusion: The different solvents may have contributed to the better effects of an alcoholic to aqueous extract of *Z. multiflora* on the growth of both bacteria. These two extracts could be used for early inhibition of the growth of the planktonic phase, as well as for better oral taste after chlorhexidine applications.

Key Words: Anti-bacterial agents, multifloral, plant extracts, *Streptococcus mutans*, *Streptococcus sanguis*

Received: 24-Jul-2022
Revised: 12-Sep-2022
Accepted: 18-Mar-2023
Published: 26-Apr-2023

Address for correspondence:
Dr. Raziyehsadat Rezvaninejad, Assistant Professor, Department of Oral Medicine, School of Dentistry, Kerman University of Medical Sciences, Kerman, Iran.
E-mail: rezvaninezhad@gmail.com

INTRODUCTION

Dental plaque is a diverse population of isolated bacteria in a matrix with a salivary origin that plays an important role in dental caries and gingivitis.[1] Extracted acid from carbohydrate metabolism causes

Access this article online

Website: www.drj.ir
www.drjjournal.net
www.ncbi.nlm.nih.gov/pmc/journals/1480

How to cite this article: Baradaran Noveiri P, Rezvaninejad R, Azarm A, Rezvaninejad R. Antibacterial effects of aqueous and alcoholic extracts of *Zataria multiflora* in comparison with chlorhexidine mouthwash on some pathogenic oral streptococci: An *in vitro* study. Dent Res J 2023;20:48.

reduced oral pH and will lead to demineralization of hydroxyapatite and dental caries.[2] *Streptococcus mutans* and lactobacilli are the two most important factors in tooth decay. *S. mutans* is involved at the beginning of all dental caries.[3] More than half of the bacteria found in gingivitis due to dental plaque are Gram-positive pathogens. Among them, *S. mutans*, *S. sanguis*, *S. mitis*, *S. oralis*, and *S. intermedius* are more commonly found,[4] which induce dental caries, with possible following cardiovascular complications.[5]

The use of chemical complementary methods such as oral rinses combined with mechanical tooth cleaning can be effectively used in the control of supragingival dental plaque, gum problems, and improvement of oral ulcers.[4,6,7] Therefore, the use of mouthwash because of its anti-inflammatory and antiplaque properties and the prevention of the formation or spread of microbial plaque is highly recommended.[8,9] Desirable oral rinses should have broad-spectrum antimicrobial effects, low pharmaceutic resistance, and have no effect on oral normal flora.[4] Chlorhexidine is a broad-spectrum antiseptic and mouthrinse that has been approved by the Food and Drug Administration (FDA) and American Dental Association (ADA).[10,11] Due to its cationic properties, it attaches to the cell walls of bacteria and destroys them. It can have various side effects such as discoloration of teeth, taste change, burning of the oral mucosa, dry mouth, and if swallowed negative systemic effects.[11-13]

In recent years, the increasing resistance of pathogenic microorganisms to various antibiotics and the high cost of obtaining new drugs has attracted the attention of many researchers around the world to the use of medicinal plants.[14,15] These include plants such as *Aloe vera*,[16] *Glycyrrhiza glabra*,[17] *Matricaria chamomilla*,[18] *Mellisa officinalis*,[5] and *Satureja khuzestania*.[1] *Zataria multiflora Boiss* is one of the *Lamiaceae* family species which has been considered the most important medicinal plant in Iran after *Foeniculum vulgare*.[19] It has attracted the attention of dentists.[1,20] Its essence contains a high concentration (64%–70%) of the two substances thymol and carvacrol.[1,21] The strong anti-bacterial properties of this plant are due to these two phenolic isomers. The effect of *Z. multiflora* essence on *S. mutans*, *S. sanguinis*[22] and the effect of methanolic extract of this plant on *S. mutans*[23,24] and also the effects of its alkaline extracts on *Staphylococcus aureus* and *Staphylococcus epidermidis*[25,26] have been studied previously.

According to Jafari *et al*., the *in vitro* antibacterial effect of commercially available *Z. multiflora* extract (Barig Essence Company) against *S. mutans* colonized on orthodontic elastic rings was compared with chlorhexidine.[20] In another *in vitro* study, the previous commercial extract was compared with sodium hypochlorite, hydrated calcium hydroxide, and normal saline as a canal irrigating solution against some streptococci in 1, 5, and 15 min.[22] Based on our search, there are limited studies on other *Z. multiflora* extracts such as the determination of minimal inhibitory and minimal bactericidal concentrations for other aqueous and alcoholic extracts with different time range on other oral streptococci.

Hence, in this *in vitro* study, the antibacterial effects of aqueous and alcoholic extracts of *Z. multiflora* on *S. mutans* and *Streptococcus sanguis* compared with chlorhexidine mouthwash are reported.

MATERIALS AND METHODS

This is an *in vitro* research aiming assessment of the antibacterial effects of aqueous and alcoholic extracts of *Z. multiflora* on *S. mutans* and *S. sanguis* compared with chlorhexidine mouthwash which was ethically approved by Hormozgan University of Medical Sciences (Approval ID: IR.HUMS.REC.1400.021).

