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Evaluation of Antibacterial Effect of Propolis and its Application in Mouthwash Production

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A B S T R A C T

Objectives: Our purpose was to determine the antibacterial properties of propolis and to evaluate its use as an antibacterial mouthwash with minimal complications.

Materials and Methods: In this experimental laboratory study, an alcoholic propolis extract was prepared. The minimum inhibitory concentration (MIC) was calculated for four bacterial species including *Staphylococcus aureus* (*S. aureus*), *Streptococcus mutans* (*S. mutans*), *Lactobacillus acidophilus* (*L. acidophilus*), and *Enterococcus faecalis* (*E. faecalis*) using agar dilution. According to the MIC, a propolis antibacterial mouthwash was produced and compared to water, chlorhexidine (CHX), and Listerine using laboratory rats for clinical examination. Salivary specimens of rats were collected at 12 hours, 1 week, and 2 weeks after using the mouthwash and examined by real-time polymerase chain reaction (RT-PCR). Data were analyzed using one-way analysis of variance (ANOVA) and repeated measures ANOVA (α=0.05).

Results: The results of agar dilution by the number of colony-forming units showed the lowest MIC for *S. aureus* and the highest for *L. acidophilus*. Our RT-PCR findings indicated that water alone had no effect on the level of oral bacteria. Propolis mouthwash showed a significant difference with CHX and Listerine ($P<0.05$) in terms of the number of *S. mutans*, *E. faecalis*, and *L. acidophilus* colonies, while CHX and Listerine were less efficient. There was no significant difference between CHX and propolis ($P=0.110$) regarding *S. aureus* colonies, but Listerine had a lower efficacy than either ($P<0.05$).

Conclusion: According to the results, propolis mouthwash was more efficient against the studied oral bacteria compared to CHX and Listerine.

Keywords: Anti-Bacterial Agents; Propolis; Mouthwashes

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INTRODUCTION

The mechanical methods used for oral health maintenance and gingivitis control can be challenging for most people [1,2].

Oral biofilms are the primary cause of gingivitis, periodontitis, caries, halitosis, and systemic disease [3]. Although tooth brushing is the most effective way to clean teeth and to control dental plaque, mouthwashes are widely used to complement tooth brushing. Researchers have shown the therapeutic effects of some commercial mouthwashes [4,5]. The Canadian Dental Hygienists Association (CDHA) considers oral cleansing as an important part of oral hygiene [4].

Chlorhexidine (CHX) and Listerine are two popular types of mouthwash frequently prescribed by dentists [5]. CHX is the golden standard antiplaque treatment and is effective in the treatment of gingivitis and periodontitis [6]. Its side effects include staining, dysgeusia, painful mucous membranes, and burning sensation during mouth washing [6]. Therefore, its regular and extended use should be avoided [7]. Listerine is a mouthwash made in an attempt to reduce the side effects of CHX; it is effective in

controlling halitosis and caries, but as it contains alcohol, there have been complaints about its unpleasant taste [5,8].

On the other hand, it has been suggested that some natural compounds may not need to be used in combination with alcohol, which can be considered as an advantage [9]. Other factors that encourage research on natural compounds include the side effects of commercial products, such as the systemic effects and antibiotic resistance [4,10]. In addition, people are more interested in using natural compounds as they are safer and healthier [11,12]. However, there is a need to raise public awareness about natural products since their usage is limited due to scattered research on their effects [13]. Today, many non-commercial formulations are under development. Nevertheless, few commercial mouthwashes contain natural compounds [11].

Propolis has long been used for healing oral ulcers [14,15], and its antibacterial, antifungal, antiviral, antioxidative, antitumor, and anti-inflammatory properties have been proven [16, 17]. Immunomodulation, stimulation of cellular and humoral immunity, and soft tissue enhancement are among the other properties of propolis [18].

