In Vitro and *In Vivo* Determination of Antimicrobial Activity of Mouthwash Solutions against Resident and Pathological Oral Microflora

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DOI: 10.60988/p.v36i4.43

ABSTRACT

The aim of this study was to investigate the bactericidal properties of four commercially available antiseptic mouthwashes containing chlorhexidine, cetylpyridinium chloride, alcohol with essential oils, propolis, menthol, xylitol and hydroxyapatite nanoparticles. We conducted a series of *in vitro* studies to monitor the antimicrobial properties of four mouthwashes with different active ingredients against representatives of the normal oral microflora (*S. mutans ATCC 35668)*, and potential pathogenic and conditionally pathogenic bacteria (*S. pneumoniae ATCC49619* , *S. epidermidis ATCC12228*, *S. aureus ATCC 29213*). In the second stage, we conducted *in vivo* tests with 24 volunteers, tracking the dynamics of the bactericidal effects of the four mouthwashes on the normal aerobic and facultatively anaerobic oral microflora. All mouthwashes demonstrated antimicrobial activity against Gram-positive bacteria tested. In most of the cases, an inhibitory effect was observed at all concentrations tested. Exceptions were mouthwash MW4 (P+M) mouthwash against *S. mutans* – MIC 1:2 and MW3 (HAN+X) against *S. epidermidis ATCC12228*– MIC 1:2. The results of our research show that the combinations of chlorhexidine digluconate + cetylpyridinium chloride and alcohol + essential oils have the most powerful antimicrobial action against aerobic and facultatively anaerobic oral microflora. With these combinations, we observed complete inhibition of microbial growth in a minimum of 30 minutes, after which the CFU/mL gradually increased, but within 8 hours of follow-up did not reach the initial baseline value (sample 0). Mouthwashes containing combinations of chlorhexidine digluconate/cetylpyridinium chloride, alcohol/essential oils, hydroxyapatite nanoparticles/xylitol and propolis/mentha viridis oil have strong antimicrobial effects against *S. aureus ATCC 29213*, *S. mutans ATCC 35668*, *S. pneumoniae ATCC49619,* and *S. epidermidis ATCC12228*– MICs from 1:2 to ≥1:8.

KEYWORDS:
mouthwash; antimicrobial properties; oral microflora

ARTICLE INFO:
Received: March14,2024
Revised: September 11, 2024
Accepted: September 14, 2024
Available on line: January 3, 2025

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Introduction

The normal oral microflora is presented by the species *Streptococcus*, *Lactobacillus*, Gram-negative *diplococcus*, *Actinomyces*, *Bacteroides*, *Fusobacterium,* and others. These strains have a protective role against pathogens from outside. However, colonization of oral bacteria on the surfaces of teeth can generate dental plaque formation, beginning with the accumulation of Gram-positive streptococci, developed by the aggregation of Gram-negative anaerobic bacteria[1]. Subsequently, the bacteria biofilm induces an inflammatory response and causes gingivitis, caries, and destruction of teeth and periodontal tissues[2]. The health of the oral cavity is not associated with achieving complete sterility; the ideal condition is to remove most cariogenic periodontopathic agents from dental plaque[1].

The primary method of preventing disease and maintaining good oral hygiene is to control plaque and mechanically prevent its accumulation on the teeth and adjacent gingival surfaces. Although mechanical methods such as brushing and flossing are considered the basis of plaque control, some antibacterial mouthwashes with topical or systemic effects can be prescribed as an alternative therapeutic aid. Mouthwashes can also inhibit dental plaque and are widely used to maintain oral hygiene. Mouth rinses containing antimicrobial compounds can inactivate bacteria that remain in the mouth after brushing and inhibit their regrowth and reattachment to the surface of the teeth[3]. A variety of antimicrobial formulations have been produced that incorporate different types of ingredients, such as chlorhexidine, ethanol, essential oils, propolis, and others.

