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ORIGINAL RESEARCH

In Vitro Effectiveness of Antimicrobial Properties of Propolis and Chlorhexidine on Oral Pathogens: A Comparative Study

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ABSTRACT

Periodontal disease is one of the most prevalent infectious oral conditions in the present century, and it is necessary to conduct research to find a solution to overcome these diseases. A variety of microbial strains of bacteria and fungi are involved in the pathogenesis of periodontal disease. The use of chemical agents such as mouthwashes is one of the strategies to control these diseases. The purpose of the present study was to compare the antimicrobial effects of propolis and chlorhexidine gluconate (CHX) on the bacterial strains of *Streptococcus mutans*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and the yeast strain of *Candida albicans* using the broth microdilution method. The results showed the inhibitory and microbicidal activities of the two substances against the tested microbial strains. The antibacterial and antifungal effects of CHX were more effective reported in this study than that of propolis against the studied pathogens. The results of this study also indicated that the propolis was less effective in inhibiting bacterial growth than the CHX. In addition, the combination of these two solutions had a synergistic effect on inhibition of other studied strains, with the exception of *C. albicans* and *S. aureus*. There is a need for further research on strains isolated from oral biofilm to achieve complementary results.

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Introduction

Oral health reflects the systemic health of the human body, and also the health of oral system as a whole affects the general health of the body (Hagh Negahdar et al., 2017). There are more than 700 types of microorganisms living in the mouth, most of which are part of the normal flora, and some of which are pathogenic microorganisms (Mehdipour et al., 2018). Microbial plaque accumulation is the most important stage of pathogenicity of these microorganisms in the teeth and their supporting tissues. Therefore, microbial plaque removal is the best strategy to prevent periodontal disease and dental caries. The first and most effective approach to maintain oral health and prevent oral and dental diseases and systemic diseases of oral disease origin is to remove this microbial plaque using mechanical methods such as toothbrush and floss (Newman et al., 2011).

Periodontitis is a microbial and inflammatory disease that begins with microbial biofilm formation and leads to loss of connective tissue attachment on the root surface. Inflammation and the immune response are associated with bacteria that have been colonized in the periodontium and surrounding tissues (Camacho-Alonso et al., 2017).

Microbial cells that grow in a biofilm are physiologically distinct from the planktonic cells. Planktonic bacteria are free-living bacteria, which grow in test tubes and culture media in the microbiology laboratory. Accumulation of planktonic strains may be involved in the development of periodontal disease. They are needed for biofilm formation (O'Toole & Kolter, 1998).

The goal of periodontal treatment is the elimination of microorganisms and their products and the factors causing periodontal disease spread. Scaling, root planing and health education are the foundation of periodontal treatment (Bhandari et al., 2014). There are many surgical and non-surgical methods for treating periodontal disease. Subgingival scaling is the most important clinical procedure whose effectiveness has been proven in studies (Haffajee et al., 1997; Herrera et al., 2002; Saha et al., 2015).

However, the removal of microbial plaque and oral pathogens using toothbrushes and floss for prevention, as well as the use of scaling and root planing to treat periodontal disease are often not performed completely (Lang & Brecx, 1986; Martins et al., 2019). Studies show that the use of chemical agents can increase the efficiency of mechanical methods through antimicrobial effects (Ebrahimi et al., 2014; Maza et al., 2002; Shen et al., 2010).

Products with antibacterial activity, such as mouthwashes, can be used as an adjunct to oral hygiene measures due to their ability to control biofilm formation (De Luca et al., 2017). The mouthwashes are the most common topical chemicals to prevent the microbial biofilm formation. Chlorhexidine is a broad-spectrum antiseptic solution effective against gram-negative and -positive bacteria and has been applying in the treatment of periodontal disease for many years. The chlorhexidine has a cationic component capable of binding to some negatively charged areas of the cell membrane causing cell lysis (Costa et al., 2012).

The effect of chlorhexidine on the prevention of growth, proliferation and adhesion of Candida albicans has been proven in many studies (Langslet et al., 1974; Pereira-Cenci et al., 2008), while some studies ruled out its effectiveness (Barkvoll & Attramadal, 1989; Ferretti et al., 1987). The chlorhexidine has been commonly used in the community due to its broad-spectrum antibacterial effects, excellent biocompatibility (at low concentrations and short-term consumption periods), reasonable price, availability and controlled release (Xiao et al., 2014).

