

DETERMINATION OF THE MAXIMUM INHIBITORY DILUTION OF CETYLPYRIDINIUM CHLORIDE-BASED MOUTHWASHES AGAINST *STAPHYLOCOCCUS AUREUS*: AN *IN VITRO* STUDY

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ABSTRACT

The aim of this *in vitro* study was to determine the maximum inhibitory dilution (MID) of four cetylpyridinium chloride (CPC)-based mouthwashes: CPC+Propolis, CPC+Malva, CPC+Eucaliptol+Juá+Romã+Propolis (Natural Honey[®]) and CPC (Cepacol[®]), against 28 *Staphylococcus aureus* field strains, using the agar dilution method. Decimal dilutions ranging from 1/10 to 1/655,360 were prepared and added to Mueller Hinton Agar. Strains were inoculated using Steers multipoint inoculator. The inocula were seeded onto the surface of the culture medium in Petri dishes containing different dilutions of the mouthwashes. The dishes were incubated at 37°C for 24 h. For readings, the MID was considered as the maximum dilution of mouthwash still capable of inhibiting microbial growth. The obtained data showed that CPC+Propolis had antimicrobial activity against 27 strains at 1/320 dilution and against all 28 strains at 1/160 dilution, CPC+Malva inhibited the growth of all 28 strains at 1/320 dilution, CPC+Eucaliptol+Juá+Romã+Propolis inhibited the growth of 2 strains at 1/640 dilution and all 28 strains at 1/320 dilution, and Cepacol[®] showed antimicrobial activity against 3 strains at 1/320 dilution and against all 28 strains at 1/160 dilution. Data were submitted to Kruskal-Wallis test, showing that the MID of Cepacol[®] was lower than that determined for the other products ($p < 0.05$). In conclusion, CPC-mouthwashes showed antimicrobial activity against *S. aureus* and the addition of other substances to CPC improved its antimicrobial effect.

Key words: Bacteria. Mouthwashes. Cetylpyridinium chloride.

INTRODUCTION

Currently, a wide range of options in oral antiseptics and toothpastes is available in the market. These products contain synthetic and/or natural compounds with antimicrobial activity¹².

Among these synthetic compounds is cetylpyridinium chloride (CPC), a quaternary ammonium compound included in the group of the cationic surface-active agents¹⁸. It acts primarily by penetrating the cell membrane, causing leakage of cell components, disruption of the bacterial metabolism, inhibition of cell growth, and finally, cell death³.

Natural extracts, such as propolis, *Malva sylvestris*,

Punica granatum, *Zizyphus joazeiro*, *Eucalyptus globulus*, and *Salvadora persica* are included in the formulation of commercially available oral hygiene products. The addition of these substances aims to improve the antibacterial action, since these natural extracts have demonstrated effect against a wide range of microorganisms²³. The antibacterial action of CPC-based mouthwashes is variable and depends on the product's formulation¹⁰.

Staphylococcus aureus is a major human pathogen, responsible for a number of hospital-acquired infections¹⁷. This microorganism is able to colonize several locations in the human body, but mouth, hands, and nasopharynx are the main reservoirs for propagation of this germ in the

hospital environment¹⁵. Therefore, control of *S. aureus* is extremely relevant for the determination of the antiseptic properties of hygiene products. The increased occurrence of methicillin-resistant *S. aureus* strains (MRSA), as well as of other strains resistant to different broad-spectrum antimicrobial agents, represents a therapeutic challenging situation⁵. Among the microorganisms present in the oral cavity, the reduction in the number of *S. aureus* prior to surgical procedures has been associated with a lower incidence of infective endocarditis and postoperative infections¹.

The purpose of this *in vitro* study was to determine the maximum inhibitory dilution (MID) of CPC-based mouthwashes and other products containing natural extracts in addition to CPC against 28 *S. aureus* field strains.