Some of the most available commercially mouth rinse preparations contain *chlorhexidine digluconate at different concentrations in combination with cetylpyridinium chloride*. *Chlorhexidine* is a cationic antiseptic with broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, yeasts, and lipid-enveloped viruses. It increases the permeability of the cell membrane followed by coagulation of cellular macromolecules, it does not interact with any microbial enzymes or receptors, and therefore does not lead to resistance from organisms[4]. Chlorhexidine is the 'gold standard' or positive control compared to other substances due to its proven efficiency. However, with prolonged use of mouth rinses, some side effects such as oral mucosal erosion, brown discoloration, and bitter taste[5], increased rate of supragingival calculus formation have been reported[4]. Therefore, an alternative mouthwash is needed that could negate these side effects but is still effective equivalent to it.

Cetylpyridinium chloride is a broad spectrum antimicrobial agent used for both the reduction of malodor (halitosis) as well as for the treatment and prevention of plaque and gingivitis. His action is expressed in inhibiting enzymes associated with bacterial metabolism and alteration of gene expression of select pathogens. According to Sreenivasan [6], ex vivo tests on supragingival plaque microorganisms demonstrated significantly greater antimicrobial activity by the cetylpyridinium chloride-based mouth rinses (>90% killing, $p < 0.001$) and chlorhexidine rinse (>98% killing, $p<0.05$) compared to fluoride control mouthwash. Cetylpyridinium chloride 0,05% formulated with or without alcohol demonstrated broad-spectrum antimicrobial activity against laboratory strains and supragingival plaque bacteria.

Mouthwashes containing *ethanol and a combination of essential oils* are part of the home-care oral hygiene regimen. *Essential oils* kill microbes by disrupting their cell walls and inhibiting their enzymatic activity. They prevent bacterial aggregation, slow bacterial multiplication, and extract endotoxins[7]. Previous studies have found this type of mouthwash as an effective solution in the reduction of dental plaques and oral bacterial counts[8, 9, 10, 11].

Some mouthwashes formulated through innovative technology are available on the pharmaceutical market containing hydroxyapatite nanoparticles, xylitol, and sodium benzoate. Hydroxyapatite nanoparticles integrate with tooth enamel and fill irregular tooth surfaces, with a repairing and whitening action. It does not consist of typical antimicrobial compounds and during our investigation no in vivo studies on antibacterial efficacy were found. Xylitol reduces *Streptococcus mutans* levels in plaque and

saliva by disrupting their energy production processes, leading to futile energy cycle and cell death[12]. Reduce the adhesion of these microorganisms to the teeth surface, reduces their acid production potential, and breaks up contaminants biofilm[13, 14]. Sodium benzoate, which disperses carbohydrates, fats, and proteins, thereby weakens the attachment of plaque, which can then be easily removed by brushing the tooth.

Herbal mouthwashes do not contain alcohol and/or sugar, two of the most common ingredients found in other similar products. These ingredients can be fermented from microorganisms that cause bad breath and halitosis[15]. Some of these mouthwashes contain propolis and menthol, which is used in mouthwashes and toothpastes to prevent caries and treat gingivitis and stomatitis. It is commercially available in mouthwash solutions and in many purified products from which wax was removed. Due to its antimicrobial, antiviral and antioxidant properties, propolis is widely used in medicine, pharmacology, and cosmetics[16].

The aim of this study was to investigate the bactericidal properties of four commercially available antiseptic mouthwashes containing chlorhexidine, cetylpyridinium chloride, alcohol with essential oils, propolis, menthol, xylitol and hydroxyapatite nanoparticles. The in vitro study aimed to follow the inhibitory properties of mouthwashes against Gram-positive cocci: *S. mutans ATCC 35668*, *S. epidermidis ATCC12228*, *S. pneumoniae ATCC49619* and *S. aureus ATCC 29213,* and through an in vivo study, we followed the duration of these antimicrobial effects on the salivary aerobic and facultative anaerobic microflora.