As far as the authors of the present study are informed, there is no study about the antibacterial properties of propolis mouthwash in Iran. Also, it has not been compared to other common mouthwashes such as CHX and Listerine. Therefore, the aim of the present study was to determine the antibacterial properties of propolis in a laboratory environment and to use it to produce an antibacterial mouthwash with minimal complications.

MATERIALS AND METHODS

This experimental laboratory study has been approved by the Ethics Committee of AJA University of Medical Sciences (code: 9000010). All animal experiments in this study were conducted according to the Helsinki Protocol.

Preparation of propolis extract:

In the present study, propolis was obtained from the western region of Isfahan province, Iran, in Spring 2017. First, 100 g of propolis was cut into small pieces and was frozen in a freezer at -80°C. Next, the pieces were crushed and dissolved in an 80% alcohol (Sigma-Aldrich, St. Louis, MO, USA) at a 1:5 ratio in an ultrasonic bath at 40°C for 2 hours (the ratio of the alcohol was much lower than that of ethanol and was reduced from 20:1 to 5:1). The resulting solution was filtered using a Whatman filter (Sigma-Aldrich, St. Louis, MO, USA) and was kept in a dark place for three days. Next, it was stored for one day in the refrigerator and then filtered by a No.1 Whatman filter to remove the wax (Fig. 1).

The resulting 20% w/w solution was kept in open space for two days in order for its alcohol content to evaporate [19,20]. The remaining crude extracts were dissolved in approximately 500 mg/ml of dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) and were stored at -20°C until the treatment [20].

Microbial culture and in-vitro experiments:

The bacterial species under study were *Staphylococcus aureus* (*S. aureus*; ATCC 29213), *Enterococcus faecalis* (*E. faecalis*; ATCC 29212), *Streptococcus mutans* (*S. mutans*; ATCC 35668), and *Lactobacillus acidophilus* (*L. acidophilus*; ATCC 314) obtained from the cell bank of the Pasteur Institute of Iran.

Tryptose agar culture medium (Difco Laboratories, Detroit, MI, USA) was used to culture *S. aureus*, *S. mutans*, and *L. acidophilus*, while for the culturing of *E. faecalis*, blood agar medium (Difco Laboratories, Detroit, MI, USA) was used. The method of culturing the bacteria is shown in Table 1.

Table 1. Primers used for *AIF* and *ACT1* gene in PCR

Bacteria	Temp (°C)	CO2 Conc	CT (h)
Staphylococcus aureus	37	---	48
Streptococcus mutans	37	5%	48
Lactobacillus acidophilus	37	10%	72
Enterococcus faecalis	37	---	48

Temp: Temperature; CO2 Conc: Carbon dioxide Concentration; CT: Cultivation Time

After the stock culture was obtained, the bacteria were transferred to the abovementioned culture media and were incubated (Teifazmateb Co., Tehran, Iran) at 35°C for 48 hours. The culture media were used to produce a suspension with the appropriate number of cells. A suspension of the studied bacteria at a concentration of 10.5×10^8 colony-forming units (CFU)/ml was prepared in the culture media [19].

Fig. 1: The wax removed by the Whatman filter

Methods of testing the antimicrobial activity:

Agar dilution was used to evaluate the antimicrobial activity of propolis extract. The minimum inhibitory concentration (MIC) was calculated based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [21,22]. The MIC is the lowest concentration that prevents significant bacterial growth. Serial dilutions of the ethanolic extract of propolis (EEP) were prepared, ranging from 50 µg/ml to 600 µg/ml, under aseptic conditions and were added to the culture media. Seven culture media were prepared for each bacterial species, and the alcoholic extracts at the concentrations of 50, 75, 150, 200, 300, 450, and 600 µg/ml were mixed with the culture media and were incubated at 35°C for 48 hours. For each culture medium and each concentration, three replicates were used to minimize the test error. The antimicrobial effects of different dilutions were investigated, and the 300-µg/ml concentration was determined as the MIC of the EEP, which resulted in no bacterial growth [21,22].