MATERIAL AND METHODS

The following CPC-based products were evaluated: *Cepacol*[®] (Aventis Pharma Ltda., São Paulo, SP, Brazil), *Natural Honey CPC + Propolis* (Skill Brothers Indústria e Comércio Ltda, São Paulo, SP, Brazil), *Natural Honey CPC*

+ *Malva* (Skill Brothers Indústria e Comércio Ltda.), and *Natural Honey CPC+Eucaliptol+Juá+Romã+Propolis* (Skill Brothers Indústria e Comércio Ltda.) (Table 1).

MID determination was performed in duplicate by double serial dilution (from 1/10 through 1/655,360) in test tubes (20x200 mm) with 2.0 mL of sterile distilled water. After dilutions, 18.0 mL of Mueller Hinton Agar culture medium (Difco[®], USA) were added to each tube, and the resulting solutions were poured onto Petri dishes (20x100 mm).

The microbial inoculum (~10⁸ cfu/mL) with turbidity equivalent to a #0.5 McFarland standard was prepared in test tubes (15x125 mm) with saline, using 28 *S. aureus* field strains obtained from nasal and oral cavities. The strains were conserved on a collection cultured on Ni agar medium. The identification of *S. aureus* was based on the production of catalase and coagulase. Regardless of the results of the coagulase test, all catalase positive Gram-positive cocci were submitted to the API-Staph system (bioMérieux, France) for biochemical identification.

Microorganisms were seeded using a Steers multipoint inoculator²⁷. The Steers inoculator consists of two metallic plates. One plate has 25 wells onto which 200 µL of each standardized microbial inoculum were transferred. The other

TABLE 1- Chemical composition of the antiseptic solutions evaluated

Antiseptic	Composition	
Cepacol [®]	- Cetylpyridinium chloride - Disodium EDTA - Sodium saccharin - Polysorbate 80 - Glycerin - Water -Sodium phosphate monobasic anhydrous	- Eucalyptol - Menthol - Methyl salicylate - Mint oil - Chinese cinnamon flavor - Yellow tartrazine - Ethyl alcohol 96GL
Natural Honey CPC+Propolis	-Natural propolis extract - Sodium fluoride 0.05% (226ppmF) - Cetylpyridinium chloride (CPC) - Sorbitol - Sodium phosphate monobasic - Sodium phosphate dibasic	- Ethanol - Sorbitan monolaurate - Sodium saccharin - CI42.053 green - Mint flavor - Demineralized water
Natural Honey CPC+Malva	- Natural <i>Malva sylvestris</i> extract - Sodium fluoride 0.05% (226ppmF) - Cetylpyridinium chloride(CPC) - Sorbitol -Sodium phosphate monobasic -Sodium phosphate dibasic	- Ethanol - Sorbitan monolaurate - Sodium saccharin - CI42.090 blue - Mint flavor - Demineralized water
Natural Honey CPC + Eucaliptol + Juá + Romã + Propolis	-Natural extracts of pomegranate, propolis, and <i>Zizyphus joazeiro</i> -Eucalyptol -Methyl salicylate -Sodium fluoride 0.05% (226ppmF) -Cetylpyridinium chloride(CPC) -Sorbitol	- Sodium phosphate dibasic - Ethanol - Sorbitan monolaurate - Sodium saccharin - CI 19.140 and 15.985 color - Pomegranate flavor - Demineralized water

TABLE 2- Percentage of *S. aureus* strains inhibited (from a total of 28 strains) by each dilution of the tested mouthwashes

Dilution	CPC+Propolis				CPC+Malva				CPC+Eucaliptol+Juá+Romã+Propolis				CPC*			
	i. s.	i. s.	c. d.	c. d.	i. s.	i. s.	c. d.	c. d.	i. s.	i. s.	c. d.	c. d.	i. s.	i. s.	c. d.	c. d.
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
1/640	0	0.0	0	0.0	0	0.0	0	0.0	2	7.1	2	7.1	0	0.0	0	0.0
1/320	27	96.4	27	96.4	28	100.0	28	100.0	26	92.9	28	100.0	3	10.7	3	10.7
1/160	1	3.6	28	100.0									25	89.3	28	100.0