Dentists often prescribe chlorhexidine to prevent plaque from progressing (Overholser et al., 1990). However, the cytotoxic properties at high concentrations and long-term usage (Gürgan et al., 2006; Mariotti & Rumpf, 1999; Varoni et al., 2012) and side effects are the main disadvantages of chlorhexidine that limit the utilization of this drug. In addition, the chlorhexidine causes discoloration in tooth (Al-Tannir & Goodman, 1994; Prasanna & Lakshmanan, 2016) and composite restorations (Ardu et al., 2010; Diab et al., 2007). Some manufacturers try to use naturally plant-derived ingredients and extracts to fabricate oral care products to prevent the side effects of synthetic products. Among these natural products, propolis stands out due to its antimicrobial activity against a wide range of gram-positive and gram-negative pathogenic microorganisms (Koo et al., 2000; Koru et al., 2007; Mehdipour et al., 2019; Santos et al., 2002).

Propolis is a naturally occurring resinous-like substance produced by bees by combining wax and saliva with resins collected from plants (Coleman et al., 2010). Various studies have reported antibacterial, antifungal and antitumor properties for the propolis. Due to the differences in plants in different regions, the composition of propolis varies in different regions, which causes various properties of the substance (Dettenkofer et al., 2004; Prospero et al., 2003).

Although this product has been accepted in traditional medicine, it has recently been reconsidered by researchers (Burdock, 1998). The use of propolis against a wide range of oral bacteria may be beneficial for improving oral health. In addition, the current view is that the use of standard propolis mouthwash is safer and less toxic than many other synthetic mouthwashes (Björkner, 1994; Dobrowolski et al., 1991; Hausen et al., 1987; Ikeno et al., 1991; Sonmez et al., 2005). This substance is used for surgical procedures, endodontic treatment, direct and indirect pulp capping procedures, removal of dentin hypersensitivity, etc. (Jaiswal et al., 2017). The present in vitro study aimed to compare the effectiveness of the antimicrobial properties of propolis and chlorhexidine.

According to studies (Akca et al., 2016; Carbajal Mejía, 2014; Cockerill, 2011; Gebara et al., 2002; Jaiswal et al., 2017; Santos et al., 2002; Singh et al., 2019), both chlorhexidine and propolis have antimicrobial effects. Due to the prevalence of periodontal disease and chlorhexidine-induced side effects and considering the differences in propolis compositions (thus differences in its antimicrobial properties) in different regions, this in vitro study investigates the effectiveness of antimicrobial properties of native propolis and chlorhexidine. If the antimicrobial effects of native propolis on oral pathogens are successful, it can be considered as an alternative to chlorhexidine.

Materials and methods

Preparation and cultivation of microbial strains

In order to prepare standard strains, the lyophilized bacterial and yeast samples were ordered from the National Center for Genetic and Biological

Reservoirs of Iran and the National Cell Bank of the Pasteur Institute of Iran. The identification code for each strain is shown in Table 1.

Table 1. List of microbial strains used and their identification code.

Identification Code	Microbial strains
ATCC 29212	*Enterococcus faeclis*
ATCC 19615	*Streptococcus pyogenes*
ATCC 35668	*Streptococcus mutans*
ATCC 9027	*Pseudomonas aeruginosa*
PTCC 1112	*Staphylococcus aureus*
ATCC 10231	*Candida albicans*

Each strain was cultured under standard conditions. All strains were first inoculated onto Tryptic Soy Broth (TSB) medium to activate their physiological conditions and growth. *Streptococcus pyogenes* and *Streptococcus mutans* were incubated under anaerobic conditions (5-10% Co2) and other strains were incubated under aerobic conditions for 24 hours at 37°C. Determination of aerobic or anaerobic conditions goes back to the nature of the microorganism. The conditions mentioned for each strain are essential for the proper growth of that strain. After initial incubation, by confirming the growth of the strains (turbidity of the broth medium), the culture medium containing the grown strains was cultured as a loop full of inoculated medium onto the Tryptic Soy Agar (TSA) medium under incubation conditions to confirm the purity of the strains.

To maintain the strains, a colony of each strain was first inoculated in 5 ml of Nutrient Broth (NB) or TSB medium, and the inoculated culture medium was placed in an incubator shaker to obtain the optical density (OD) of 0.4-0.6 nm at a wavelength of 600 nm. Then, 200 µl of sterile glycerol was poured into the test tube, and 800 µl of bacterial suspension was added, and kept at -70°C.

