

Original Article:

Effects of Propolis and Persica Mouthwashes on Three Common Oral Streptococci: A Comparative Study



Mohammad Sadegh Alemrababi¹, Mehdi Dastorani², Hadi Hamidi¹, Saeed Mohammadian³, Maryam-Sadat Sadrzadeh-Afshar^{*}

1. Department of Oral & Maxillofacial Medicine, School of Dentistry, Aja University of Medical Sciences, Tehran, Iran.

2. Department of Endodontics, School of Dentistry, Aja University of Medical Sciences, Tehran, Iran.

3. Department of Dentist, Oral & Maxillofacial Medicine, School of Dentistry, Aja University of Medical Sciences, Tehran, Iran.

* Corresponding Author:

Maryam-Sadat Sadrzadeh-Afshar, PhD.

Address: Department of Oral & Maxillofacial Medicine, School of Dentistry, Aja University of Medical Sciences, Tehran, Iran.

Phone: +98 (912) 2140257

E-mail: m_sadrzade@alumnus.tums.ac.ir



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ABSTRACT

Background: Dental caries and periodontal diseases are among the most common oral diseases, and research to achieve an effective strategy to overcome these diseases is necessary. One of these strategies is to use anti-septics and disinfectants, including mouthwashes. Although chlorhexidine was the first and most common mouthwash and the gold standard of anti-plaque treatments, it bears many side effects. However, herbal mouthwashes with anti-microbial properties and fewer side effects can effectively treat many of these diseases.

Objectives: This study aimed to compare the efficiency of the two herbal types of mouthwash produced in Iran.

Methods: The present in vitro study was conducted to investigate the anti-bacterial effects of persica and propolis mouthwashes on three strains of oral streptococci. The Zone of Inhibition (ZOI) was measured by the Disk Diffusion Method (DDM). The Minimum Inhibitory Concentration (MIC) value of each mouthwash was determined for all microorganisms using the macrodilution method.

Results: Statistical analysis of data of DDM showed that the anti-bacterial effect of persica was significantly higher than propolis against *Streptococcus. salivarius* and *Streptococcus. mutans* ($P<0.001$), and these two types of mouthwash had similar anti-bacterial effects on *Streptococcus. sanguis*. Local propolis exhibited better MIC results than persica against *S. salivarius* and *S. mutans*, and these two types of mouthwash showed similar results against *S. sanguis*.

Conclusion: Local propolis was more potent than persica in preventing the growth of oral streptococci.

Keywords:

Propolis, Salvadoraceae, Mouthwashes, Minimum inhibitory concentration, *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*

Introduction

The oral cavity mirrors the body, and its health reflects systemic health [1]. More than 700 strains of microorganisms live

in the oral cavity, of which 400 strains have been isolated from the periodontal pockets and 300 strains from other parts [2]. Most of these microorganisms are part of the normal flora of the oral cavity, which can develop infections under weakened immune conditions [3]. Me-

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chanical plaque removal is the first and most effective way to maintain oral health and prevent periodontal disease, dental caries, and systemic diseases with oral microbial origin [4].

Despite trying to maintain oral hygiene, many people cannot fulfill mechanical plaque removal to the desired level [5]. As a result, chemical plaque control is applied to complete mechanical plaque removal [6, 7]. Mouthwashes are one of the most common topical methods in chemical plaque control. Chlorhexidine was the first and most common mouthwash and the gold standard of anti-plaque treatments, but it has side effects, such as a change in the sense of taste, pain, burning sensation, and tooth discoloration [7-10]. Indiscriminate use of broad-spectrum antibiotics also leads to increased drug sensitivity, antibiotic resistance of bacteria, and drug toxicity [11]. Despite many advances and technologies in hygiene production, there is a great interest in using natural products and compounds because of the side effects of synthetic products, including their systemic effects. However, public awareness of natural products must be promoted. Besides, the use of these natural products is limited due to little research on them [12-14].