After the MIC was determined, a 3% propolis mouthwash was prepared, which contained propolis extract, alcohol (as a solvent), menthol (as a breath freshener), sodium benzoate (as a preservative), sodium saccharin and sorbitol (as flavoring agents), and water. Every 100 ml of the mouthwash contained 70 mg of water, 30 mg of alcohol, 6 mg of propolis extract, 1 mg of menthol, 1 mg of sodium benzoate, and 1 mg of sodium saccharin and sorbitol [7,23,24].

Animal experiments and the real-time polymerase chain reaction (RT-PCR):

Since the mouthwash produced here has a different composition and a new concentration of propolis compared to the types prepared before [23,24] and available in the market (30%; Soren Tech Toos Co., Mashhad, Iran), the clinical trial was conducted on animals. The animal study was conducted on the oral microbial flora of laboratory rats. A total of 52 one-month-old female rats (Wistar rats) with weights of 80 to 120 g and ages of 6-8 weeks were selected. The rats were put in special cages and were coded and kept at 25°C and 55% humidity for 12 hours in light and for 12 hours in darkness. The animal cages were cleaned twice a day. The rats had full access to food during the day, but they were only allowed to drink three times per day. First, saliva was collected from all rats before using the mouthwash. The saliva was collected from the sublingual areas and the oral mucosa using a 2-ml syringe. One ml of saliva was transferred to a microtube, centrifuged at 1000×g at 4°C, and washed twice with phosphate-buffered saline (PBS) [25]. The DNA of the bacteria was isolated according to the manufacturer's instructions using the EZ1 DNA Tissue Kit (Qiagen, Hombrechtikon, Switzerland). The resulting compound was used as a sample for measuring the number of bacteria using the RT-PCR method.

Quantitative RT-PCR (qRT-PCR) was conducted on a volume of 20 µl containing 10 µl of 2×SYBR Premix Dimer Eraser (Takara Bio Inc., Otsu, Shiga, Japan), 0.4 µM of forward primer, 0.4 µM of reverse primer, 0.4 µl of 50X ROX™ Reference Dye (Takara Bio Inc., Otsu, Shiga, Japan), and 2.5 µl of template DNA. All qRT-PCR tests were repeated three times [26,27]. The thermal cycles for all evaluations were as follows:

A 2-minute cycle at 95°C, followed by 40 cycles of 5-second denaturation at 95°C, primer annealing for 30 seconds at 60°C for *S. aureus* and *E. faecalis* and at 57.5°C for *S. mutans* and *L. acidophilus*, and finally, a 30-second expansion at 60°C. The results of the qRT-PCR test were reported as the logarithm of the CFU/ml [28].

The rats were randomly assigned to four groups of 13 such that the supervisor and the person conducting the test were blind to the grouping of animals. Each rat used drinking water only three times a day.

On the third day, in the first group, drinking water was included in all meals (the control group). In the second group, 50 ml of 0.12% CHX (Vi-One, Rozhin Co., Tabriz, Iran) was used once a day as the drink for rats [29].

In the third group, 50 ml of Listerine (TOTAL CARE, Johnson & Johnson S.p.A., Pomezia, Italy)

was used once as the drink for rats. In the fourth group, 50 ml of the produced propolis mouthwash was used as the drink for rats. After 12 hours, one week, and two weeks of using the mouthwashes, saliva was sampled again.

Statistical analysis:

Data were entered into SPSS 20 software (SPSS Inc., Chicago, IL, USA), and the numbers of the bacteria at each stage after the use of the studied mouthwashes were compared using analysis of variance (ANOVA). One-way ANOVA and repeated measures ANOVA were used to compare the changes in the number of each bacterium at each stage of the study and for each mouthwash. The animals were weighed daily, and the changes in their weight were assessed by one-way ANOVA.

RESULTS

The MICs determined for each bacterium are shown in Table 2.

