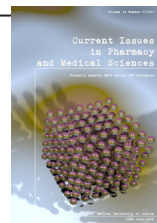


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Evaluation of antibacterial and antifungal properties of selected mouthwashes: *in vitro* studies

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ABSTRACT

Introduction. Mouthwashes should include antimicrobial compounds to inhibit microorganism multiplication, thus the formation and development of dental plaque.

Aim. The aim of the study was to evaluate the antibacterial and antifungal effectiveness of mouthwashes depending on their active ingredients.

Material and methods. In the study, the effect of antibacterial and antifungal properties of mouthwashes on reference bacterial and yeast-like fungi strains was examined. The first type of the assessed mouthwashes contained only sodium fluoride or sodium fluoride and amine fluoride as active ingredients, while the second type contained sodium fluoride and cetylpyridinium chloride.

In the study, a well diffusion method was used to test microorganisms constituting natural or pathogenic microflora of oral mucosa. The used reference microorganisms came from the ATCC: *L. acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 53103, *S. mutans* ATCC 25175, and *Candida* yeasts: *C. albicans* ATCC 2091, *C. albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 14243.

Results. The mouthwashes containing sodium fluoride and cetylpyridinium chloride showed an inhibitory effect against a greater number of reference strains used in the study than did mouthwashes that contained only sodium fluoride (or sodium fluoride and amine fluoride) as active ingredients. Against the four reference strains of *Candida* genus, the mouthwashes with an inorganic and organic fluoride compound showed no or minimum inhibitory effect or were much less effective than the mouthwashes that also contained cetylpyridinium chloride.

Conclusion. Mouthwashes containing multiple ingredients with different antimicrobial mechanisms show synergistic action against the bacterial and fungal microflora responsible for the accumulation of dental plaque.

INTRODUCTION

The presence of dental plaque, which is in fact, bacterial biofilm, is a source of dental caries, gingivitis, periodontitis and mucositis. The spots where dental plaque mainly accumulates are teeth surfaces along the gum line, in the form of supragingival and subgingival plaque, as well as anatomical teeth furrows and cavities, and carious lesions. Other spots that should also be mentioned are crowded teeth areas, inappropriately made fillings, prosthetic restorations and orthodontic braces – fixed and removable [1].

Supragingival plaque is dominated by Gram-positive bacteria, including *Streptococcus sanguinis*, *Streptococcus*

mutans, *Streptococcus mitis*, *Streptococcus salivarius* and lactic acid bacteria, and it is a cause of dental caries. Subgingival plaque is responsible for gingivitis and periodontitis, and mainly involves anaerobic Gram-negative bacteria, such as *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter* spp., *Capnocytophoga* spp., *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and oral spirochetes including *Treponema denticola* [2].

Scientific evidence shows the possibility of participation of *Candida* yeast-like fungi in a cariogenic process, and biofilms formed by *S. mutans* and *Candida albicans* indicate increased cariogenic potential compared to biofilms of one species [3].

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The hidden cariogenic potential of *Candida* species is connected with their virulence characteristics, such as acidogenicity and acidoduricity, ability to form abundant biofilms, to ferment and absorb sugars from food, as well as to create collagenolytic proteinases. Data suggest that *Candida* yeast-like fungi can play a key role as a secondary factor preserving cariogenic process, especially in dentine caries [4].

The principal method of preventing the formation of bacterial biofilm in the oral cavity is hygiene based on regular mechanical removal of bacterial plaque with a toothbrush, toothpaste and dental flosses. This process is complemented by using mouthwashes to reach areas inaccessible to toothbrushes. Mouthwashes should contain antimicrobial compounds, thereby inhibiting microorganisms multiplication, thus formation and development of dental plaque. Routine mouth rinsing after teeth brushing should be performed once-twice a day.

Mouthwashes contain agents with antibacterial properties, most frequently chlorhexidine, triclosan, and cetylpyridinium chloride (CPC) [5].

Most toothpastes, mouthwashes, dental flosses and chewing gums contain fluorine compounds, and most frequently they are sodium fluoride, stannous fluoride, sodium monofluorophosphate, and aminofluorides. The principal activity of fluorine is that of anticariogenic [6], aiming at decreasing enamel demineralization and favoring remineralization of damaged surfaces of hard dental tissues [7]. Moreover, fluorine decreases formation of bacterial plaque and reduces the quantity of cariogenic bacteria (*S. mutans*, *L. acidophilus*). It influences the metabolism of bacterial cells, modifying carbohydrate metabolism by inhibition of enolase – the fluoride ion, through connecting with the magnesium ion enolase molecule, inhibits its activity [8]. Mouthwashes evaluated in the own studies contained sodium fluoride in various concentrations.