Materials and Methods

The study was carried out at the Medical University-Varna, Bulgaria, Training sector "Medical Laboratory Assistant" in September 2023. (Protocol/approval decision number 115/31.03.2022- Ethics Committee for Scientific Research at the Medical University of Varna)

The research we conducted involved two stages. In the first of these, we conducted a series of *in vitro* studies to monitor the antimicrobial properties of four mouthwashes with different active ingredients against representatives of the normal oral microflora (*S. mutans ATCC 35668*) and potential pathogenic and conditionally pathogenic bacteria (*S. pneumoniae ATCC49619* , *S. epidermidis ATCC12228* , *S. aureus ATCC 29213*). For this purpose, we used the method of serial dilutions to determine the minimum inhibitory concentration (MIC), as well as the determination of the minimum bactericidal concentration (MBC) in agar medium. In the second stage, we conducted *in vivo* tests with 24 volunteers, tracking the dynamics of the bactericidal effects of the four mouthwashes on the normal aerobic and facultatively anaerobic oral microflora. All participants are declared informed written consent for participation in the study and publication of the data for research and educational purposes to be mentioned.

The mouth rinses chosen for the study are shown in Table 1, together with the active ingredients named on the package.

In vitro determination of MIC and MBC against test organisms

The MIC values of four mouthwashes against four microbial strains were determined by the broth dilution method. 1 ml of each mouthwash was serially diluted in Brain heart infusion broth up to a final dilution of 1:8. Bacterial culture containing 0.5 McFarland (1.5×108 colony forming units/ml) of organisms is added to each concentration of mouth rinses. Control cultures were also prepared. Tubes were incubated for 24 hours at 37°C and growth was examined by observing the presence or absence of turbidity in the solution. The MIC of each mouthwash was defined as the lowest concentration that inhibits the visible growth of bacteria.

Confirmation of growth inhibition from the serial dilution method was obtained by subculture of 0,1 ml of each broth culture on blood agar and incubation for 24 hours at 37°C. The MBC was determined as the lowest concentration of a mouthwash that results in the killing of 99.9% of the testing bacteria.

Table 1. Active ingredients of the four types of mouthwash testing.

Mouthwash	Active ingredients with antimicrobial activity
MW1 (CHD+CP)	*Chlorhexidine digluconate 0,12%*
	Cetylpyridinium chloride 0,05%
MW2 (AL+EO)	*Alcohol 21,60%*
	Essential oils:
	Thymol 0.064%
	Eucalyptol 0.092%
	Methyl Salicylate 0.060%
	Menthol 0.042%
MW3 (HAN+X)	*Hydroxyapatite nanoparticles (unknown activity against oral bacteria)*
	Xylitol
MW4 (P+M)	*Propolis Extr. 2,00%*
	Mentha Viridis Oil 0.042%

Selection of the study group and protocol

These clinical trials utilized an open, randomized design. Subjects completed an informed consent form after explaining the nature of the study to them. The selected participants initially received oral hygiene instruction at the point where the same types of toothpaste and toothbrushes were distributed, and were instructed to brush their teeth twice a day for 1 minute immediately after meals, and to use 10 ml of one of the following mouthwashes for 30 seconds.

The study group was made up of 24 healthy adult volunteers between 27 and 49 years of age who had no obvious oral pathology and presented a minimum of 24 permanent teeth. Subjects were required to abstain from oral hygiene, eating, and drinking prior to and during the study. Exclusive criteria are borrowed from *Quintas et al.*[7] and applied in our study: smoker or formal smoker, presence of dental prostheses or orthodontic devices, antibiotic treatment or routine use of oral antiseptics in the previous three months, and presence of any systemic disease that could alter the production or composition of saliva. On the day of the experiment, volunteers were not allowed to eat or drink during the course of the tests.

In vivo salivary bacterial study

The duration of antimicrobial activity of the four mouthwashes against normal oral microflora was measured *in vivo* with the help of 24 volunteers. The tests with the four mouthwashes were carried out on five consecutive Mondays, i.e., each at an interval of six days. Regarding the day of each study, we pre-instructed the volunteers to brush their teeth with the toothbrush and toothpaste provided by us no later than 6 AM, then not to consume food and drink. The first control saliva sample of each volunteer was collected at 8:00 AM, before oral treatment with mouthwash. The next seven test samples were taken at 2 min, 30 min, 1 h, 2 h, 4 h, 6 h and 8 h after using the mouthwash.