Materials and bacteria strains

S. mutans PTCC 1683 and *S. sanguis* PTCC 1449 were purchased as lyophilized from the local center of the Iranian Research Organization for Science and Technology. Chlorhexidine was also prepared in a 0.2% (2 mg/ml) solution purchased from Nazho Pharmaceutical Company (Tehran, Iran).

Extracts preparation

Alcoholic extract preparation

Z. multiflora alcoholic extract was purchased from Soha-Jissa company (Salmanshahr, Mazandaran, Iran) with a primary concentration of 20 mg/ml (Batch No. IEE059.01).

Aqueous extract preparation

To prepare the aqueous extract of *Z. multiflora*, 30 g of the dried leaves of the plant were soaked, cleaned, and then pulverized by a mill.[27] The dried powder of the leaves was poured into 300 ml of sterile deionized distilled water (ratio 1:10) and stirred for 24 h in a dark chamber at room temperature on a shaker at 100 rpm. The extract was then filtered with Whatman No. 1 filter paper (made in England). The filtered

extract was placed in an oven at 40°C for 24 h until complete evaporation of the solvent. The dried extract was collected from the glass surface and stored in a sterile, dark glass container at 4°C for later use.[28]

Disk diffusion assay

The prepared bacteria were cultured on blood agar for 24 h at 37°C to achieve the most suitable cultivation.[29] Then samples were taken from the cultured bacteria with a sterile swab, added to 10 ml of physiological saline, and mixed well to obtain a uniform bacterial suspension. This bacterial suspension was set into the standard density of 0.5 McFarland turbidity with a spectrophotometer (2100 Unico, China) at a wavelength of 625 nm resulting in (1.5×10^8 CFU/ml) bacterial concertation.[30,31] Then, comparison of growth inhibition zone with disk diffusion method was performed in triplicate by measuring the diameter of inhibition zone in primary concentrations of extracts with an antibiogram ruler after 48 h of incubation in the incubator at 37°C and comparison with Chlorhexidine 0.2% (as the positive control).[32]

Minimal inhibitory concentration and minimal bactericidal concentration

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by the serial dilution method in triplicate. At this stage, 200 µL of sterile physiological serum was added to 10 sterile microtubes, then 200 microliters of extracts were added to the first microtube. After mixing the extract with physiological serum, 200 µl of this microtube was removed and added to microtube number 2. This operation was continued until the last one when 200 µl of solution was discarded from it.[33] Therefore, a serial dilution was created from microtube one to microtube ten. Then, 200 µl of the bacterial suspension previously prepared at a turbidity of 0.5 McFarland was added into each microtube. 100 µl was taken from each sample and used for surface culture on Mueller–Hinton agar in a 37°C incubator for 24 h.[34] Chlorhexidine 0.2% was used as the positive control and other dilutions were used as the tests.

The lowest concentrations of the extract with visible colonies and no visible colonies on culture media were defined as MIC and MBC, respectively. The number of colonies was also counted for each sample, and its means were reported. We also note that these tests were performed in triplicate[35].

Statistical analysis

A one-way analysis of variance (ANOVA) and comparison of the data with Duncan's multiple domain test were performed using SPSS software (version 26.0; SPSS Inc., Chicago, IL, USA). An independent *t*-test was used to compare the antibacterial effects of each extract on two bacteria. The level of significance of the results in all cases was considered to be 5% ($P < 0.05$).

RESULTS

Primary PH determination

Results of primary PH determination of Chlorhexidine, alcoholic extract and aqueous extract are shown in Table 1.

Results of disk diffusion assay

After performing the disk diffusion test and ensuring that it has antibacterial properties and comparing the diameter of the growth inhibition zone, it was found that the bacterial growth inhibitory properties for chlorhexidine mouthwash are higher than alcoholic extract and for alcoholic extract are higher than aqueous extract as shown in Table 2.

Minimal inhibitory concentration and minimal bactericidal concentration

The results of the bacterial colony count of *S. mutans* and *S. sanguis* culture on Mueller–Hinton agar culture after the effect of compounds are shown in Table 3.

No identical Latin lowercase letters in each column indicate a significant difference in each sample and non-identical Latin uppercase letters indicate significant differences in each row ($P < 0.05$).

