



COMPARISON OF ANTIMICROBIAL EFFICACY OF THREE TYPES OF MOUTHWASH, CONTAINING CHLORHEXIDINE-CHLORBUTANOL, ALCOHOL-ESSENTIAL OILS AND PROPOLIS-MENTHA OIL COMBINATIONS

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ABSTRACT

Mouthwash is an antiseptic solution intended to reduce the microbial load in the oral cavity, although other mouthwash might be given for other reasons such as for their analgesic, anti-inflammatory or anti-fungal action.

A study was carried out to compare the antibacterial and antifungal properties of three mouthrinse preparations - both containing commercial used antiseptic combinations (chlorhexidine 0,100% + chlorbutanol 0,500% and alcohol 21,60% + essential oils) and one with natural active ingredients (propolis 2,00% + mentha oil 0,042%).

The antibacterial and antifungal activity of three types of mouthwash were tested on three microbial strains - *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* by two alternatives of agar diffusion tests – "cup plate" technique and disc-diffusion test.

The results showed the highest antimicrobial activity of the chlorhexidine-chlorbutanol combination. Mouthwash containing propolis with mint oil demonstrated activity only against *S. aureus* strain.

It turned out that testing the antimicrobial activity of mouthwash with alcohol with essential oils with the agar diffusion method is not representative. For 24 hours with this mouthwash, no inhibition zones were observed in none of the strains at any concentration. Our assumption is that alcoholic solutions are exuding and bacteria starts to grow.

Keywords: mouthwash, antimicrobial activity, chlorhexidine, chlorbutanol, alcohol, propolis, essential oils,

INTRODUCTION

Usually, mouthwashes are antiseptic solutions intended to reduce the microbial load in the oral cavity, although other mouthwashes might be given for other reasons such as for their analgesic, anti-inflammatory or anti-fungal action. Additionally, some rinses act for neutralizing acid and keep the mouth moist in xerostomia (dry mouth) [1, 2, 3]. Nowadays, mouthwash use is a part of a

daily dental hygiene routine. There are many types and brands of mouthwash on the market. They combine ingredients to treat a variety of oral conditions. Most of the mouthwash offers a set of benefits, such as antibacterial action against the bacteria that produce plaque and bad breath, strengthening tooth enamel, and improving gum health.

Potential mouthwash users are apparently one of the great untapped markets this undoubtedly leads to the continuous growth of the range of products offered in the markets. Factors driving the demand for mouthwashes include accretive awareness among consumers for dental hygiene, increasing recommendations from dental physicians and growth in spending power of consumers.

The most commonly used active ingredients in therapeutic mouthwash include:

• Chlorhexidine

Of the antimicrobial agents available for dental use, chlorhexidine is the most thoroughly researched and most used one. In the pharmacies in Bulgaria mouthwash containing chlorhexidine are available in different concentrations and combinations with other substances. Most commonly, chlorhexidine is in combination with cyclopiRIDYL chloride or chlorbutanol.

It is an antiseptic compound which is used for disinfection of the skin before surgery, as well as for the sterilization of surgical instruments. It is also used to prevent dental plaque to treat fungal infections in the oral cavity [4]. It is active against Gram-positive and Gram-negative bacteria, facultative anaerobes, aerobes, and yeasts. It acts against gram-positive bacteria at concentrations $\geq 1 \mu\text{g/l}$. Gram-negative bacteria and fungi require significantly higher concentrations of 10 to more than $73 \mu\text{g/ml}$. Chlorhexidine is ineffective against polioviruses and adenoviruses. Its effectiveness against herpes viruses has not yet been fully elucidated.

• Alcohol (Maximum content - up to 27%)

It is used as a carrier for the active ingredients, rarely

due to bactericidal action. There is evidence of a carcinogenic effect of mouthwashes containing alcohol, but there is still no conclusive scientific evidence to that effect [5].

• **Essential oils and phenols**

Phenolic compounds include essential oils such as phenol, thymol, eugenol, eucalyptol or menthol. They show antibacterial properties and are effective in reducing halitosis. Due to its properties, they are used in a number of mouthwashes.

• **Propolis**

Propolis is used in mouthwashes and toothpaste to prevent caries and to treat gingivitis and stomatitis. It is commercially available in the mouthwash solutions and in many purified products from which the wax was removed. Due to its antimicrobial [6, 7], antiviral, and antioxidant properties, it is widely used in medicine, pharmacology, and cosmetics.

Other active ingredients that may be used in therapeutic mouthwash include benzydamine, cetylpyridinium chloride, fluoride, peroxide. **Benzydamine hydrochloride** is a locally acting anti-inflammatory drug, its action is to relieve the pain and inflammatory in the mouth. Studies have shown that benzydamine has a noticeable in vitro antibacterial activity against the antibiotic-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. **Cetylpyridinium chloride** is an antiseptic compound, mainly applied in some types of mouthwashes, toothpaste and has been found to be effective in preventing dental plaque and reducing gum inflammation [5]. **Fluoride** is used as a prevention of caries [8]. It is included in mouthwash for patients at high risk of dental caries or people with xerostomia. **Hydrogen peroxide** has a bactericidal effect on anaerobic bacteria and also has a mechanical cleaning action. It is often used to treat acute necrotizing ulcerative gingivitis.