Cetylpyridinium chloride (CPC) shows bactericidal activity against Gram-positive bacteria and activity (albeit, weaker) against Gram-negative. It also reveals fungicidal activity (*C. albicans*). It is not active against acid-resistant mycobacteria and viruses. Due to its antibacterial properties, it is widely used as an ingredient in mouthwashes [9]. It works by absorption to negatively charged teeth surfaces and bacterial plaque, and by decreasing surface tension of the bacterial cell membrane, what consequently results in its rupture and the destruction of the bacterial cell. What is more, it influences bacteria metabolism and significantly reduces adhesion of microorganisms to teeth surfaces [10]. By way of its properties, CPC is well and quickly absorbed by teeth surfaces – this considerably decreases adhesion of bacteria to hard dental tissues, reducing amount of bacterial plaque. Apart from fluorine compounds, it is the most frequently used mouthwash ingredient [7].

AIM

The aim of the study was to evaluate the antibacterial and antifungal effectiveness of mouthwashes depending on their active ingredients.

MATERIALS AND METHODS

The influence of six mouthwashes on the reference bacterial and yeast-like fungi strains was evaluated. The impact of the following mouthwashes was assessed: Colgate Plax Cool Mint (Colgate-Palmolive®), Listerine Professional (Listerine®), Oral B Pro-Expert (Procter & Gamble®), Mint Perfekt Sensitiv (Ziaja®), Elmex (Gaba International AG®), and Smile 3D Protection “Multi Care” (Smile 3D Protection®). The listed products are the most popular among the patients who were under preventive and therapeutic care of the authors’ department. All the selected mouthwashes are widely available in pharmacies, drug stores, and shops.

The mouthwashes containing sodium fluoride and amine fluoride (olafur) include: Mint Perfekt Sensitiv by Ziaja (250 ppm F), Elmex (250 ppm F), and Listerine Professional (220 ppm F).

Sodium fluoride and cetylpyridinium chloride are ingredients of the following mouthwashes: Smile 3D Protection “Multi Care” (500 ppm F), Colgate Plax Cool Mint (225 ppm F) and Oral B Pro-Expert Clinic Line (98 ppm F) (Tab. 1).

Table 1. Ingredients of the evaluated mouthwashes

Mouthwash	Ingredients
Colgate Plax Cool Mint (Colgate-Palmolive®)	water, glycerin, sorbitol, cetylpyridinium chloride, sodium fluoride 0.05% (225 ppm F), menthol, propylene glycol, poloxamer 407, potassium sorbate, saccharin, patent blue (CI 42051)
Listerine Professional (Listerine®)	dipotassium oxalate 1.4%, water, sorbitol, propylene glycol, phosphoric acid, aroma, poloxamer 407, sodium benzoate, sodium methyl cocoyl taurate, sodium lauryl sulfate, sodium fluoride (220 ppm F), sucralose, sodium saccharin
Oral B Pro-Expert (Procter & Gamble®)	water, glycerin, aroma, polysorbate 20, cetylpyridinium chloride (0.05%), sodium fluoride (98 ppm F), saccharin, methylparaben, sodium benzoate, propylparaben, patent blue (CI 42051)
Mint Perfekt Sensitiv (Ziaja®)	water, hydrogenated castor oil, betaine, olafur, sodium fluoride (0.025%), potassium nitrate, sodium saccharin, sodium benzoate, aroma, dipentane, eugenol, citric acid, propylene glycol, fuchsia (CI 17200)
Elmex (Gaba International AG®)	water, hydrogenated castor oil, aroma, sodium fluoride (0.025%), olafur, potassium acesulfame, polyaminopropyl biguanide, hydrochloric acid
Smile 3D Protection Multi Care (Smile 3D Protection®)	water, glycerin, hydrogenated castor oil, sodium fluoride (500 ppm F), cetylpyridinium chloride, aroma, saccharin, sodium benzoate, citric acid, patent blue (CI 42051), tetrasodium EDTA, polysorbate 20, sodium citrate, dipentane, quinolone yellow (CI 47005)

The following kinds of media were used in the study:

- to grow *Lactobacillus* spp. and to evaluate antiseptic activity of the studied mouthwashes against reference bacteria strains – Rogosa Agar or MRS Agar;
- to grow *S. mutans* and to evaluate antiseptic activity of the studied mouthwashes against reference bacteria strains – Mitis Salivarius Agar with 5% of sheep blood;
- to grow *Candida* spp. and to evaluate antiseptic activity of the studied mouthwashes against reference yeasts strains – Sabouraud agar.