As shown in Table 3, MIC values for the effect of aqueous extract, alcoholic extract, and chlorhexidine

Table 1: Measured pH of aqueous and alcoholic extracts of *Zataria multiflora* and chlorhexidine mouthwash

Solution	Chlorhexidine	Alcoholic extract	Aqueous extract
pH	5.28	5.03	5.83

Table 2: Measured inhibition zone of aqueous and alcoholic extracts of *Zataria multiflora* by disk diffusion method shown in millimeters

Solution Bacteria	Aqueous extract (100 mg/mL)	Alcoholic extract (20 mg/mL)	Chlorhexidine
S. mutans	26.8±1.64	35.8±1.92	52.6±1.94
S. sanguis	25.8±2.05	33.2±2.58	50.8±0.84

S. mutans: *Streptococcus mutans*; *S. sanguis*: *Streptococcus sanguinis*

Table 3: Comparison of growth of two different bacteria in Mueller–Hinton agar at different dilutions of aqueous, alcoholic, and chlorhexidine extracts (×10^8 CFU/mL) (mean±standard error) (*n*=3)

Dilutions	Chlorhexidine		Alcoholic extract		Aqueous extract	
	S. sanguis	*S. mutans*	*S. sanguis*	*S. mutans*	*S. sanguis*	*S. mutans*
1/2	0^A	0^A	0^A	0^A	20±3^A	0B
1/4	0^A	0^A	0^A	0^A	36,800±1505^A	11,666±2886^B
1/8	0^A	0^A	0^A	0^A	70,400±1457^A	73,600±2500^A
1/16	0^A	0^A	0^A	0^A	124,217±7229^A	92,526±5851^B
1/32	0^A	0^A	0^A	0^A	>10$^{5\,A}$	136,300±7738^B
1/64	0^A	0^A	0^A	0^A	>10$^{5\,A}$	>10$^{5\,A}$
1/128	0^A	0^A	8050±162^A	4330±44^B	>10$^{5\,A}$	>10$^{5\,A}$
1/256	0^A	0^A	33,880±987^A	5240±50^B	>10$^{5\,A}$	>10$^{5\,A}$
1/512	0^B	20±5^A	74,263±1643^A	24,433±1353^B	>10$^{5\,A}$	>10$^{5\,A}$
1/1024	0^B	14,183±310^A	>10$^{5\,A}$	27,380±2085^B	>10$^{5\,A}$	>10$^{5\,A}$

No identical Latin lowercase letters in each column indicate a significant difference in each sample and nonidentical Latin uppercase letters indicate significant differences in each row (P<0.05). *S. mutans*: S*treptococcus mutans*; *S. sanguis*: *Streptococcus sanguinis*

on *S. mutans* were obtained in 1/4, 1/128, and 1/512 dilutions, also MBC values of these samples were evaluated as 1/2, 1/64, and 1/256.

On the other hand, the MIC values for the effects of aqueous extract and alcoholic extract on *S. sanguis* used in this study were obtained in dilutions of 1/2 and 1/128. This index for chlorhexidine should be seen at concentrations lower than 1/1024, which was not within the range of the samples in this study. Furthermore, the values set for MBC of these samples were evaluated as 1, 1/64, and 1/1024.

The colony count results showed that the difference in the number of colonies grown in all concentrations of the aqueous extract in *S. sanguis* was significantly higher than in *S. mutans* ($P < 0.05$).

About alcoholic extract, it was also found that the ability of this extract to induce growth inhibition against *S. sanguis* in all dilutions <1/64 is significantly lower than *S. mutans* ($P < 0.05$) [Table 3].

Comparison of the means of bacterial colony counts counted under different dilutions of chlorhexidine mouthwash showed that this substance had the ability to kill *S. sanguis* in all dilutions, but this effect against *S. mutans* was observed up to a dilution of 1/256 ($P > 0.05$) and lesser dilutions were deficient. Furthermore, in both dilutions of 1/512 and 1/1024, the ability of this substance to kill *S. sanguis* was significantly higher than that of *S. mutans* ($P < 0.05$).

DISCUSSION

In the present study, the effects of different concentrations of aqueous and alcoholic extracts of *Z. multiflora* leaves on the growth of *S. mutans* and *S. sanguis* were studied, and the culture results were compared with those of 0.2% chlorhexidine mouthwash. Examination of this effect by disc diffusion showed that all three compounds have the ability to limit the growth of both bacteria. The statistical comparison showed that the effect of the alcoholic extract of *Z. multiflora* on *S. mutans* and *S. sanguis* is significantly more effective in inhibiting the growth of both bacteria compared to the aqueous extract of this plant. But what is important is the higher ability of chlorhexidine to inhibit the growth of both bacteria compared to aqueous and alcoholic extracts. The findings of this method indicate that for all three compounds used, there is no significant difference in their effects on two different bacteria. That is, chlorhexidine, alcoholic extract, and aqueous extract have shown the same effect in inhibiting the growth of both bacteria with this method. MIC is the gold standard for measuring the resistance of a variety of bacteria to a variety of antibiotics. MBC, is also the minimum lethal concentration, the lowest concentration of an antibiotic that inhibits the growth of the target bacterium after repeated cultures in antibiotic-free media.[36] The findings of MIC and MBC for applied standardization in the pharmaceutical industry are some of the most important indicators that should be considered in the research of new materials and compounds. In general, the results of the growth inhibition mentioned in this study are consistent with the results of growth inhibition in culture medium. That is, in both methods, 0.2% chlorhexidine showed the greatest inhibitory effect on both bacteria, followed by alcoholic extract and finally aqueous extract [Table 3]. The ability to inhibit

growth in the samples used is determined under aqueous extract < alcoholic extract < chlorhexidine for both bacteria, respectively. This effect increases with increasing concentrations of alcoholic and aqueous extracts on both bacteria [Table 3].