Propolis is a resinous-like natural substance produced by bees through combining wax and saliva with resins collected from plants [15]. Anti-bacterial, anti-fungal, and anti-tumor properties have been demonstrated in propolis. Because of the differences in plants present in various regions of the world, the composition of propolis is diverse, which causes different properties of the substance [16, 17].

Persica herbal mouthwash contains the active ingredients of toothbrush tree (*Salvadora persica*), mint, and yarrow. The main ingredients in this mouthwash are tannins, calcium, and chloride. Unlike other mouthwash solutions, this solution can be swallowed, and its use is allowed in children and pregnant women. The chemical composition of persica mouthwash, including sodium chloride, silica, and sulfur, causes anti-microbial, anti-fungal, anti-plaque, and anti-caries effects. Large amounts of chloride in this plant reduce the calculus formation and prevent tooth discoloration by the thiocyanate component [18]. This component releases cyanide in contact with saliva, which prevents the growth of oral bacteria. Fluoride is another component of the toothbrush tree that can affect bacterial glycosyltransferase function and acid production or intercellular polysaccharides [19]. Various studies have shown the effectiveness of propolis mouthwash on *Streptococcus mutans* [20, 21]. The studies report a better improvement of

gingival conditions than [22, 23] chlorhexidine mouthwash. *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius* are the most common and main pathogenic bacteria of the oral cavity [24]. Accordingly, the present in vitro study was conducted to compare the efficacy and anti-bacterial effect of two propolis and persica mouthwashes produced by domestic manufacturers in Iran on three common oral streptococci.

Materials and Methods

This in vitro study was performed in 2019 on *S. mutans*, *S. salivarius*, *S. sanguis*, which were prepared as lyophilized ampoules from the collection center of industrial microorganisms (the Scientific and Industrial Research Organization of Iran). These organisms included *S. mutans* with Persian type culture collection (PTCC=1683), *S. salivarius* (PTCC=1448), and *S. sanguis* (PTCC=1449) (Table 1). These bacteria are part of the microbial flora of the oral cavity and are involved in the development of oral diseases. Herbal mouthwashes also included native propolis of Mashhad and Persica, which were official drugs purchased from Iranian manufacturing companies (Table 2, Figure 1). Each of the studied bacteria of *S. salivarius*, *S. sanguis*, and *S. mutans* were identified by the numbers 49, 48, and 83, respectively, which corresponded to the last two digits of the company code of these bacteria (Figure 2). Each mouthwash was also labeled with its own brand. Culture media also included Muller-Hinton agar (MHA), prepared according to the manufacturer's instructions and then autoclaved. After cooling, these solutions were dispensed into 10-cm Petri dishes and stored in the refrigerator (Figure 3).

First, lyophilized ampoules containing powdered microbial strains (Figure 4) were scraped from a location higher than the available cotton. After disinfection with 70% alcohol-soaked gas, they were broken from the scratch site. After removing the cotton in the ampoule by sterile forceps, 0.3 to 0.4 mL of distilled water was added to the dry powder in the ampoule using a sterile Pasteur pipette. After uniform suspension, the required amount was removed from each ampoule containing the suspension using a 2-mL syringe and cultured on Tryptic Soy Broth (TSB) medium for initial propagation. After incubation at 37°C, they were transferred onto MHA medium. Then, to confirm the cultured *S. mutans*, *S. salivarius*, *S. sanguis*, some of their colonies were taken by a sterilized loop for smear preparation and Gram staining as follows (Figures 5, 6):

1. Preparing a smear of the desired bacteria on the slide



Figure 1. Propolis and persica mouthwashes

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2. Air drying the smear
3. Fixing the smear using flame heat
4. Pouring a few drops of crystal violet dye on the smear for 1 to 2 minutes
5. Washing the smear under a gentle stream of water
6. Pouring a few drops of Lugol solution on the smear for 1 min
7. Washing the smear under a gentle stream of water
8. Pouring a few drops of decolorizing agent (ethanol, 95%) on the smear until the last purple drops come out of the smear (17 s)