Saliva samples were mixed on a Vortex mixer and 1 ml was serially diluted in Ringers solution – from 10-1 to 10-6. Subsequently, a 0.1 ml sample of each dilution was inoculated on a blood agar plate using a sterile spreader. Petri dishes were aerobically incubated for 24 h at 37°C. On the next day the resulting colonies were counted and the number of bacteria in millions per milliliter of saliva was calculated.

Results

Chavarría-Bolaños et al.,[17] report high inhibitory activity of chlorhexidine gluconate mouthwashes against *C. albicans*, *S. aureus,* and *E. coli*. *Shah et al., 2022*[18] also report a strong antimicrobial effect against *S. mutans*, while simultaneously comparing the action of mouthwashes with alcohol and herbal

Table 2. MICs of four mouthwashes with different active ingredients against Gram-positive bacterial strains.

	Active ingredients	***MICs (serial dilution)***			
		S. aureus *ATCC29213*	***S. epidermidis*** *ATCC12228*	***S. pneumoniae*** *ATCC49619*	***S. mutans*** *ATCC35668*
MW1	Chlorhexidine digluconate 0,12% Cetylpyridinium chloride 0,05%	≥1:8	≥1:8	≥1:8	≥1:8
MW2	Alcohol 21,60% Thymol 0.064% Eucalyptol 0.092% Methyl Salicylate 0.060% Menthol 0.042%	≥1:8	≥1:8	≥1:8	≥1:8
MW3	Hydroxyapatite nanoparticles Xylitol	≥1:8	1:2	≥1:8	≥1:8
MW4	Propolis Extr. 2,00% Mentha Viridis Oil 0.042%	≥1:8	≥1:8	≥1:8	1:2

active ingredients, announcing that these mouthwashes also have a powerful effect against *S. mutans*.

In our studies, we determined MICs of four MWs with different active ingredients against *S. mutans ATCC35668*, *S. pneumoniae ATCC49619*, *S. aureus ATCC29213,* and *S. epidermidis ATCC12228*. All mouthwashes demonstrated antimicrobial activity against Gram-positive bacteria tested. In most of the cases, an inhibitory effect was observed at all concentrations tested. Exceptions were mouthwash MW4 (P+M) mouthwash against *S. mutans* – MIC 1:2 and MW3 (HAN+X) against *S. epidermidis* – MIC 1:2. The results are presented in Table 2.

The determination of MW1 MBC (chlorhexidine digluconate and cetylpyridinium chloride) and MW2 (alcohol and essential oils) demonstrated MBCs ≥ 1:8 against all Gram-positive microorganisms tested. MW3 with xylitol and hydroxyapatite nanoparticles demonstrated only bacteriostatic antimicrobial effects; no MBC was presented. MW4 with propolis and mentha oil demonstrated MBC ≥ 1:8 against *S. aureus ATCC 29213*, *S. epidermidis ATCC12228*– MBC 1:4 and lack of bactericidal effects against *S. pneumoniae ATCC49619* and *S. mutans ATCC 35668*.