Ahmadi *et al*., by investigating the effect of ethanolic extract of *Z. multiflora* on inhibiting the growth of *S. aureus* and *E. coli* by the plate propagation method, reported a growth inhibition zone of 22 mm and 16 mm, respectively.[33] In the present study, the index for the effect of alcoholic extract on the growth inhibition of *S. mutans* and *S. sanguis* was 35.8 and 33.2 mm, respectively. In the study by Zomorodian *et al*., it was reported that the MIC of *Z. multiflora* essential oil for both *S. mutans* and *S. sanguis* was 1/4.[1] In the present study, this index was the same for *S. mutans* in the aqueous extract, but the alkaline extract used in this study showed a MIC of 1/128 for both bacteria. This indicates that the alcoholic extract has more antibiotic ability than the essential oil used in the study by Zomorodian *et al*.

The higher efficiency of alcoholic extract than aqueous extract should be found in the nature of extraction of phenolic materials with these two solvents. The presence of large amounts of thymol and carvacrol in Shirazi thyme and other thyme species has been confirmed.[37] Various studies on the antibacterial properties of thymol and carvacrol have been shown to be due to their ability to alter the structure of bacterial cell membranes. According to the findings, the ability of these materials to bind to the fat membrane of the cell wall increases the curvature of this membrane. The hydrophilic portion of these molecules attaches to the polar part of the membrane, while the hydrophobic part of the benzene ring of these molecules sinks into the inner part of the membrane. This causes instability of the fat layers and changes in the structure of the membrane, leading to a decrease in elasticity and an increase in membrane fluidity. This process increases the permeability of potassium and hydrogen ions.[22] These compounds also lower the pH by passing through the membrane and thus act as proton exchangers. These compounds, which contain a hydroxyl radical, are released from the membrane into the cytoplasm, where they release their protons. It then returns to the cell membrane to remove a potassium ion from the cytoplasm. This cation is released and thymol or carvacrol can trap another proton again and this cycle will be repeated. This process is associated with the depletion of cellular ATP stores and causes the loss of large amounts of cellular energy and ultimately the death of bacteria.[24] The entry of these compounds into the bacterial cell also affects the activity of inner membrane proteins such as enzymes and receptors.

Thymol and carvacrol, by binding to membrane proteins, cause their deformation and consequently, their inefficiency. Therefore, the two factors of changing the cell membrane elasticity and changing the function of membrane proteins, and membrane depolarization[36] are the main factors affecting the effects of these molecules on bacterial cells. The unique nature of thymol and carvacrol, i.e., the hydrophobic property of the benzene ring together with the hydrophilic property of the hydroxyl agent (OH), causes the mentioned processes and creates a special ability for them to destroy different bacteria.[36]

The solubility of thymol and its isomer (carvacrol) in alcoholic solvents is much higher than in water.[38,39] The solubility of thymol in ethanol is reported to be 90%,[39] and the solubility of this substance in water is reported to be 0.1%.[39] On the other hand, the study by Ultee *et al*. showed that the solubility of carvacrol in octanol is 10,000 times higher than water.[40] This difference in the extraction of thymol and carvacrol, which are the most important antibacterial substances of the extracts studied in this study, could be the main reason for the greater ability of the alcoholic extract to inhibit the growth of both *S. mutans* and *S. sanguis*. The study by Chen *et al*. showed that by increasing the concentration of alcohol from 2% to 5%, the solubility of thymol increased from 0.52 to 0.62 mg/ml and the solubility of carvacrol at the same concentrations. It also increased from 0.46 to 0.57 mg/ml. A similar increase has been observed in other compounds such as eugenol and trans-cinnamaldehyde.[41] Hydroalcoholic extraction of thymol from *Z. multiflora* with different percentages of alcohol (26%, 37%, and 72%) also showed that the amount of thymol extracted was equal to 2.7, 3.7, and 6 mg/g, respectively. That is, the content of extracted thymol depends entirely on the percentage of ethanol used, and with increasing alcohol concentration, more thymol is extracted.[42] The concentration-dependent effect of the alcoholic extract of *Z. multiflora* on the growth inhibition of methicillin-resistant *S. aureus* has also been confirmed by Yadegar *et al*.[26] The effect of *Z. multiflora* essential oil on cultured samples of *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus*

mirabilis, *Klebsiella pneumoniae*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*, showed that in all cases, with increasing essential oil concentration, halo diameter growth has also increased.[43]

A similar effect has been previously reported by Faraji *et al*. (2018) in increasing the growth inhibitory property of zinc and oxycinose by increasing the annual concentration of methane (methanolic and ethanolic) in melissa (*Melissa officinalis*).[5] The extraction percentage of each of these materials is very diverse according to the efficiency of the extraction method[44] and, as a result, will be very effective in the subsequent use of these extracts.