Figure 2. Lyophilized ampoules containing microbial strains

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9. Washing the smear under a gentle stream of water
10. Pouring a few drops of fuchsine dye on the smear for 45 s
11. Washing the smear under a gentle stream of water
12. Air drying the slide



Figure 3. Cultured media environments

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Figure 4. Work table under the hood and adjacent to the flame

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Figure 5. Gram coloring kit

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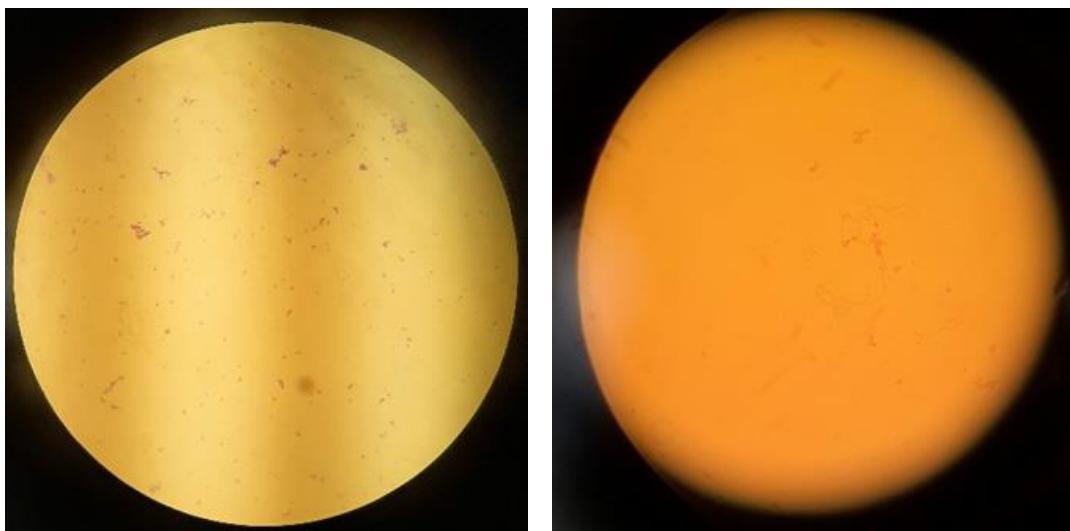


Figure 6. Stained blades under a light microscope

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13. Microscopic observation with a magnification of 100x using immersion oil

A single colony of bacteria cultured onto the MHA was taken and transferred into normal saline to prepare the bacterial suspension. According to the Kirby-Bauer method [25], the turbidity of these pure bacteria reached 0.5 McFarland (1.5×10^8 CFU/mL). The bacterial suspension was cultured by the lawn culture method. Thus, a sterile cotton swab was first dipped into the bacterial suspension, and excess fluid was removed by pressing on the inner edge of the test tube. Bacterial-impregnated swabs were then cultured on the surface of the plate containing MHA so that the entire agar surface was impregnated with the bacteria. Three empty sterile plates were

taken. Subsequently, the propolis and persica mouthwashes with therapeutic concentrations (without dilution) were poured into the two plates, and distilled water was poured into the third plate as a control. Next, three sterile 6.4-mm diameter paper disks (manufactured by Padtan Teb Co., Tehran, Iran) were dipped into each plate via sterile forceps to be completely wetted (Figure 7). After draining the excess solution, the plates were placed in an oven at 40°C for about 10 min to allow excess mouthwash to evaporate. The dried disks were carefully seeded by sterile forceps onto the culture medium inside the plate (containing bacteria) and pressed gently on the agar surface until the entire disk was in contact with the agar. Blank disks (disks containing distilled water) were also used as