Salivary bacterial counts study

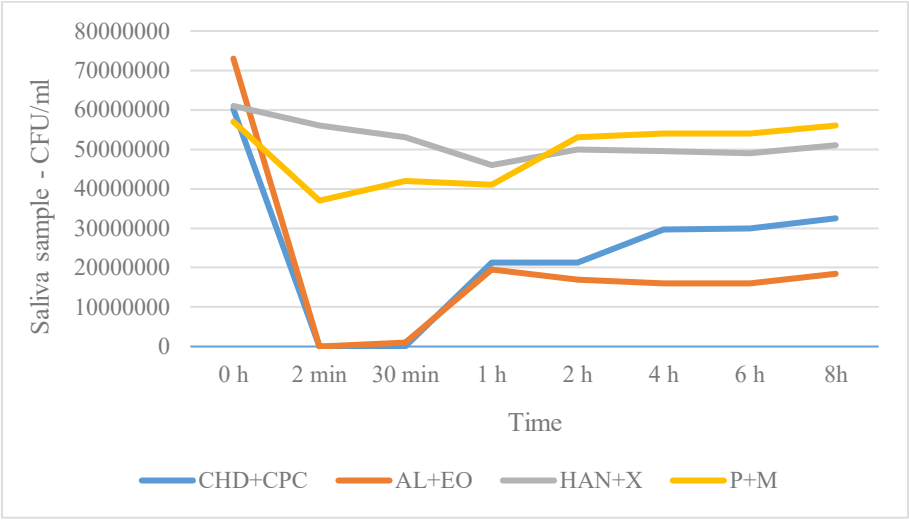
In vivo antibacterial determinations aimed to follow the dynamics of microbial multiplication of the normal oral microflora under the antimicrobial action of mouthwashes with different compositions. For this purpose, we tested the four mouthwashes with different compositions in four different weeks with the help of 24 volunteers. The sampling with each mouthwash lasted for 8 hours - 0th control sample and subsequent seven saliva samples after the use of the respective mouthwash (at the second minute, the 30th minute, 1, 2, 4, 6 and 8 hours after use). We performed a serial dilution of all samples, and after cultivation, the aerobic and facultative anaerobic microorganisms grown were enumerated. The survival of the oral microflora, relative to baseline, was calculated and averaged for each time point, and the so-called microbial numbers (as described in Materials and Methods). The results are presented in Figure 1.

The results of our research show that the combinations of chlorhexidine digluconate + cetylpyridinium chloride and alcohol + essential oils have the most powerful antimicrobial action against aerobic and facultatively anaerobic oral microflora. With these combinations, we observed complete inhibition of microbial growth in a minimum of 30 minutes, after which the CFU/mL gradually increased, but within 8 hours of follow-up did not reach the initial baseline value (sample 0). These data also overlap with the obtained results of determination of MIC and MBC against *S. mutans ATCC 35668, S. pneumonia ATCC49619 , S. aureus ATCC 29213* and *S. epidermidis ATCC12228*. After the mouthwash with ingredients of hydroxyapatite nanoparticles + xylitol and propolis extr. + Mentha viridis oil, the microbial density of the oral flora decreases to a certain extent and within an hour completely (MW4) or almost completely (MW3) restores its initial values. The results of the determination of MBC against the tested Gram positive bacteria with mouthwashes MW3 and MW4 confirm these results.

Discussion

The antimicrobial action of chlorhexidine and its combination with cetylpyridinium chloride is well known[19, 20, 21]. They exhibit a long-lasting bactericidal effect against a wide range of microbes, both representatives of the normal microflora of the oral cavity and obligate pathogens. However, chlorhexidine-containing mouthwashes also have their known side effects, which limits their widespread use. The use of chlorhexidine by patients under 12 years is not recommended (under 18 years in the US). It is also recommended for short-term use only; 2-4 weeks, only licensed for 30 days of use in the UK[22, 23]. Mouthwashes containing a combination of alcohol and essential oils also have a well-studied and marked antimicrobial effect against oral cocci[7, 24]. Compared to chlorchexidine-based mouth rinses[9], Listerin® (alcohol and essential oils) had a similar antibacterial effect; however, after 4 hours from rinsing, chlorchexidine preparation showed further reduction in microbials. The advantage of Listerin® is that the mouthwash has no proven side effects[25, 26]. In our in vivo study we observed complete inhibition of microbial growth in the oral cavity in a minimum of 30 minutes, after which CFU/mL gradually increased, but within the 8-hour follow-up did not reach the initial baseline value. *Tahan N, 2018* also reports on a 6-hour in vivo follow-up in which CFU/ml of normal oral flora did not recover to baseline after using chlorhexidine mouthwash[27]. Tests for determination of MICs of MW1 and MW2 also improved strong antimicrobial effects against *S. aureus ATCC 29213 , S. epidermidis ATCC12228 , S. pneumonia ATCC49619,* and *S. mutans ATCC 35668* (MIC ≥ 1:8). The action of MW2 against *S. epidermidis ATCC12228*was weaker - MIC 1:2.