On the other hand, Haghighati *et al*. on the effect of extracts of 10 medicinal plants on growth inhibition of *Candida albicans*, *S. mutans*, and *Actinobacillus* showed that discs containing alcoholic extract of *Z. multiflora* were able to create a growth inhibition zone of 11.6 mm.[23] This barrier diameter is smaller than the findings of the present study (35.8 mm). This discrepancy can most likely be related to the discrepancy between the materials used in these two studies. The alcoholic solvent used in the research of Haghighat *et al*. was methanol alcohol, while the solvent used in this research was ethanol. The group also noted that by increasing the purity of methanol from 80% to 100%, the inhibitory capacity of *Z. multiflora* extract on all three pathogens increases.

Thymol of three species of thyme (*Thymus vulgaris, Thymus zygis, and Thymus citriodorus*) was extracted using 3 different solvents (ethanol, limolin, and ethyl lactate) and it was found that the ability to extract thymol in the ethanol solvent was significant (1/1). Higher by 12% than limolin solvent (9.4%) and ethyl lactate (9.2%).[44] Furthermore, Gas Chromatography Mass Spectrometry (GC/MS) studies have shown great diversity in 32 different compounds in the extracts of these three species, which can have a different effect on the performance of each of the same pathogens.[45]

In different studies, significant differences in the ability to inhibit the growth of different essential oils and alcoholic extracts (ethanolic or methanolic) compared to the aqueous extract of the same plant on different pathogens have been reported in different medicinal plants. Goudarzi *et al*., by examining the effects of aqueous and alcoholic (ethanolic) extracts of *Z. multiflora* on hemorrhagic *E. coli* by the well-drying method, stated that the MIC of alcoholic extract for this bacterium was 1/64 in dilution and aqueous extract, even with zero dilution, had no effect on the lack of growth of this bacterium.[46] This group showed that with increasing the concentration of the extract in the well, the diameter of the growth inhibition zone of this extract also increases. In another study, Kamkar found that ethanolic dill extract has more antioxidant capacity than its essential oil.[47] A comparison of the properties of different essential oils and extracts of ethanol, acetone, and aqueous on the inhibitory effect of the growth of 50 medicinal plants on the fungus *Candida albicans* showed that the effect of essential oils of Thymus kotschyanus and *Z. multiflora* is more effective than ethanolic and acetoic extracts of these two plants.[47] A noteworthy point in this study was the three-fold effect of Shirazi thyme ethanolic extract compared to mountain thyme. The effect of solvent on the extraction of plant compounds has already been reported in the case of phenolic substances extracted from potato peel.[48] By extracting the extract with five different solvents, including water, methanol, ethanol, hexane, and acetone, this group showed that the highest amount of phenolic substances is present in methanolic extract. By preparing fenugreek extract by two methods of extraction, with pure methanol and with a methanol/water mixture (ratio of 1: 1), it was shown that the extract prepared with pure methanol has more phenolic compounds and its antioxidation effect is also higher.[49] A comparison of the effects of aqueous and alcoholic extracts prepared from turbid (Daphne oleoides) plants also showed that the ability to inhibit the growth of alcoholic extract of this plant (with a diameter of no growth equal to 20.55 mm) on *S. mutans* is greater than that of aqueous extract.[50]

Saoudi *et al*. investigated that *Thymus capitatus* essence has more antiacanthamoeba (Acanthamoeba) effects than its alcoholic extract.[51] This finding is not consistent with the results of the present study, and the reason is the difference in the studied pathogens. Other studies on the effects of some medicinal plants on various pathogens have reported a greater effect of aqueous extracts than alcoholic extracts. As mentioned, the variety of compounds present in the organs of different plants and the species of the pathogen under study make the mode of unique action of each compound against a particular pathogen.[51]

Due to the presence of different compounds in *Z. multiflora*, especially thymol and carvacrol, the inefficiency of the aqueous extract used in this study

compared to the alcoholic extract can be attributed to the difference in the solubility of these two substances in ethanol compared to water.

The ability of chlorhexidine to inhibit the growth of pathogens in oral diseases has been confirmed in many studies.[13,20,23] However, it has been reported that it also has a great ability to destroy the natural flora of the mouth.[4] According to the obtained findings, it can be stated that the ability of chlorhexidine to inhibit the growth of *S. mutans* and *S. sanguis* is significantly higher than alcoholic and aqueous extracts. It was proved by both the plate propagation method and the culture method and by counting colonies in the culture medium. The aqueous and alcoholic extracts used in this study had a greater inhibitory effect on *S. mutans* than *S. sanguis*. The interpretation of this issue should be related to the characteristic of more acid production by *S. mutans* than by *S. sanguis*.[6] Under the conditions of multiplication of these two bacteria, the environment is made more acidic by *S. mutans*. A comparison of the antibacterial effects of thyme extract at different pH showed that its effect at pH 5.5 is greater than pH 6.5. This property is due to the interaction of the cytoplasmic membrane, aqueous medium, and phenolic content of the extract.[52]

It should be noted that the use of plant extracts in prophylaxis should not necessarily be due to their bactericidal properties, but rather to the ability of the substance used to prevent the growth of the desired bacteria, which can be used in pre-medicine. Such effects may include changes in the pH of the bacterial cytoplasm, increased permeability of the bacterial membrane to ions and metabolites, inhibition of intracellular or extracellular enzymes in bacteria, and destruction of bacterial metabolic pathways. They can reduce the uptake of other bacteria onto the biofilm, destroy plaque, and prevent the biofilm from spreading to the teeth.[37] Furthermore, nontherapeutic approaches such as eliminating the burning sensation in the mouth and unpleasant odor previously used in the use of peppermint mouthwash to reduce the unpleasant effects of chlorhexidine[52] as well as reducing the indicators of gingivitis and plaque, using mouthwash made from the essential oils of three plants (balsam herb, peppermint, and thyme)[53] is another use of plant extracts.