* (p<0.05)

i.s. Inhibited strains
c.d. Cumulative data

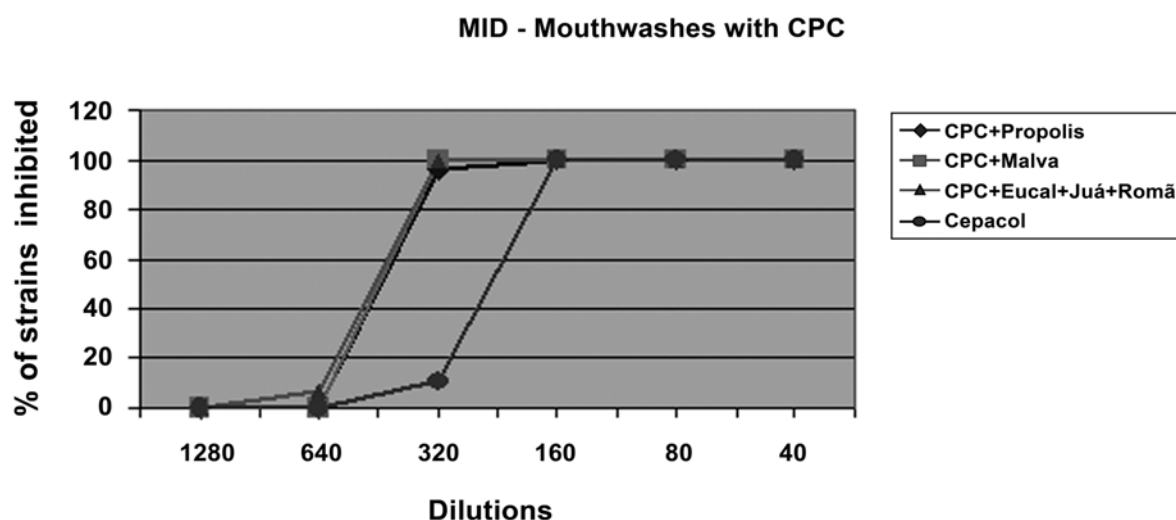


FIGURE 1- Graph depicting the MID values obtained for each mouthwash

plate has 25 metallic needles that fit into the wells. Using these needles, the inocula were seeded onto the surface of the culture medium in Petri dishes containing different dilutions of the mouthwashes. Since the Steers inoculator has 25 wells and 28 strains were evaluated, three inocula (5 µL) were seeded equidistantly from each other, approximately 1 cm from dish periphery, using an automatic pipette.

The dishes were then incubated overnight at 37°C and readings were performed considering the MID as the greatest dilution of mouthwash capable of inhibiting the growth of all test strains, following the methodology proposed by Roberts and Addy²¹.

Statistical Analysis

Results were expressed as scores determined from minimum to maximum dilution. Comparisons among the groups were performed by Friedman’s nonparametric test. When this test indicated significant difference between the groups, Dunn’s multiple-comparison test, which allows two-by-two comparison between groups, was applied. Significance level was set at 5%.

RESULTS

The mouthwashes evaluated in this study presented different MID values (Table 2 and Figure 2).

Statistical analysis demonstrated no statistically significant differences (p>0.05) among CPC+Propolis, CPC+Malva, and CPC+Eucaliptol+Juá+Romã+Propolis. However, the MID for Cepacol® was lower than that determined for all other three products (p<0.05).

DISCUSSION

CPC is a cationic compound used in oral antiseptics. It has a broad action against bacteria present in the oral cavity²¹. Over 99% of the microorganisms associated with biofilm/dental plaque formation and gingivitis are eliminated by solutions containing 0.065% CPC³⁰. A reduction of 39% in biofilm/dental plaque formation has been observed in brushed surfaces, while this percentage is 25% in non-brushed surfaces^{6,9}. Roberts and Addy²² (1981) reported a residual effect for 180 to 300 min following the use of CPC-

based oral products.