The biomimetic active ingredient hydroxyapatite is used in various fields of oral care[28, 29]. It remineralizes early caries lesions[30, 31], reduces initial bacterial attachment to enamel similar to 0.2% chlorhexidine[32], and acts as buffer and a calcium and



Legend:

***CHD+CPC** – MW1- Chlorhexidine digluconate 0,12% and cetylpyridinium chloride 0,05%;*
***AL+EO** – MW2 - Alcohol 21,60% and four essential oils;*
***HAN+X** – MW3 - Hydroxyapatite nanoparticles and xylitol;*
***P+M** – MW4 - Propolis Extr. 2,00% and 0.042% Mentha viridis oil.*

***Figure 1.** Dynamics of microbial density (number of viable cells/milliliter of saliva sample – CFU per mL) after using mouthwashes with different compositions*

phosphate reservoir in biofilms[33]. To extend these preventive effects, hydroxyapatite can be used together with xylitol to achieve an antibacterial effect and to prevent/reduce gingivitis[34].

In the scientific literature, we found no data from in vitro and in vivo studies on the antimicrobial activity of mouthwashes containing the combination of hydroxyapatite nanoparticles/xylitol and propolis/menthol. Our tests for MIC determination of MW3 improved antimicrobial effects against *S. aureus ATCC 29213* , *S. pneumoniae ATCC49619* and *S. mutans ATCC 35668*- MIC ≥ 1:8 and *S. epidermidis ATCC12228* - MIC 1:2. In the in vivo test to track the dynamics of CFU/ml of normal flora, this combination reduced the total microbial count of aerobic and facultative anaerobic bacteria to a very small extent. Similar results were observed in MB4 with propolis and menthol. When determining the MIC against *S. mutans ATCC 35668* (the predominant microbe in the mouth), it is found that the antimicrobial effect is weaker (MIC = 1:2), compared to the other microbes tested - MIC = 1:8. According to *El-sayed SR et al., 2016* the use of propolis mouthwash achieved a mean reduction of total bacterial count. The effect of 0.2% chlorhexidine was greater than the effect of propolis in the reduction of total bacterial count[35].

The correlation between salivary bacteria (such as *Streptococcus mutans*) and the selected gram-positive cocci (*Streptococcus pneumoniae*, *Staphylococcus epidermidis* and *Staphylococcus aureus*) is based on their role in infections affecting both the oral cavity and other parts of the human body. *Streptococcus mutans* are typical oral bacteria and major con-

tributors to dental caries and periodontal diseases. These bacteria form dental plaque, which leads to the production of acids that degrade tooth enamel and cause cavities[36].

S. epidermidis, S. pneumoniae and *S. aureus*, while not primarily associated with the oral cavity, can colonize it and are recognized as opportunistic pathogens. They can cause infections in immunocompromised individuals or lead to complications such as endocarditis when they enter the bloodstream. Studying these bacteria together is important because *Streptococcus* species from the oral cavity can lead to systemic infections, similar to *Staphylococcus*, particularly in individuals with compromised immune systems. Both types of bacteria can interact during inflammatory processes, especially in oral infections, where contamination with Staphylococcus from external sources is also possible. Therefore, investigating the antimicrobial activity against these pathogens could indicate whether a given treatment is effective not only against oral bacteria but also against systemic infections caused by gram-positive cocci[37, 38].

Conclusions

Mouthwashes containing combinations of chlorhexidine digluconate/cetylpyridinium chloride, alcohol/essential oils, hydroxyapatite nanoparticles/xylitol and propolis/mentha viridis oil have strong antimicrobial effects against *S. aureus*, *S. mutans*, *S. pneumoniae,* and *S. epidermidis* with MICs ranging from 1:2 to ≥1:8. Our *in vivo* study of the inhibitory effect of these combinations against normal microflora has shown that they significantly reduce CFU/mL compared to mouthwashes containing hydroxyapatite nanoparticles/xylitol and propolis/mentha viridis oil. □

Acknowledgements: *This work was supported by the institutional Science Fund (Project №21012) of the Medical University of Varna, Bulgaria.*

Conflict of interest: *The authors declare no conflict of interest.*

Data Availability statement: *The authors confirm that the data supporting the findings of this study are available within the article.*

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