Evaluation of the antimicrobial effect of propolis and chlorhexidine 0.2% on the study microbial strains

Disk diffusion test was performed to evaluate the susceptibility of selected standard strains to conventional antibiotics (amoxicillin, penicillin, metronidazole and nystatin) in the treatment of oral infections (including periodontitis), and the antibiotic sensitivity and resistance patterns were determined according to the CLSI M100-S29 standards (Gebara et al., 2002).

Determination of minimum inhibitory concentration (MIC) of planktonic cells by broth microdilution method

Microdilution in microtiter plate was used to determine the MIC of propolis and chlorhexidine 0.2% on the growth of planktonic cells. The MIC value was determined according to the CLSI M100-S29 standards (Gebara et al., 2002).

The microbial strains were removed from the freezer of -70°C and cultured onto Mueller Hinton Agar (MHA) medium.

After overnight incubation period at 37°C, several colonies were taken from the culture medium of each bacterium by loop and incubated in 5 ml of Mueller Hinton Broth (MHB) medium overnight at 37°C and 150 rpm. Using sterile MHB medium, a dilution equivalent to 0.5 McFarland Turbidity Standard was obtained from each of the studied strains (the OD of suspension was set in the range of 0.08 to 0.13 using a spectrophotometer at 600 nm). The obtained dilutions were diluted 150 times using sterile normal saline.

Each microtiter plate well was added by 100 µl of sterile MHB medium, and of chlorhexidine and propolis solutions at concentrations of 2 mg / ml and 30 mg / ml in the dilution series of 1-500 µg/ml and 1.83-7500 µg/ml, respectively from the desired solutions was prepared in each row from the plate (10 wells). Then, the suspension equivalent to 0.5 McFarland Turbidity Standard, diluted 150 times, was added to each well with a volume of 100 µl. The wells 11 and 12 were considered as bacterial growth control (positive control) and medium sterility control (negative control), respectively. After the incubation time (24 hours at 37°C), the bacterial growth rate in the wells was examined. The lowest dilution of solution that inhibited bacterial growth was considered as MIC (Gebara et al., 2002).

Determination of minimum bactericidal concentration (MBC) of planktonic cells

To determine the MBC value for the study bacteria, 10 µl of contents from the turbidity-free wells were cultured onto MHA medium and incubated at 37°C for 37 hours (Gebara et al., 2002).

Evaluation of the synergistic effect using Checkerboard method in inhibiting the growth of planktonic cells of the studied strains

The synergistic effect of chlorhexidine and propolis on inhibiting the growth of planktonic cells of strains was evaluated by Checkerboard dilution

technique. This method has been developed to investigate the interaction of antibiotics. In this method, each horizontal row was assigned to a density of chlorhexidine and each vertical row to a density of propolis. The desired dilutions of chlorhexidine and propolis were prepared separately as diluents using TSB culture medium. The obtained MIC was used for each of the solutions as the dilution center and the dilutions of 1.2, 1.4, 1.8 and 1.16 and 2, 4 and 8 times of the desired MIC was presumed as the synergy test (Figure 1). Each microtiter plate well was added by 50 µl of each dilution of the two solutions examined. Finally, 100 µl of bacterial suspension was added to the wells. Each plate contained positive and negative controls. The prepared microtiter plates were incubated at 37°C for 24 hours and then used to determine the MIC value.

	1	2	3	4	5	6	7	8	9	10	11	12
A	GC	A 0.5	A 1	A 2	A 4	A 8	A 16	A 32	A 64	A 128	A 256	A 512
B	B1	A 0.5 + B1	A 1 + B 1	A 2 + B 1	A 4 + B 1	A 8 + B 1	A 16 + B 1	A 32 + B 1	A 64 + B 1	A 128 + B 1	A 256 + B 1	A 512 + B 1
C	B2	A 0.5 + B2	A 1 + B 2	A 2 + B 2	A 4 + B 2	A 8 + B 2	A 16 + B 2	A 32 + B 2	A 64 + B 2	A 128 + B 2	A 256 + B 2	A 512 + B 2
D	B4	A 0.5 + B4	A 1 + B 4	A 2 + B 4	A 4 + B 4	A 8 + B 4	A 16 + B 4	A 32 + B 4	A 64 + B 4	A 128 + B 4	A 256 + B 4	A 512 + B 4
E	B8	A 0.5 + B8	A 1 + B 8	A 2 + B 8	A 4 + B 8	A 8 + B 8	A 16 + B 8	A 32 + B 8	A 64 + B 8	A 128 + B 8	A 256 + B 8	A 512 + B 8
F	B16	A 0.5 + B16	A 1 + B 16	A 2 + B 16	A 4 + B 16	A 8 + B 16	A 16 + B 16	A 32 + B 16	A 64 + B 16	A 128 + B 16	A 256 + B 16	A 512 + B 16
G	B32	A 0.5 + B32	A 1 + B 32	A 2 + B 32	A 4 + B 32	A 8 + B 32	A 16 + B 32	A 32 + B 32	A 64 + B 32	A 128 + B 32	A 256 + B 32	A 512 + B 32
H	B64	A 0.5 + B64	A 1 + B 64	A 2 + B 64	A 4 + B 64	A 8 + B 64	A 16 + B 64	A 32 + B 64	A 64 + B 64	A 128 + B 64	A 256 + B 64	BC