In different compounds tested in thyme extracts, high levels of other compounds such as p-Cymene, a precursor to carvacrol, have also been reported.[51,54-56] These compounds are hydrophobic substances that cause water retention and swelling of the bacterial membrane. It also changes the structure of membranes due to its lipophilic properties and increases their permeability to thymol and carvacrol.[56] This is why the use of essential oils or extracts of any of the medicinal plants shows their antibacterial effects in much higher amounts than when each of their constituents is separately.[1]

CONCLUSION

In this study, it was found that aqueous and alcoholic extracts of *Z. multiflora* have antibacterial properties against two bacteria, *S. mutans* and *S. sanguis*. Therefore, we suggest conducting other controlled studies in *in vivo* conditions as a mouthwash for investigating streptococcal bacteria reduction.

Acknowledgments

We would like to thank the staff of this university. We also sincerely thank Kaveh Madhoush, director of ViroMed medical laboratory, and his crews (Rasht), for their kind help on bacterial purchasing and cultures.

Financial support and sponsorship

Nil.

Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

REFERENCES

1. Zomorodian K, Ghadiri P, Saharkhiz MJ, Moein MR, Mehriar P, Bahrani F, *et al.* Antimicrobial activity of seven essential oils from Iranian aromatic plants against common causes of oral infections. Jundishapur J Microbiol 2015;8:e17766.
2. Carroll KC, Hobden JA, Miller S, Morse SA, Mietzner TA, Detrick B. Jawetz, Melnick & Adelberg's Medical Microbiology. 27th ed. New York: McGraw-Hill Education; 2016.
3. Moezzi Ghadim N, Taghibakhsh M, Godarzi H, Liravinezhad Hoseini N, Alirezaei S. Evaluation of the effect of four herbal extracts on growth of *Streptococcus mutans* and *Lactobacillus*. J Res Dentomaxillofac Sci 2018;3:7-13.
4. Azizi A, Fath Elahzadeh B, Maleknezhad P, Shamspour A. Evaluation of effects Irsha antiseptic mouthwash on pathogen streptococcus and oral normal microflora. J Isfahan Dent Sch 2009;5:24-9.
5. Faraji AA, Issazadeh KH, Rouhi S, Parvareh M, Zaboli F. A survey on the antibacterial effects of mouthwash Cetylpyridinium chloride and alcoholic extract of *Melissa officinalis* L. on *Streptococcus mutans* and *Streptococcus sanguinis*. J Rafsanjan