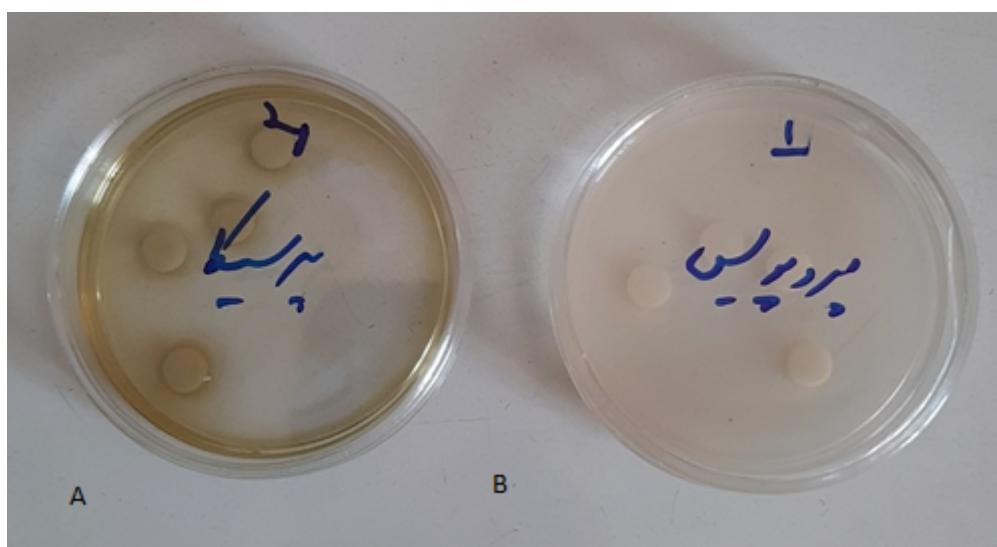


Figure 7. Mouthwash impregnated disks

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A: Persica; B: Propolis.



Figure 8. Inoculation of bacteria using sterile sampler

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controls. The incubation was performed at 37°C for 24 h, and the next day the diameter of the Zone of Inhibition (ZOI) (in mm) was measured by an accurate ruler and recorded in the information form. To increase the accuracy, each of the samples and disks in the control group was tested in triplicate on the three bacteria. The MHA medium was used to obtain a single colony, and the Disk Diffusion Method (DDM) [26] was employed to determine the susceptibility of bacteria to the studied mouthwashes. The TSB medium was applied to determine the Minimum Inhibitory Concentration (MIC) value of each mouthwash using the macrodilution method. It should be noted that all test steps were performed aseptically under the hood and in the vicinity of the flame.

To measure the MIC value of each mouthwash, 2 mL of the prepared TSB medium was poured into test tubes and autoclaved. Thus, 10 tubes were provided to dilute each mouthwash. The preparation of dilutions of 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256 using a pipette was started by adding 2 mL of mouthwash to tube No. 1/2. After stirring the contents of this tube, 2 mL of it was transferred to the next tube. This process was continued until the last tube, and finally, 2 mL of solution was discarded from the last tube. Tube No. 0 was considered positive control (containing only medium and 20 µL of bacteria), and tube No. 1 negative control (containing mouthwash and bacteria only). The microbial suspension was prepared separately from the desired bacteria with a turbidity

of 0.5 McFarland (checked using a spectrophotometer). Then, 20 μ L of this bacterial suspension was inoculated into all test tubes using a sterile sampler ([Figure 8](#)). Immediately after inoculation, each tube was thoroughly mixed with the sampler so that the inoculated bacteria were evenly distributed in the broth medium [27]. All steps of preparing mouthwashes and inoculating the tubes under the hood and adjacent to the flame were performed under aseptic conditions. These steps were performed separately for each bacterium. The MIC results were read and recorded after 24 h and 72 h by examining the presence or absence of growth in each tube, which in most cases was visually evident. It should be noted that each culture medium with clear transparency was considered a negative sample (non-growth) and each culture medium with clear turbidity a positive sample (growth).