Mouthwashes containing 0.05% CPC promote reduction in the amount of salivary microorganisms for 3 h following use²¹. One-minute rinsing with 15.0 mL of CPC-based mouthwash (0.05% CPC) for two weeks resulted in an inhibition of biofilm/dental plaque formation when associated with mechanical cleaning²⁴. Rawlinson, et al.²⁰ (2008) determined the plaque inhibition properties of two formulations of alcohol-free mouthwash with 0.1% or 0.05% CPC. They showed that the use of both CPC mouthwashes resulted in less plaque accumulation compared to the control (placebo).

According to the FDA Plaque Subcommittee, CPC is a safe antimicrobial agent for prevention of biofilm formation and gingivitis, when used in concentrations ranging from 0.05 to 0.1%⁸. In the present study, the products evaluated had a CPC concentration of 0.05%.

According to Albuquerque Jr., et al.¹ (2004), mouthwashes containing CPC are capable of inhibiting *S. aureus* strains *in vitro* at a 1/20 dilution. However, in this study, the MID was 1/160 for CPC+Propolis and Cepacol[®] and 1/320 for CPC+Malva and CPC+Eucalipto+Juá+Romã+Propolis.

Several natural extracts have been incorporated to the formulation of oral antiseptics¹³, such as *Jupinerus communis*, *Urtica dioica*, *Achillaea millefolium*²⁹ and *Salvadora persica*¹⁴. In the present experiment, three out of the four mouthwashes evaluated contain natural extracts. Based on the obtained results, addition of these extracts to CPC improved its antimicrobial action, as reported elsewhere¹⁰.

Propolis, a substance present in two of the CPC-based solutions evaluated in the present study, is described as a natural antibiotic produced by bees, and has shown activity against *S. aureus*²⁶. However, Silici and Kutluca²⁶ (2005) reported only one-weak activity of propolis against the Gram-negative microorganisms *Escherichia coli* and *Pseudomonas aeruginosa*.

Malva sylvestris, found in one of the products evaluated in the present study, has been shown to have antiinflammatory action¹¹, as well as antimicrobial activity against *E. coli* and *P. aeruginosa*². However, according to Coelho de Souza, et al.⁷ (2004), extracts from aerial portions of *M. sylvestris* do not demonstrate activity against *S. aureus*, *S. epidermidis*, *E. coli*, *Micrococcus luteus*, and *C. albicans*. Still according to the same study, only a slight activity was observed against *Saccharomyces cerevisiae*. Extract from the seed of *Malva moschata*, a plant found in Scotland, is active against *S. aureus*, *S. epidermidis*, *Proteus mirabilis*, and *E. coli*. Nevertheless, extracts obtained from leaves, roots, and seeds may present different effects due to the different concentrations of the active principle in the solutions produced from each of these parts of the plant¹⁶.

Another natural ingredient added to one of the CPC-based products evaluated is *Zizyphus joazeiro* extract, which has shown antimicrobial activity against Gram-positive microorganisms²⁵.

Eucalyptus, another source of natural extract present in

one of the solutions evaluated in the present study, is a genus comprising approximately 600 species of trees, native from Australia. *Eucalyptus globulus* is the most commonly cultivated species in subtropical and Mediterranean regions, and its natural extract has shown effectiveness against *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*¹⁹. *Eucalyptus globulus*, *Eucalyptus maculata*, and *Eucalyptus viminalis* were able to inhibit the growth of several Gram-positive bacteria (*S. aureus*, MRSA, *Bacillus cereus*, *E. faecalis*, *Alicyclobacillus acidoterrestris* and *Propionibacterium acnes*) and one yeast (*Trichophyton mentagrophytes*). On the other hand, these extracts showed little effectiveness against Gram-negative bacteria (*E. coli* and *P. putida*). Nevertheless, *Eucalyptus botryoides* and *Eucalyptus nitens* extracts inhibited the growth of both Gram-positive and Gram-negative microorganisms²⁸.

Punica granatum (pomegranate) extract is another compound reported as being active against *S. aureus*, *P. aeruginosa*, *C. albicans*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*⁴.

CONCLUSIONS

According to the proposed methodology and based on the obtained results, it may be concluded that the addition of natural extracts to CPC enhanced its antimicrobial effect.

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