Figure1. Sample of Microdilution checkerboard.

Statistical analysis

Significant differences were determined by one-way analysis of variance (ANOVA) with pairwise comparisons using Tukey's test. The $p \leq 0.05$ was considered statistically significant. The statistical analysis was performed by Prism5 and Minitab17 software.

Results

Results of determining the antibiotic susceptibility pattern of selected strains using disk diffusion test

The disk diffusion test was performed to evaluate the susceptibility of selected strains to conventional antibiotics in the treatment of oral infections, and the antibiotic sensitivity and resistance pattern was determined according to CLSI M100-S29 (Gebara et al., 2002). The results showed that the studied strains were sensitive, resistant or intermediate resistance to antibiotics. Among these, all bacterial strains were sensitive and intermediate resistance to the antibiotics of amoxicillin, penicillin and metronidazole. The bacterial strains were resistant to the nystatin, while the fungal strains were sensitive to nystatin, penicillin and metronidazole and resistant to amoxicillin.

Results of determining the MIC value for planktonic cells using broth microdilution method

Table 2 shows the results of this study.

Results of determining the MBC value for planktonic cells

Table 3 shows the MBC values in the turbidity-free wells onto the MHA mediums.

Results of determining the synergistic effects using checkerboard method on MIC and MBC values for planktonic cells of selected strains

The tests were performed according to certain concentrations and ΣFMIC value was calculated for wells with no growth. The results of determining the synergy of chlorhexidine and propolis are shown in Table 4. Moreover, the content of wells with no growth was cultured and ΣFMBC value was calculated. The results showed that ΣFMBC = ΣFMIC. Additionally, no antagonistic effect was observed in any of the tests performed on the studied microorganisms.

Table 2. MIC value of Propolis and Cholorhexidine in MHB medium for studied strains.

Anti microblal agent	***Concentration range µg/ml***	***MIC (µg/ml)***					
		P.aeroginosa	***E.faecalis***	***S.mutans***	***S.pyogenes***	***S.aureus***	***C.albicans***
cholorhexidine	1-500	7.81	3.9	31.25	125	62.5	125
propolis	1.83-7500	7500	3750	1875	937.5	937.5	937.5

Table 3. MBC values of antibiotics and lyase enzyme for studied strains.

Anti microblal agent	***Concentration range µg/ml***	MBC(**µg/ml**)					
		P.aeroginosa	***E.faecalis***	***S.mutans***	***S.pyogenes***	***S.aureus***	***C.albicans***
cholorhexidine	1-500	62.5	31.25	=MIC	=MIC	125	=MIC
propolis	1.83-7500	=MIC	=MIC	=MIC	1875	3750	=MIC

Table 4. Results of synergy in MIC value of chlorhexisine and propolis in studied strains.

Microorganisms	**MIC value of studied solutions alone**	**MIC value of studied solutions in combinations**	**Synergic effect**
P.aeroginosa	Chlorhexidine 7.81µg/ml	1/95µg/ml	Yes
	Propolis7500µg/ml	937.5 µg/ml	
E.faecalis	Chlorhexidine 3.9 µg/ml	0.97 µg/ml	Yes
	Propolis 3750 µg/ml	1875 µg/ml	
S.mutans	Chlorhexidine 31.25 µg/ml	7.81 µg/ml	Yes
	Propolis 1875 µg/ml	234 µg/ml	
S.pyogenes	Chlorhexidine 125 µg/ml	15.62 µg/ml	Yes
	Propolis 937.5 µg/ml	234.3 µg/ml	
S.aureus	Chlorhexidine 62.5 µg/ml	31.25 µg/ml	Indifferent
	Propolis 937.5 µg/ml	468.75 µg/ml	
C.albicans	Chlorhexidine 125 µg/ml Propolis 937.5 µg/ml	125 µg/ml 937.5 µg/ml	Indifferent