In cases where, for reasons such as the turbidity of mouthwashes, it was difficult to judge whether the bacteria had grown or not, sampling was performed by sterile loop and cultured on an MHA medium. Cultivation was performed on each plate in the form of several serial lines of the corresponding MIC tubes marked with the coding on the back of the plate. Incubation was then performed at 37°C for 24 hours. The MIC value was considered the lowest concentration of each substance that caused a 90% decrease in turbidity compared to the control group (MIC90).

Statistical analysis

The data collection tool in this study was a checklist. In data analysis, the normality of data distribution was first examined by the 1-sample Kolmogorov-Smirnov test. The inter-group variables were compared by independent samples t test and Mann-Whitney U test for normal and non-normal data distribution, respectively. The intra-group variables were compared by paired-samples t test and Wilcoxon test for normal and non-normal data distribution, respectively, and repeated measures analysis of variance (ANOVA).

Results

This in vitro comparative study was designed to investigate the effect of propolis and persica mouthwashes on three common oral streptococci using two culture media (MHA and TSB) and 20 samples for each mouthwash (n=20). In a statistical study of the effects of different mouthwashes on diverse bacteria, three various effects were evaluated:

1. The effect of bacterial species on ZOI

2. The effect of mouthwash type on ZOI

3. The co-effect of mouthwash and bacteria on ZOI ([Table 3](#))

Statistically, the measured variable and the final variable were the ZOI diameters. It should be noted that the efficacy of microbe type, mouthwash type, and co-effect of both on ZOI diameter was compared based on the results of the linear regression Post Hoc Test.

As shown in [Table 3](#), *S. salivarius* was significantly more sensitive than other tested strains ($P<0.001$). Other sensitive microorganisms to mouthwash were *S. mutans* and *S. sanguis*, respectively. *S. salivarius* exhibited the highest diameter of ZOI compared to other streptococci, and the difference between them was significant. It is noteworthy that the difference between *S. salivarius* and *S. mutans* with *S. sanguis* was statistically significant ($P<0.001$). The anti-bacterial effect of persica and propolis mouthwashes was significantly higher than the negative control group ($P<0.05$) ([Figure 9](#)).

The results revealed that 60% of the observed ZOI diameter changes were due to the co-effect of microbe and mouthwash types ($P<0.001$), indicating that the growth inhibitory effect of mouthwashes on oral streptococci depends on both the type of microorganism and the type of mouthwash used. Among the two types of mouthwash, the results showed that persica had more effects than propolis.

In addition, about 50% of the observed ZOI diameter changes were due to the effect of the type of mouthwash, which was statistically significant ($P<0.001$). Comparing the ZOI diameter of persica mouthwash with propolis mouthwash, it was found that persica mouthwash was more significant and showed better effects in terms of changes in zone of inhibition compared to propolis mouthwash ($P<0.05$).

The results showed that the growth inhibitory effect of mouthwashes against studied streptococci had a total impact of 65% based on the Kruskal-Wallis analysis method compared to each other ([Table 3](#)), meaning that about 65% of the observed ZOI diameter changes were due to the type of microorganism, which was statistically significant ($P<0.001$).

After repeating the experiments of this method several times, since similar results were obtained, the MIC value of each mouthwash in the presence of microorganisms was reported as a number. Therefore, analysis of variance