Global Mouthwash Market is anticipated to grow significantly in the years to come due to the rise in demand for mouthwashes that contain natural ingredients and increasing demand for alcohol-free mouthwashes.

It is presumed that mouth contains approximately 550 to 600 species of bacteria, besides viruses and fungus. Among the bacteria, there are the *Actinomyces israeli*,

Capnocytophaga spp., *Staphylococcus spp.*, *Streptococcus mitis* and others [9, 10]. Although *Staphylococcus spp.*, particularly, is part of individuals' normal microbiota, indeterminate conditions it can cause infections, very frequent in hospitals [11]. *Escherichia coli* is a gram-negative bacterium that has a major virulence factor, lipopolysaccharide, present in the outer membrane. This endotoxin can cause endodontic diseases such as pulp/periapical inflammation and periodontitis, osteomyelitis of the jawbone and abscesses of the soft tissues around them.

Candida albicans is commonly found in mucosal layers, can be pathogenic in cases of immunodeficiency, such as in patients with human immunodeficiency virus (HIV), cancer, transplants, and/or prolonged hospitalization. In the oral cavity, *C. albicans* is responsible for clinical manifestations of both pseudomembranous and erythematous candidiasis [12].

AIM

To investigate the antibacterial and antifungal properties of three mouth rinse preparations - both containing commercial used antiseptic combinations (chlorhexidine with chlorbutanol and alcohol with essential oils) and one with a natural active ingredient (propolis with mentha oil).

Within the study, the demonstrated inhibition zone was compared in two types of agar diffusion test - "cup plate" technique and disk-diffusion test.

MATERIALS AND METHODS

This experimental study was performed in Medical College – Varna, Bulgaria in September 2019. Sample processing and all other laboratory procedures were done in the Training sector "Medical Laboratory Assistant".

The antimicrobial activity of three types of mouthwash was evaluated. Two of them containing commercial combinations of active ingredients - chlorhexidine with chlorbutanol; alcohol with essential oils and one - only with natural ingredients - propolis extract with mentha oil. The active ingredients with antimicrobial activity are presented in Table 1.

Table 1. Active ingredients of the three testing types of mouthwashes and testing concentrations

Mouthwash	Active ingredients with antimicrobial activity	Testing concentrations (%)		
MW1	<i>Chlorhexidine gluconate 0,100%</i>	0,1	0,05	0,025
	<i>Chlorbutanol hemihydrate 0,500%</i>	0,5	0,25	0,125
MW2	<i>Propolis Extr. 2,00%</i>	2	1	0,5
	<i>Essential oil (Mentha Viridis Oil 0.042%)</i>	0,042	0,021	0,011
MW3	<i>Alcohol 21,60%</i>	21,6	10,8	5,4
	Essential oils:			
	<i>Thymol 0.064%</i>	0,064	0,032	0,016
	<i>Eucalyptol 0.092%</i>	0,092	0,046	0,023
	<i>Methyl Salicylate 0.060%</i>	0,06	0,03	0,015
	<i>Menthol 0.042%</i>	0,042	0,021	0,011

MW – Mouthwash

Bacteria and fungi strains

The following bacterial and yeast strains were purchased and used in the study: *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. All strains were passed three times to assure purity and viability before each experiment.

Tube dilution method

Mouth rinses were evaluated in their pure form as well as in two dilutions ranging from 1:2 and 1:4. 2-fold dilutions were made up in sterile phosphate buffer and antimicrobial activity of each concentration was tested with two alternatives of the agar diffusion test.

Agar diffusion tests – Disk-diffusion test and “Cup plate” technique

The antibacterial and antifungal activity of the three types of mouthwash was tested on three microbial strains - *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* by two types of agar diffusion test – in cups and with discs.

The bacterial strains were reactivated in Brain heart infusion broth and incubated overnight aerobically at 37°C. Aliquots of standardized cell suspension were spread on Mueller-Hinton agar (20 ml per plate), and the plates were dried at 27°C for 15 min. Each plate was separated into four equal zones – three with cups and one position for a sterile disc. Cups were made on the solidified agar layer with the help of a sterile borer at 8-mm diameter. The cups, as well as the sterile disc, are filled with 100 µl of the dilutions. The Petri plates were incubated aerobically at 37°C for 24 hours and the inhibition zone diameters were determined in mm. “Cup plate” technique was performed in triplicate.

The data is processed with the SPSS Statistics 19 statistical program.

RESULTS AND DISCUSSION

The results of the antimicrobial activity tests are presented in Table 2.