Discussion

According to the results of this study, the chlorhexidine was more successful than the propolis, but both solutions showed the inhibitory and microbicidal effect against the studied microorganisms. The results showed that the combination of these two solutions had a synergistic effect on inhibition of other strains under study, with the exception of *C. albicans* and *S. aureus*. The results of this study showed that the propolis and the chlorhexidine have a wide range of inhibitory effects on the planktonic strains tested. The planktonic bacteria are free living bacteria. They are populations that grow in test tubes and culture media in a microbiology laboratory, and are not taken from the oral biofilms.

The antibacterial effects of propolis against microorganisms can be complex, leading to the degradation of the cytoplasm, cytoplasmic membrane and cell wall, partial decomposition of bacteria and inhibition of protein synthesis (Takaisi-Kikuni & Schilcher, 1994). Mello et al reported that the pH and concentration of propolis may change due to the action of solvents, and that acidic propolis solutions are more effective on bacteria (Mello & Hubinger, 2012). In addition, the cell wall of bacteria and their biofilm properties have been suggested as adjunctive factors, which determine the antibacterial effect of propolis (Duarte et al., 2003; Lemos & Burne, 2008; Quivey Jr et al., 2000). Therefore, the propolis can act against any microorganism in different ways.

Slight differences between our MIC and MBC values and those found in other studies may be due to differences in tolerance of strains or the origin of propolis specimens, as the composition of propolis depends on regional variation (Marcucci, 1995). Samples of propolis taken from poplar sprouts, which seem to be the predominant sources of propolis, in temperate regions (Asia, Europe, North America, etc.) mainly contain phenolic compounds, including several flavonoids, aromatic acids and their esters (Tomás-Barberán et al., 1993).

The MIC and MBC values determined in our study were consistent with other studies, which indicate that the planktonic gram-positive bacteria are more

susceptible to damage by propolis compared to the gram-negative bacteria. Kujumgiev (Kujumgiev et al., 1999) and Nieva (Moreno et al., 1999) stated that propolis can only be active against gram-positive bacteria and some fungi. Sforcin (Sforcin et al., 2000) and Dobrowolfski (Dobrowolski et al., 1991) reported less effect of propolis against gram-negative bacteria.

The results of this study demonstrated that propolis is effective on the pathogenic bacteria in concentrations tested less than CHX. Our results revealed that the current concentration of propolis may not be sufficient to show an antibacterial effect on the pathogenic bacteria. EralpAkca (Akca et al., 2016) found that the propolis acted similarly to chlorhexidine in inhibiting gram-positive strains. Carbajal (Carbajal Mejía, 2014) concluded that the propolis had a greater antifungal effect against *C. albicans* than the chlorhexidine. In a study of Elaine (Gebara et al., 2002), all strains tested were more sensitive to the propolis. The discrepancy between the results of our study and other cases may be related to factors confirming a relationship between the composition of propolis, their activity in different structures of the bacterial cell wall, and the cellular activity of cariogenic bacteria.

In the present study, all strains were sensitive to the propolis and the chlorhexidine. In the studies of Santos (Santos et al., 2002) and Argaval (Agarwal et al., 2012), all strains tested were sensitive to the propolis. These and many other studies examining the effects of the propolis on the periodontal pathogens have reported similar results to our study. Similar cell wall structures and extracellular polymeric substance (EPS) of the periodontal pathogens may explain why propolis has shown the same values of MIC and MBC.

In our study, the propolis showed a strong antifungal activity against Candida strains, consistent with a study of Kujumgiev (Kujumgiev et al., 1999). The CHX, which is a gold standard, was selected as an antiseptic agent in this study because of its broad effect on several microorganisms and its properties. The MBC values observed for bacteria and fungi showed a significant difference (P-value <0.05) in the resistance of microorganisms to the CHX or the propolis.

Conclusion

The results of this study revealed that propolis was less effective in inhibiting bacteria than CHX in their planktonic state and it was suggested that propolis could not be as effective as CHX on oral microorganisms in their planktonic state. Further research on species isolated from oral biofilm is needed to obtain complementary results.

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Conflict of interest

The authors declare no conflict of interest.

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