In the study of antibacterial and antifungal activities of six selected mouthwashes, the well diffusion method was used in reference to microorganisms constituting natural or pathogenic microflora of oral mucosa. The used reference microorganisms came from the ATCC (American Type Culture Collection). It included the following bacteria: *L. acidophilus* ATCC 4356, *Lactobacillus rhamnosus* ATCC 53103,

S. mutans ATCC 25175, and *Candida* yeasts: *C. albicans* ATCC 2091, *C. albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 14243.

Initially, microorganisms were multiplied (1-5 days, temperature 35-37°C, in the case of lactic acid – additionally in anaerobic conditions) on the appropriate propagation media. Next, suspensions were prepared out of them in the sterile physiological fluid (0.85% NaCl) with a strictly defined density of 0.5 McFarland. The following stage was seeding with sterile swab in three different directions on the proper selective media. When the whole surface of plate was covered with microorganisms suspension, wells were cut in the medium with a sterile 10 mm diameter punch. After that, 100 µl of each of the studied mouthwashes was distributed to the wells and all was pre-incubated for 1 hour in order to diffuse fluid to the medium. Thereafter, plates were incubated in appropriate conditions, i.e., *Candida* spp. – 35-37°C, 2 days, aerobic conditions; *S. mutans* – 35-37°C, 3 days, aerobic conditions; *L. acidophilus* – 35-37°C, 3-5 days, anaerobic conditions. After the above-mentioned time, the plates were taken out and inhibition zone diameters around the wells with studied mouthwashes were observed. Diameters were measured in mm and compared. The most representative results were used to evaluate the antibacterial and antifungal activity of the tested mouthwashes.

RESULTS

As it is shown in Table 2, growth in microorganisms around the wells with the studied antiseptic rinses was to various degrees inhibited. Each of the selected mouthwashes had a number allocated, as follows:

- no. 1 – Colgate Plax Cool Mint (Colgate-Palmolive®),
- no. 2 – Listerine Professional (Listerine®),
- no. 3 – Oral B Pro-Expert (Procter & Gamble®),
- no. 4 – Mint Perfekt Sensitiv (Ziaja®),
- no. 5 – Elmex (Gaba International AG®),
- no. 6 – Smile 3D Protection “Multi Care” (Smile 3D Protection®).

Inhibition zones varied from 0 to 30 mm in the case of bacteria and from 0 to 24 mm in the case of yeasts. The studied mouthwashes showed antimicrobial activity, both antibacterial and antifungal, except for mouthwashes no. 4 (Mint Perfekt Sensitiv) and no. 2 (Listerine Professional), the spectrum of which was only limited to bacteria. Growth in *L. acidophilus* ATCC 4356, *L. rhamnosus* ATCC 53103 and *S. mutans* ATCC 25175 was inhibited by mouthwash no. 4 (Mint Perfekt Sensitiv). The product also inhibited *Candida* yeasts (*C. albicans* ATCC 10231) to the minimum level. In turn, mouthwash no. 2 (Listerine Professional) had inhibitory effect only on growth of *L. acidophilus* ATCC 4356 and *L. rhamnosus* ATCC 53103, and it did not exhibit inhibiting influence on *S. mutans* bacteria and *Candida*

yeasts. The following mouthwashes: no. 6 (Smile 3D Protection “Multi Care”), no. 3 (Oral B Pro-Expert), no. 1 (Colgate Plax Cool Mint) and no. 5 (Elmex) showed inhibiting influence on all the selected microorganisms.

The greatest inhibiting impact on growth of *L. acidophilus* ATCC 4356 was shown by mouthwash no. 6 (Smile 3D Protection “Multi Care”), followed by no. 4 (Mint Perfekt Sensitiv). Similar activity was indicated by mouthwashes no. 3 (Oral B Pro-Expert), no. 1 (Colgate Plax Cool Mint) and no. 5 (Elmex). Product no. 2 (Listerine Professional) was the least effective in inhibiting *L. acidophilus* ATCC 4356 growth.