Table 2: Minimum inhibitory concentration (MIC) of the alcoholic propolis extract for each bacterium

Bacteria	MIC (µg/ml)
Staphylococcus aureus	150
Streptococcus mutans	300
Lactobacillus acidophilus	600
Enterococcus faecalis	300

Figures 2 to 5 show the culturing of bacteria with and without exposure to propolis extract. Figure 6 shows the changes in the average number of *S. aureus* colonies in each group, and Table 3 shows the results of repeated measures ANOVA for *S. aureus* in different groups at different time points. Figure 7 shows the changes in the number of *S. mutans* colonies in each group, and Table 4 shows the results of repeated measures ANOVA for *S. mutans* in different groups at different time points. Figure 8 shows the changes in the number of *L. acidophilus* colonies in each group, and Table 5 shows the results of repeated measures ANOVA for *L. acidophilus* in different groups at different time points. Figure 9 shows the changes in the number of *E. faecalis* colonies in each group, and Table 6 shows the results of repeated measures ANOVA for *E. faecalis* in different groups at different time points.

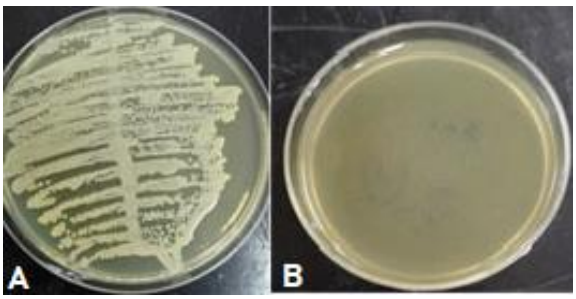


Fig. 2: (A) Cultivation of *Staphylococcus aureus* under normal conditions without propolis extract. (B) Cultivation of *Staphylococcus aureus* in a medium containing 150 µg/ml of ethanolic extract of propolis (EEP)

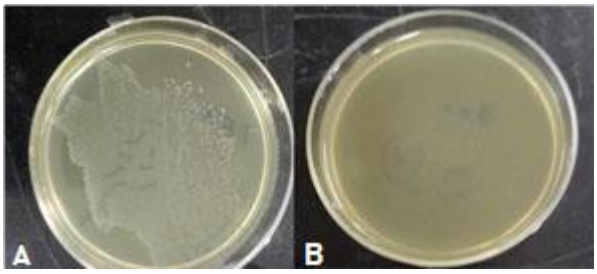


Fig. 3: (A) Cultivation of *Streptococcus mutans* under normal conditions without propolis extract. (B) Cultivation of *Streptococcus mutans* in a medium containing 300 µg/ml of ethanolic extract of propolis (EEP)

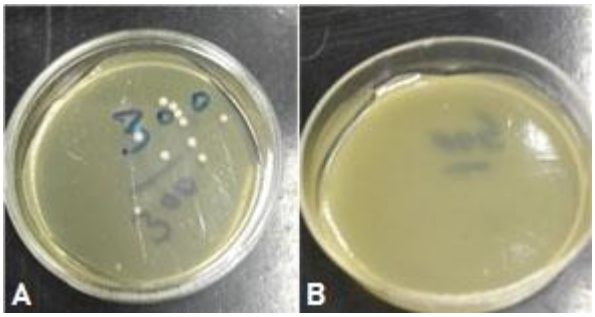


Fig. 4: (A) Cultivation of *Lactobacillus acidophilus* under normal conditions without propolis extract. (B) Cultivation of *Lactobacillus acidophilus* in a medium containing 300 µg/ml of ethanolic extract of propolis (EEP)

One-way ANOVA did not show any significant difference in the baseline level of *S. mutans* (P=0.843), but the difference in the baseline levels of *S. aureus*, *E. faecalis*, and *L. acidophilus* was significant among the groups (P=0.001, 0.002, and 0.008, respectively). Water did not reduce the number of the bacteria, and there was a significant increase in bacterial levels (P<0.05). CHX caused more reduction in the number of *S. aureus* than did Listerine (P=0.027), but the difference was not significant with propolis (P=0.110).