Univ Med Sci 2018;17:319-30.
6. Daboor SM, Masood FS, Al-Azab MS, Nori EE. A review on *Streptococcus mutans* with diseases dental caries, dental plaque and endocarditis. Indian J Microbiol Res 2015;2:76-82.
7. Magaz VR, Llovera BF, Martí M, Garre A. Clinical impact and cosmetic acceptability of chlorhexidine-enriched toothpaste and mouthwash application on periodontal disease: A randomized clinical study. J Contemp Dent Pract 2018;19:1295-300.
8. Ravanshad SH, Basiri EA, Mohammadzadeh M. *In vitro* evaluation of the antimicrobial effectiveness of *Zataria multiflora* as an irrigant in infected root canals with *Enterococcus faecalis*. Shiraz Univ Dent J 2009;10:92-8.
9. Yousefimanesh H, Amin M, Robati M, Goodarzi H, Otoufi M. Comparison of the antibacterial properties of three mouthwashes containing chlorhexidine against oral microbial plaques: An *in vitro* study. Jundishapur J Microbiol 2015;8:e17341.
10. Azizi A, Fath Elahzadeh B, Maleknezhad P, Shamspour A, Lavaf S. Evaluation of the effects of chlorhexidine 0.12% mouthwash on mouth pathogene *Streptococcus* and normal microflora. Shiraz Univ Dent J 2008;9:299-303.
11. Maghareh Abed A, Yaghini J, Fallah A. Comparison of the side effects of two common Iranian-made chlorhexidine mouthwashes. J Isfahan Dent Sch 2011;6:458-63.
12. Jabbari Ghanati M, Haghighi Moghadam Y, Valizadeh Hassanloei MA, Ghraaghaji Asl R. Comparison of the effect of two methods mouthwash (Chlorhexidine and Chlorhexidine combined with Hydrogen peroxide solution) on frequency of oral plaques in patients undergoing mechanical ventilation in the Intensive Care Unit. J Urmia Nurs Midwifery Fac 2018;16:508-16.
13. Ali Mohammadi I, Nasr Esfahani M, Hakimaneh SM, Talei D, Bafandeh MA, Shayegh SS. Comparison of the effect of herbal mouthwashes and Chlorhexidine on gingival healing after crown lengthening surgery (A clinical trial). J Mash Dent Sch 2020;44:248-58.
14. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence 2011;2:435-44.
15. Singh S, Singh SK, Chowdhury I, Singh R. Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. Open Microbiol J 2017;11:53-62.
16. Rezvaninejad R, Nabavi N, Khoshroo SM, Torabi N, Atai Z. Herbal medicine in treatment of recurrent aphthous stomatitis: A literature review. J Iran Dent Assoc 2017;29:127-34.
17. Zare P, Saeedi M, Akbari J, Morteza-Semnani K. A review on herbal oral care products. J Mazandaran Univ Med Sci 2016;26:394-410.
18. Sadeghi M, Bahramabadi R, Assar S. Antibacterial effects of Perisca and Matrica herbal mouthwashes on common oral microorganisms: An *in vitro* study. J Mashhad Dent Sch 2011;35:107-14.
19. Mirzaei K, Fathi A, Asadinejad SM, Moghadam NC. 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. Acad J Health Sci 2022;37:58-63.
20. Jafari A, Aghilli H, Herandi V. Investigating antibacterial property of the *Zataria multilflora* essence and chlorhexidine on orthodontic elastic rings contaminated with streptococcus mutant *in vitro*. J Shahid Sadoughi Univ Med Sci 2013;21:514-22.
21. Saedi Dezaki E, Mahmoudvand H, Sharififar F, Fallahi S, Monzote L, Ezatkhah F. Chemical composition along with anti-leishmanial and cytotoxic activity of *Zataria multiflora*. Pharm Biol 2016;54:752-8.
22. Khayat A, Sahebi S, Moazami F. Antimicrobial effect of NaOCl, Hydrated Ca (OH_2), thyme oil and normal saline as irrigating solutions on black pigmented and strep viridance. Shiraz Univ Dent J 2004;4:19-28.
23. Haghighati F, Jafari S, Momen Beitollahi J. Comparison of antimicrobial effects of ten Herbal extracts with chlorhexidine on three different oral pathogens; an *in vitro* study. Hakim Res J 2003;6:71-6.
24. Dalirestani Z, Aghazadeh M, Adibpour M, Amirchaghmaghi M, Pakfetrat A, Mosannen Mozaffari P, *et al*. *In vitro* comparison of the antimicrobial activity of ten herbal extract against *Streptococcus mutans* with chlorhexidine. J Appl Sci 2011;11:878-82.
25. Talei G, Meshkatalsadat M, Mousavi Z. Antibacterial activity and chemical composition of essential oils from medicinal plants of Lorestan, Iran. J Med Plant 2007;6:45-52.
26. Yadegar A, Sattari M, Bigdeli M, Bakhtiari F. Evaluation and comparison of antibacterial effects of alcoholic extracts of *Zataria multiflora* Boiss leaves, flowers and root on Methicillin-resistant *Staphylococcus aureus*. J Med Plants 2010;9:58-65.
27. Mottaghiyan Z, Aghazadeh M, Mahmoodzadeh Hosseini H, Imani Fooladi AA. Evaluation of antibacterial activity of *Zataria multiflora* against the expression of icaADB and aap gene and biofilm formation in Staphylococcus epidermidis. Arch Clin Infect Dis 2019;14:e65321.
28. Mazarie A, Mousavi-Nik SM, Fahmideh L. Assessments of phenolic, flavonoid and antioxidant activity of aqueous, alcoholic, methanol and acetone extracts of thirteen medicinal plants. Nova Biologica Rep 2018;4:299-309.
29. Oshagh M, Nazari Dashliborun Y, Ebrahimi Saravi M, Bazargan A. Evaluation of Chlorhexidine and *Zataria multiflora* essential oil in removing streptococcus viridans and candida from the surface of removable orthodontic appliances: A randomized clinical trial. J Mazand Univ Med Sci 2014;23:192-9.
30. Mansoori N, Moghaddam M, Kazemi F, Bahreini M, Aroie H. Influence of different concentrations of ethanolic extract of seven medicinal plants on three bacteria strains. Res Med 2018;41:236-43.
31. Gayathiri E, Bharathi B, Priya K. Study of the enumeration of twelve clinical important bacterial populations at 0.5 McFarland standard. Intern J Creat Res Thoughts 2018;6:880-93.
32. Iranpoor A, Bayani M, Arjomandzadegan M, Nakhostin A. Antibacterial activity and antibiofilm properties of *Satureja* essential oil against periodontal pathogens. J Arak Univ Med Sci 2019;22:16-27.
33. Ahmadi E, Abdollahi A, Najafipour S, Meshkibaf MH, Fasihi Ramandi M, Namdar N, *et al*. Surveying the effect of the phenol compounds on antibacterial activity of herbal extracts: *In vitro* assessment of herbal extracts in Fasa-Fars province. J Fasa Univ Med Sci 2016;6:210-20.
34. Yaghooti Khorasani MM, Assar S, Rezahosseini O, Assar S. Comparison of inhibitory dilutions of a thymol-based