Table 2. Antibacterial and antifungal activity of three concentrations of commercial usable types of mouthwash containing chlorhexidine with chlorbutanol; propolis extract with mentha oil and alcohol with essential oils.

Mouthwash and concentration of active antimicrobial ingredients	Zone of inhibition (mm)					
	<i>E. coli</i>		<i>S. aureus</i>		<i>C. albicans</i>	
	cups	disc	cups	disc	cups	disc
MW1 chlorhexidine gluconate 0,100% + chlorbutanolhemihydrate 0,500%						
CHX 0,100; CHB 0,500	16,7 ±3,55	12	24 ±0	18	21,7 ±0,22	16
CHX 0,050; CHB 0,250	16 ±0	10	21 ±0,66	15	18,3 ±1,55	12
CHX 0,025; CHB 0,125	15 ±0,66	10	20,3 ±0,89	13	9,33 ±0,88	No
MW2 propolisextr. 2,0% + menthaoil 0.042%						
P 2,00; MO 0,042	No	No	23,7 ±1,55	14	No	No
P 1,00; MO 0,021	No	No	15 ±0,66	10	No	No
P 0,500; MO 0,011	No	No	14,7 ±1,55	No	No	No
MW3 alcohol 21,60% + essential oils:thymol 0.064%;eucalyptol 0.092%; methyl salicylate 0.060%;menthol 0.042%						
A 21,60; T 0.064, E 0.092, MS 0,060, M 0,042	No	No	No	No	No	No
A 10,80; T 0.032, E 0.046, MS 0,030, M 0,021	No	No	No	No	No	No
A 5,40; T 0.016, E 0.023, MS 0,015, M 0,011	No	No	No	No	No	No

JoMW – mouthwash; CHX – chlorhexidine; CHB – chlorbutanol; P – propolis extract; MO –mint oil; A – alcohol; T – thymol; E – eucalyptol; MS - methyl salicylate; M – menthol; No – no zone of inhibition.

Values are presented as mean ± Standard Deviation of 3 experiments

MW1 - Ingredients in MW1 have baseline concentrations of 0.100% chlorhexidine and 0.500% chlorbutanol. Test concentrations are up to two times diluted from baseline (lowest 0.03). All tested concentrations demonstrated both antibacterial and antifungal activity against the microbial strains. The antibacterial activity against *S. aureus* is highest. The sensitivity of *E. coli* and *C. albicans* is also high at all tested concentrations except for the 0.03% solution in *C. albicans*.

MW2 - containing 2,00% propolis and 0,042% mint oil exhibits antibacterial activity only against *S. aureus*. Inhibition zones were established for all cups in the agar with the corresponding mouthwash concentrations, but at the loaded discs, 0.06% and 0.03% inhibition zones were not observed or were too small. MW2 did not demonstrate antimicrobial activity against *E. coli* and *C. albicans*.

MW3 - containing 21,6% alcohol and different concentrations of four essential oils (Table 1). In this study, no antibacterial effect was shown for MW3, differently from the findings of previous investigations [13, 14, 15] of other researchers and our previous still unpublished *in vivo* investigation (includes determination of minimal inhibitory concentration and minimal bactericidal concentration). On the next day, the entire methodology was repeated with MW3, the results were the same.

We have found other studies, including agar diffusion tests of alcoholic mouthwashes, with the same results - have not been identified zone of inhibition [16, 17, 18]. The antibacterial effect of MW3 mostly comes from its al-

cohol content, which may have evaporated by the time the zone of bacterial inhibition was measured.

In the present study, we compared the sensitivity of the strains tested by two similar methods - loading 100 microliters of the respective mouthwash concentration of a sterile disc and placing the same volume of mouthwash in a cup made with a cork borer. Our study showed that the cups were very successful made with the large portion of a 1 ml automatic pipette. The microbial strains demonstrate a larger zone of inhibition at all concentrations in the "cup plate" technique. The *S. aureus* inhibition zones around the discs were on average 6.7 mm smaller in diameter. In *E. coli* - 5.2 mm and in *C. albicans* - 4.7 mm. The study showed that both methodologies did not record unambiguous results.

CONCLUSION

Mouthwash containing chlorhexidine 0,100% and 0.500% chlorbutanol (MW1) demonstrates antimicrobial activity against *E. coli*, *S. aureus* and *C. albicans* (except for the 0.03% solution in *C. albicans*). MW2 with propolis extr. 2,00% + mint oil 0.042% demonstrates a zone of inhibition only in Petri dish with *S. aureus*. Mouthwash with alcohol and essential oils (MW3) did not show inhibitory action against the tested bacteria.

In our study, we were present two types of agar diffusion antibiotic susceptibility tests. Both methodologies did not record unambiguous results - in the "cup plate" technique the microbial strains demonstrate a larger zone of inhibition at all concentrations.

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