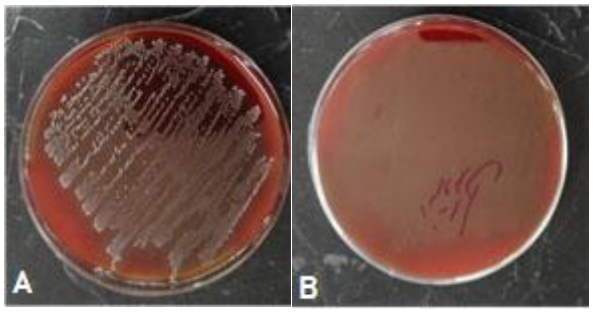
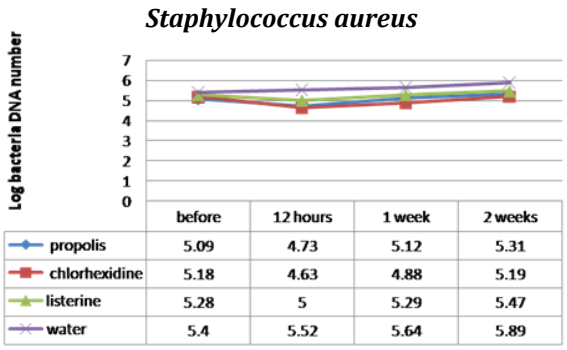
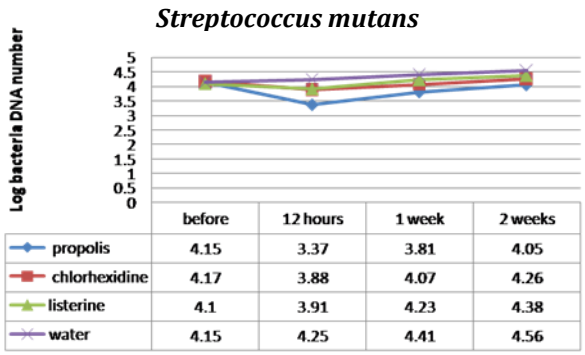


Fig. 5: (A) Cultivation of *Enterococcus faecalis* under normal conditions without propolis extract. (B) Cultivation of *Enterococcus faecalis* in a culture medium containing 300 µg/ml of ethanolic extract of propolis (EEP)



	before	12 hours	1 week	2 weeks
propolis	5.09	4.73	5.12	5.31
chlorhexidine	5.18	4.63	4.88	5.19
listerine	5.28	5	5.29	5.47
water	5.4	5.52	5.64	5.89

Fig. 6: Changes in the number of *Staphylococcus aureus* in each group according to the real-time polymerase chain reaction (RT-PCR)

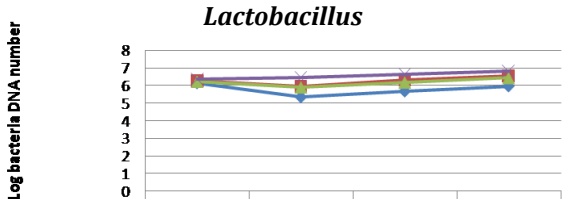


	before	12 hours	1 week	2 weeks
propolis	4.15	3.37	3.81	4.05
chlorhexidine	4.17	3.88	4.07	4.26
listerine	4.1	3.91	4.23	4.38
water	4.15	4.25	4.41	4.56

Fig. 7: Changes in the number of *Streptococcus mutans* in each group according to the real-time polymerase chain reaction (RT-PCR)

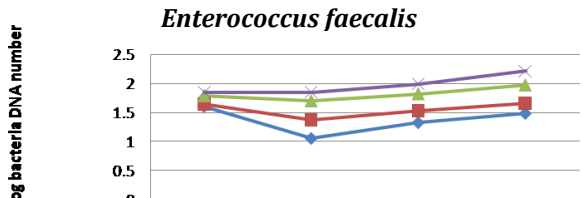
Unlike the Listerine and propolis groups, the number of *S. aureus* in the CHX group returned to the baseline level after two weeks (P=1.00; Table 2). In the Listerine and propolis groups, the number of *S. aureus* returned to the baseline level after one week (Fig. 6).