mouthwash (Orion®) with chlorhexidine on *Streptococcus mutans* and *Streptococcus sanguis*. J Isfahan Dent Sch 2011;7:122-9.
35. Rezvaninejad R, Rezvaninejad R, Ashoorian MJ, Talebi M. Comparison of Effect of Aloe vera Gel and Nystatin on Candida Species: An *in vitro* Study. Jundishapur J Health Sci. 2022;14:e122029.
36. Memar MY, Raei P, Alizadeh N, Akbari Aghdam M, Samadi Kafil H. Carvacrol and thymol: Strong antimicrobial agents against resistant isolates. Rev Med Microbiol 2017;28:63-8.
37. Abachi S, Lee S, Rupasinghe HP. Molecular mechanisms of inhibition of *Streptococcus* species by phytochemicals. Molecules 2016;21:215.
38. Nagoor Meeran MF, Javed H, Al Taee H, Azimullah S, Ojha SK. Pharmacological properties and molecular mechanisms of thymol: Prospects for its therapeutic potential and pharmaceutical development. Front Pharmacol 2017;8:380.
39. Kowalczyk A, Przychodna M, Sopata S, Bodalska A, Fecka I. Thymol and thyme essential oil-new insights into selected therapeutic applications. Molecules 2020;25:4125.
40. Ultee A, Bennik MH, Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Appl Environ Microbiol 2002;68:1561-8.
41. Chen H, Davidson PM, Zhong Q. Impacts of sample preparation methods on solubility and antilisterial characteristics of essential oil components in milk. Appl Environ Microbiol 2014;80:907-16.
42. Aghamohammadi A, Azadbakht M, Hosseinimehr SJ. Quantification of thymol content in different extracts of *Zataria multiflora* by HPLC method. Pharm Biomed Res 2016;2:8-13.
43. Shafiee A, Javidnia K, Tabatabai M. Volatile constituents and antimicrobial activity of *Zataria multiflora*, population Iran, Iran. J Chem Chem Eng 1999;18:1-5.
44. Amiri H. Essential oils composition and antioxidant properties of three thymus species. Evid Based Complement Alternat Med 2012;2012:728065.
45. Villanueva Bermejo D, Angelov I, Vicente G, Stateva RP, Rodriguez García-Risco M, Reglero G, *et al*. Extraction of thymol from different varieties of thyme plants using green solvents. J Sci Food Agric 2015;95:2901-7.
46. Goudarzi M, Sattari M, Najar Piraieh S, Goudarzi G, Bigdeli M. Antibacterial effects of aqueous and alcoholic extracts of Thyme on enterohemorrhagic *Escherichia coli*. Yafteh 2006;8:63-9.
47. Kamkar A. The study of antioxidant activity of essential oil and extract of Iranian *Anethum graveloens*. Horizon Med Sci 2009;15:11-7.
48. Mohagheghi Samarin A, Poorazarang H, Hematyar N, Elhamirad AH. Phenolics in potato peels: Extraction and utilization as natural antioxidants. World Appl Sci J 2012;18:191-5.
49. Saviz A. Effect of cultivated area treatments and alcoholic and hydroalcoholic solvent on phenolic, tocopherol and antioxidant property content of Fenugreek leaf extract. J Food Sci Technol 2018;73:153-62.
50. Mousavi F, Shirzadi Karamolah K, Mahmoudi H. Antimicrobial effect of extracts of *Daphne oleoides* on bacteria isolated from dental plaque. J Mashhad Dent Sch 2019;43:387-400.
51. Saoudi S, Sifaoui I, Chammem N, Reyes-Batlle M, López-Arencibia A, Pacheco-Fernández I, *et al*. Anti-Acanthamoeba activity of Tunisian *Thymus capitatus* essential oil and organic extracts. Exp Parasitol 2017;183:231-5.
52. Juven BJ, Kanner J, Schved F, Weisslowicz H. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Appl Bacteriol 1994;76:626-31.
53. Alaee A, Aghayan S, Kamalinejad M, Arezoomand M. The comparison of mint mouthwash effect on microbial plaque with chlorhexidine, and acceptance of persons. J Res Dent Sci 2017;14:97-102.
54. Naeini A, Naseri M, Kamalinejad M, Khoshzaban F, Rajabian T, Nami H, *et al*. Study on anti-candida effects of essential oil and extracts of Iranian medicinal plants, in vitro. J Med Plants 2011;10:163-72.
55. Burt S. Essential oils: Their antibacterial properties and potential applications in foods – A review. Int J Food Microbiol 2004;94:223-53.
56. Nieto G. A review on applications and uses of thymus in the food industry. Plants (Basel) 2020;9:961.