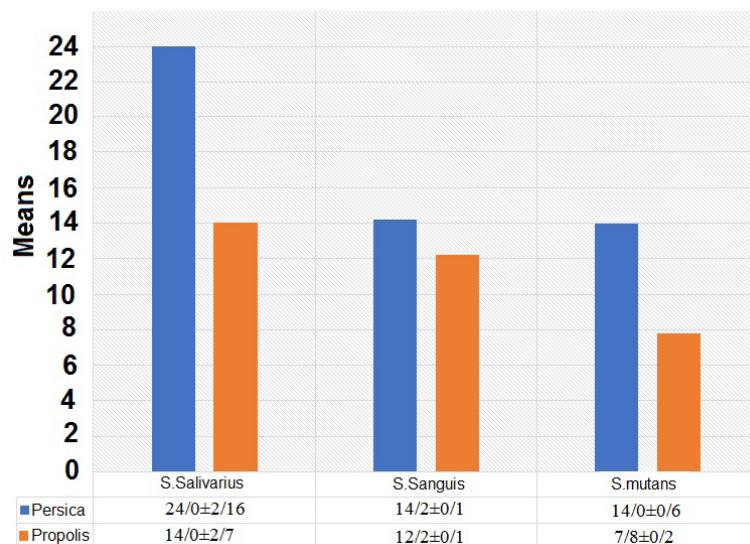


Figure 9. Mean \pm SD of Zone of Inhibition (ZOI) in millimeter for each type of bacteria

was impossible due to the zero standard deviation indexes for the data. Hence, the data obtained from this method were expressed as a descriptive comparison. It should be noted that in terms of MIC, propolis mouthwash was more effective than persica (Table 4). The results of measuring the diameter of ZOI in DDM and of MIC in the macrodilution method were the same at 24 and 48 h.

Discussion

Given the infectious nature of dental caries and periodontal disease, which are among the most common oral diseases of the century, the need for research to find a solution to overcome these diseases is evident. The use of anti-septics and disinfectants, including mouthwashes, is one of these ways [28]. An essential feature of any mouthwash is its anti-microbial properties without toxic effects on periodontal tissue. Chlorhexidine is the most common mouthwash and the gold standard of anti-plaque treatments, but it has several side effects [7-10, 29]. Hence, today the global attitude is to reduce synthetic products and use more traditional medicine [30]. Herbal mouthwashes, with their anti-microbial properties and fewer side effects, could effectively treat many diseases [17].

Since no study has compared the anti-bacterial effects of two herbal types of mouthwash of propolis and persica, the present in vitro study was conducted to compare the effectiveness and efficiency of these two herbal types of mouthwash produced in Iran against three common oral streptococci.

Propolis is a natural plant-derived resin produced by bees from the flowers, pollen, branches, and leaves of plants and used to repair hive walls and protect colonies from disease [15]. Propolis has long been used to repair wounds. It has been administered orally [23], and its anti-bacterial, anti-fungal, anti-viral, anti-oxidant, anti-tumor, and anti-inflammatory properties have been proven [31, 32]. Immunomodulatory properties, cell-mediated and humoral immune stimulation, and soft tissue strengthening are other properties of propolis. Anti-bacterial effects are considered to achieve by preventing cell division, therefore resulting in the formation of pseudo-multicellular streptococci. In addition, propolis disorganizes the cytoplasm, the cytoplasmic membrane, and the cell wall, causing a partial bacteriolysis and inhibited protein synthesis [33]. The properties of propolis suggest that it is a natural anti-bacterial agent. Although the exact mechanism of action is unknown, it is likely that cessation of RNA polymerase activity, direct damage to the cell membrane or cell wall will lead to functional and structural damage to the bacterium [34-36]. These properties are related to the flavonoid content and cinnamic acid of propolis. Other advantages include inhibiting prostaglandin synthesis, supporting the immune system by increasing phagocytic activity, enhancing healing effects on epithelial tissue, activating the thymus gland, and inducing cellular immunity. These features altogether can explain the effectiveness of propolis against bacteria involved in dental caries and periodontitis [20, 22].