Regarding *S. mutans*, propolis was more efficient than other mouthwashes and resulted in a greater reduction in the number of *S. mutans* than did CHX and Listerine (P=0.024 and 0.001, respectively).



	before	12 hours	1 week	2 weeks
propolis	6.14	5.35	5.68	5.97
chlorhexidine	6.25	5.95	6.29	6.53
listerine	6.22	5.89	6.18	6.43
water	6.37	6.48	6.64	6.82

Fig. 8: Changes in the number of *Lactobacillus acidophilus* in each group according to the real-time polymerase chain reaction (RT-PCR)



	before	12 hours	1 week	2 weeks
propolis	1.6	1.05	1.32	1.48
chlorhexidine	1.64	1.37	1.53	1.66
listerine	1.79	1.7	1.82	1.97
water	1.85	1.85	1.99	2.21

Fig. 9: Changes in the number of *Enterococcus faecalis* in each group according to the real-time polymerase chain reaction (RT-PCR)

Contrary to the Listerine group, the number of *S. mutans* in the propolis and CHX groups returned to the baseline level after two weeks (P=0.645 and 0.056, respectively; Table 3).

After one week, the number of *S. mutans* colonies did not reach the baseline level in the propolis group, while in the Listerine and CHX groups, it reached the baseline level after one week (Fig. 7).

Regarding *L. acidophilus*, propolis was more efficient than other mouthwashes and resulted in a greater reduction in the number of *L. acidophilus* colonies than did CHX and Listerine (P<0.001). Contrary to the Listerine and CHX groups, the number of *L. acidophilus* colonies in the propolis group did not return to the baseline after two weeks (P<0.001; Table 4).

The number of *L. acidophilus* colonies did not reach the baseline level in the propolis group after one week, while in the Listerine and CHX groups, it reached the baseline level after one week (Fig. 8). Regarding *E. faecalis*, propolis was more effective than other mouthwashes and

resulted in a greater reduction in the number of *E. faecalis* colonies than did CHX and Listerine (P=0.003 and <0.001, respectively). Unlike the Listerine group, the number of *E. faecalis* colonies in the propolis and CHX groups returned to the baseline level after two weeks, (P=0.198 and 1.00, respectively; Table 5). The number of *E. faecalis* colonies did not reach the baseline level in the propolis group after one week, in contrast to the Listerine group (Fig. 9).

DISCUSSION

The aim of the current study was to evaluate the antibacterial activity of propolis mouthwash against oral bacteria in rats without the use of any mechanical cleansing methods. Propolis is a natural plant-derived resin produced by bees from flowers, pollen, branches, and leaves of plants and is used for filling the pores of the hive and for protecting the colonies from diseases [30,31].

Table 3: P values of comparing *Staphylococcus aureus* in different groups between different time points

Group	Time	Before	12 hours	1 week	2 weeks	Total
Propolis	Before	---	<0.001	0.482	<0.001	0.001
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	0.482	<0.001	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	
Chlorhexidine	Before	---	<0.001	0.008	1.00	0.012
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	0.008	<0.001	---	<0.001	
	2 weeks	1.00	<0.001	<0.001	---	
Listerine	Before	---	<0.001	1.00	<0.001	0.375
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	1.00	<0.001	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	
Water	Before	---	0.032	0.020	<0.001	0.013
	12 hours	0.032	---	0.561	<0.001	
	1 week	0.020	0.561	---	<0.001	
	2 weeks	<0.001	0.001	<0.001	---	

Table 4: P values of comparing *Streptococcus mutans* in different groups between different time points