The main component of persica mouthwash is the extract of the toothbrush tree, and its anti-microbial effects can be attributed to the various ingredients in this plant, including

Table 1. Microbial samples used in research

Microbial Sample	PTCC**	Brief Bacterial Code	Conventional Culture Medium	Suitable Temperature for Incubation	Incubation Time
S. salivarius	1448	48	Muller Hinton agar	37°C	24 & 48 h
S. sanguis	1449	49	Muller Hinton agar	37°C	24 & 48 h
S. mutans	1683	83	Muller Hinton agar	37°C	24 & 48 h

**Persian type culture collection.

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Table 2. Names of mouthwashes used in research

Mouth wash	Manufacturer	Country-city	Code
Propolis	Sorentoos	Iran- Mashhad	557310
Persica	Poursina	Iran- Mashhad	97009

chlorine, trimethylamine, alkaloid resin, and sulfur compounds that release substances, such as antibodies into saliva, which prevent bacteria from colonization on the tooth surface. In addition, persica can increase pH and stimulate saliva secretion from the parotid gland [37].

The bacteria studied in this study were part of the oral microbial flora and are involved in the incidence of oral diseases. Streptococcus mutans is the most important caries-related microorganism associated with the onset of caries [38, 39]. Some researchers consider the presence of S. mutans to be a predictor of caries [40, 41]. S. mutans and S. sanguis are the most important causes of

bacterial endocarditis [42]. Several studies have reported the effectiveness of propolis mouthwash on S. mutans [20, 21] and in improving the condition of the gums and that it is more effective in improving gingivitis [22, 23] than chlorhexidine mouthwash. Therefore, the bacterial species of S. mutans, S. sanguis, and S. salivarius were selected for this study as the most common and main oral pathogenic bacteria [24].

The results of DDM and ZOI diameter measurement in this study showed that persica mouthwash was more effective than propolis mouthwash. In addition, S. salivarius formed the largest ZOI diameter, i.e., the most

Table 3. Descriptive Statistics of Zone of Inhibition (ZOI) (in mm) by type of mouthwash in terms

Bacteria	Mouthwash	Mean±SD		P (Mann-Whitney Test)	Effect Size (%)
		Propolis (n=20)	Persica (n=20)		
S. Salivarius		14.0±2.7	24.0±2.16	0.001	69
S. Sanguis		12.2±0.1	14.2±0.1	0.001	54
S. mutans		7.8±0.2	14.0±0.6	0.001	61
P (Kruskal-Wallis test)		0.001	0.001	0.001***	65
		63	13	60	

*** Repeated measures analyze groups.

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Table 4. Descriptive statistics of mic (in µg/ml) results in laboratory tubes on bacteria in two groups of mouthwash

Bacteria	Mouthwash	Propolis (n=20)	Persica (n=20)
S. Salivarius		1.16	1.4
S. Sanguis		1.8	1.8
S. mutans		1.16	1.4

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sensitive to persica mouthwash, and *S. sanguis* had the highest sensitivity to propolis mouthwash. The MIC value was measured in this study by broth microdilution test, which is a routine method for examining the anti-bacterial properties of agents and is still the most reliable and easiest method of interpreting anti-bacterial properties (despite its several limitations) [43]. Therefore, this method was selected in the present study.

In this study, the MIC results of mouthwashes showed that the effectiveness of propolis mouthwash was higher than persica. The lowest MIC values for propolis mouthwash were related to *S. mutans* and *S. salivarius*, and the highest value was reported for *S. sanguis*. In addition, the lowest MIC values for persica mouthwash were observed for *S. sanguis* and the highest values for *S. mutans* and *S. salivarius*. In terms of the effect on *S. sanguis*, both types of mouthwash worked the same, but in terms of the impact on *S. salivarius* and *S. mutans*, propolis was more effective.

Vasconcellos et al. demonstrated the effect of propolis mouthwash on *S. mutans*, *Staphylococcus aureus*, and *Enterococcus faecalis* [44].

In a clinical trial, Jajarm et al. also concluded that persica mouthwash had a significant inhibitory effect on the growth of *S. mutans* compared with placebo [45].

Mozaffari et al. found that persica mouthwash at a concentration of 50% (double diluted) had weak and transient bacteriostatic effects against *S. mutans* and *S. sanguis* [46].