Group	Time	Before	12 hours	1 week	2 weeks	Total
Propolis	Before	---	<0.001	0.007	0.645	0.008
	12 hours	<0.001	---	0.005	<0.001	
	1 week	0.007	0.005	---	0.001	
	2 weeks	0.645	<0.001	0.001	---	
Chlorhexidine	Before	---	0.003	0.017	0.056	0.001
	12 hours	0.003	---	0.004	<0.001	
	1 week	0.017	0.004	---	<0.001	
	2 weeks	0.056	<0.001	<0.001	---	
Listerine	Before	---	<0.001	0.019	<0.001	<0.001
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	0.019	<0.001	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	
Water	Before	---	0.097	<0.001	<0.001	0.014
	12 hours	0.097	---	<0.001	<0.001	
	1 week	<0.001	<0.001	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	

Table 5: P values of comparing *Lactobacillus acidophilus* in different groups between different time points

Group	Time	Before	12 hours	1 week	2 weeks	Total
Propolis	Before	---	<0.001	<0.001	0.002	<0.001
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	<0.001	<0.001	---	<0.001	
	2 weeks	0.002	<0.001	<0.001	---	
Chlorhexidine	Before	---	<0.001	0.818	<0.001	0.042
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	0.818	<0.001	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	
Listerine	Before	---	<0.001	0.902	<0.001	0.014
	12 hours	<0.001	---	<0.001	<0.001	
	1 week	0.902	<0.001	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	
Water	Before	---	<0.001	<0.001	<0.001	0.001
	12 hours	<0.001	---	0.002	<0.001	
	1 week	<0.001	0.002	---	<0.001	
	2 weeks	<0.001	<0.001	<0.001	---	

Dental caries develops due to acid production by bacteria through the dissolution of carbohydrates, which creates a cavity in the tooth, leading to the loss of the dental crown [32]. Non-restorative treatments for caries aim to disrupt the decay process, particularly on smooth dental surfaces [33], and involve chemical and mechanical disorganization of biofilms by compounds such as fluoride and antimicrobial agents [34,35]. These treatments maintain the wholeness of the tooth and demonstrate adequate efficiency [36].

On the other hand, bacterial resistance to synthetic antibiotics has encouraged researchers to use natural drugs [37]. The properties of propolis have made it a natural antibacterial agent although the mechanism of its effect is unknown. It is probable that inactivation of RNA polymerase and direct damage to the cell membrane lead to functional and structural damage to the bacteria [38-40]. Since most ingredients of propolis are soluble in alcohol, the alcoholic propolis extract is more effective [41,42].

Therefore, in the present study, the alcoholic extract of propolis was used. However, the presence of alcohol in mouthwashes is problematic due to social (religious) issues as well as certain complications such as burning sensation, mucosal sensitivity, dental discoloration, and increased risk of oral cancer [43]. Therefore, in the present study, the alcoholic extract of propolis at the lowest concentration of alcohol has been used to minimize the complications. Nevertheless, as the concentration of propolis increases in the mouthwash, the taste gets worse and the color gets blurrier, which are not appealing to the patients [23]. Therefore, in this study, we tried to use the lowest effective concentration of propolis.

S. mutans and *L. acidophilus* are the most important microorganisms associated with caries. *S. mutans* is associated with the onset of caries, whereas *L. acidophilus* is associated with its progression [44,45]. Some researchers consider the presence of *S. mutans* as a predictor of caries [46,47]. *S. aureus* and *E. faecalis* are part of the normal flora and are resistant to methicillin and vancomycin antibiotics, respectively [48]. *E. faecalis* is involved in 80% of endodontic infections and root canal therapy failures and can survive without the support of other bacteria [49]. Previous studies on the effectiveness of mouthwashes have mainly focused on plaque accumulation [50-52], whereas saliva is easier to access and can be used to clearly determine the oral microbial population [53]. It can also be used for screening caries and periodontal disease [54]. Therefore, in the present study, a combination of four bacterial species that are present in the normal flora of the mouth was studied. Since propolis at a new concentration was used in the mouthwash produced in the present study, the mouthwash was tried on rats as it was not ethical to try it on humans. The RT-PCR was used to investigate the number of bacteria as it is a reliable, fast, and sensitive method [55,56]. Although it requires a specific primer for each bacterium, it is more sensitive than the conventional culture method. Moreover, in comparison with the usual PCR, this

method requires less material, and the analysis is performed automatically. Although the agar dilution MIC test is a routine method for analyzing the antibacterial properties of materials, the interactions between various components of the culture medium prevent the correct interpretation of the results. Nevertheless, it is still the most reliable and easy method for interpreting the antibacterial properties [57].

In the present study, the effect of mouthwashes was evaluated for 2 weeks to investigate their long-term effects without the aid of mechanical methods as previous studies have shown different results for the longevity of the effect of mouthwashes [58,59].

The results of the agar dilution test showed the lowest MIC for *S. aureus* and the highest for *L. acidophilus*. These results are consistent with the findings of a study by Acka et al [19] and suggest that propolis is more effective on gram-positive bacteria. The reason for its lower effect on gram-negative bacteria is the presence of complex cell walls in these bacteria [19].

The results of the present study showed that water had no effect on the level of oral bacteria. Regarding *S. mutans*, *E. faecalis*, and *L. acidophilus*, propolis mouthwash showed a significant difference with CHX and Listerine, and after two weeks, the bacterial level in this group was still lower than the baseline level, while CHX and Listerine were less effective. As for *S. aureus*, there was no significant difference between CHX and propolis, but with CHX, the bacterial level did not reach the baseline level after two weeks, whereas in the propolis group, it reached the baseline level after one week.

Although CHX bonds to oral structures and slowly releases in the oral environment and has a long-lasting effect [60], the present study showed propolis mouthwash to have more long-lasting effects and a higher efficacy compared to CHX. Anauate-Netto et al [61] suggested that 2% propolis mouthwash is stronger than 0.12% CHX and has a 45-day lasting effect.

Suleman et al [62] demonstrated the effectiveness of the alcoholic propolis extract on *S. aureus* and *E. faecalis*. Vasconcelos et al [63] also showed the positive effect of propolis mouthwash on *S. aureus*, *S. mutans*, and E. faecalis. Santiago et al [64] showed that propolis mouthwash has antibacterial properties similar to those of CHX. These results were confirmed by Bazvand et al [65], Mohan et al [66], Carbajal Mejia [67], and Acka et al [19].

However, Nagappan and John [68], Malhotra et al [69], and Bhandari et al [70] suggested that CHX is more effective than propolis mouthwash. The difference between the results of the mentioned studies can be attributed to the difference in the formula and properties of the studied propolis. The difference in the region where propolis is collected, the season in which propolis is collected, contamination with wax, and the bee species all lead to differences in the properties of propolis. Meanwhile, differences in the microbiological examination methods, including the type of bacteria, the phase of cell differentiation, culturing conditions, the interval and the duration of drug use, and the design of the study are other reasons for the differences.

The limitations of the present study include the small sample size and considering only four species of normal bacterial flora, which might not show the full effect of mouthwashes on all bacteria. Therefore, it is recommended to conduct similar studies with larger sample sizes in order to assess the level of other oral bacteria in humans. If the results of the present study are confirmed by further studies, it can be concluded that treatment with propolis mouthwash can reduce periodontal infections, gingivitis, and primary and secondary oral infections. Considering its availability in Iran, cheap price, acceptable taste and smell, easy usage, and being non-chemical, it is well accepted among Iranian patients.

CONCLUSION

The mouthwash produced in the present study was more efficient than CHX mouthwash against *E. faecalis*, *L. acidophilus*, and *S. mutans*. It also showed similar results to CHX against *S. aureus*. Listerine was less efficient than CHX and propolis.

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