Hafaji et al. found that herbal mouthwashes have no anti-microbial potential like chlorhexidine. Still, the ingredients in herbal mouthwashes effectively prevent the growth of oral microbes so that they can help control dental plaque and gingivitis [43].

Almas and Abdolrahman concluded that chlorhexidine-containing mouthwashes had maximum anti-microbial activity, and toothbrush plant extract had low anti-microbial activity [47, 48].

Drumond et al. indicated that daily consumption of propolis 6.25% leads to a decrease in *S. mutans* in children's mouths and a decrease in plaque index and gingivitis [49].

The present study also achieved similar results, so that the findings showed that herbal mouthwash had signifi-

cant effects on oral streptococci versus placebo, which was ineffective.

The results of this study confirmed the findings reported by Vasconcellos et al. [44], Jajarm et al. [45], Mozaffari [46], Hafaji et al. [43], Almas [48] and Abdolrahman [47], Drumond et al. [20].

Santiago et al. indicated that propolis mouthwash has anti-bacterial properties similar to chlorhexidine [15].

In a clinical trial, Mohan et al. compared the effect of dental cavity disinfection with Brazilian propolis, diode laser, and chlorhexidine 2%. They concluded that propolis and diode laser were as effective as chlorhexidine on controlling *S. mutans* and *Lactobacillus acidophilus* [26].

Acka et al. compared the anti-microbial effect of the alcoholic extracts of propolis and chlorhexidine and found that propolis had anti-bacterial properties similar to chlorhexidine [27].

The present study results in all cases of DDM showed that the anti-bacterial effects of persica mouthwash were better than those of propolis. However, studies by Santiago et al. [15], and Mohan et al. [12], suggest that propolis mouthwash has stronger anti-bacterial effects (as much as chlorhexidine). The reason for the difference in the strength of different propolis mouthwashes is due to the difference in the formula and properties of the studied propolis because the difference in the region, the season of propolis collection, its contamination with wax and bee species all lead to differences in the properties of propolis. On the other hand, differences in the microbiological methods studied, including the type of microbe and the phase of cell differentiation and culture conditions and time, duration of drug use, and study design, are other causes of different results.

The present study has advantages over previous studies, such as novelty, the use of local propolis (because many articles have emphasized that the geographical area affects its properties) [27, 49], and minimal use of synthetic compounds and alcohol in mouthwash (to reduce its side effects in long-term use).

Further studies can confirm the results of the present study. It can be concluded that treatment with persica and propolis mouthwashes can mitigate oral diseases, such as caries and possibly gingivitis, periodontal infections, and primary and secondary oral infections. Given the anti-bacterial properties of herbal mouthwashes and the fact that they have fewer side effects than chemical

mouthwashes, they may be considered anti-bacterial agents. As studies on these mouthwashes are few and their anti-microbial spectrum is unclear, it is recommended to study more and more broadly on them before recommending their use as anti-bacterial agents.

Persica mouthwash in the present study showed better results in the disk diffusion method than local propolis mouthwash against *S. salivarius* and *S. mutans*. These two types of mouthwash exhibited almost similar conditions as for *S. sanguis*.

Local propolis mouthwash in the present study displayed better results in the MIC method than persica mouthwashes against *S. salivarius* and *S. mutans*. The two types of mouthwash had similar conditions as for *S. sanguis*.

Conclusion

Given the limitations of this in vitro study, the results of the MIC method are more reliable than those of the ZOI method according to CLSI (Clinical & Laboratory Standard Institute) standards. Thus, the herbal mouthwash of local propolis has stronger than persica in preventing the growth of these oral bacteria. According to the study results, in choosing a mouthwash to treat the infection, besides the type of mouthwash and its anti-septic properties, the sensitivity of the pathogenic microorganism to the mouthwash should also be considered. In other words, the growth inhibitory effect depends on both the type of microorganism and the type of mouthwash used.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Aja University of Medical Sciences (Code: IR.AJAUMS.REC.1398.170).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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