The greatest inhibiting impact on growth of *L. rhamnosus* ATCC 53103 was evidenced by mouthwash no. 4 (Mint Perfekt Sensitiv), followed by no. 6 (Smile 3D Protection “Multi Care”). To a lesser degree, growth in those bacteria was inhibited by mouthwashes no. 1 (Colgate Plax Cool Mint), no. 5 (Elmex) and no. 3 (Oral B Pro-Expert), while the least effective was mouthwash no. 2 (Listerine Professional).

The greatest inhibiting impact on growth of *S. mutans* ATCC 25175 was demonstrated by product no. 1 (Colgate Plax Cool Mint). To a lesser degree, inhibiting activity against *S. mutans* was shown by mouthwashes no. 3 (Oral B Pro-Expert), no. 6 (Smile 3D Protection “Multi Care”), no. 4 (Mint Perfekt Sensitiv) and no. 5 (Elmex). As mentioned before, mouth rinse no. 2 (Listerine Professional) did not reveal antibacterial activity against *S. mutans* at all. The mouthwashes no. 6 (Smile 3D Protection “Multi Care”), no. 3 (Oral B Pro-Expert) and no. 1 (Colgate Plax Cool Mint) exhibited similar inhibiting influence on growth of all the studied yeas-like fungi strains. Product no. 5 (Elmex) indicated weaker activity against *Candida* yeasts. Mouthwash no. 4 (Mint Perfekt Sensitiv) showed negligible impact on inhibition of *C. albicans* ATCC 10231 growth and did not inhibit growth of remaining studied *Candida* yeasts. Product no. 2 (Listerine Professional) showed inhibiting property against none of the studied yeasts.

DISCUSSION

In vitro studies of susceptibility of reference bacteria and fungi strains to activity of antimicrobial compounds allow

Table 2. Antimicrobial activity of mouthwashes against selected reference bacteria and fungi strains

Microorganisms	Inhibition zone of microorganisms growth around a well with mouthwash (mm)					
	Colgate Plax Cool Mint (Colgate-Palmolive®)	Listerine Professional (Listerine®)	Oral B Pro-Expert (Procter & Gamble®)	Mint Perfekt Sensitiv (Ziaja®)	Elmex (Gaba International AG®)	Smile 3D Protection “Multi Care” (Smile 3D Protection®)
	1	2	3	4	5	6
<i>Lactobacillus acidophilus</i> ATCC 4356	20-21	16-17	20-22	22-23	21-22	25-26
<i>Lactobacillus rhamnosus</i> ATCC 53103	20-21	12-13	19-20	28-30	19-21	24-25
<i>Streptococcus mutans</i> ATCC 25175	23-24	0	20-21	15-17	15-16	19-20
<i>Candida albicans</i> ATCC 2091	19-20	0	18-23	0	15-16	20-22
<i>Candida albicans</i> ATCC 10231	19-20	0	17-19	7-9	13-14	20-21
<i>Candida parapsilosis</i> ATCC 22019	20-21	0	19-20	0	14-17	20-21
<i>Candida glabrata</i> ATCC 90030	21-22	0	20-21	0	17-18	20-21
<i>Candida krusei</i> ATCC 14243	23-24	0	20-21	0	14-15	21-22

for evaluation of influence of active substances on selected microorganisms. As far as mouthwashes are concerned, it is vital to establish optimal lists of ingredients, which, due to active substance content, would most effectively induce a reduction of the bacteria and fungi constituting dental plaque.

In our work, we demonstrated the significantly greater antibacterial properties against *S. mutans* of the mouthwashes containing sodium fluoride and cetylpyridinium chloride, compared to the mouthwashes containing only fluoride compounds. In the evaluation of various antibacterial substances efficacy against *S. mutans* grown on ready-made enamel slabs, Savas *et al.* also observed that a mouthwash containing both compounds was more effective in reducing the quantity of *S. mutans* colonies [11].

Evidence of the antibacterial synergistic activity of sodium fluoride and cetylpyridinium chloride against *S. mutans* strains was indicated in the 100% reduction of bacteria in oral bacterial biofilm after the use of mouthwash containing both ingredients. In contrast, after the use of mouthwash involving only NaF, the number of *S. mutans* present in dental plaque was reduced by only 16.82% [12].

Similarly to own studies, in Brazilian studies, no positive influence of Listerine® (NaF) mouthwash on the reduction of *S. mutans* was found. However, a similar impact of Colgate Plax® and Oral B® (NaF and CPC) mouthwashes on decrease in the quantity of *S. mutans* was stated. This confirms the effectiveness of synergistic activity of both ingredients against *S. mutans* bacteria [13].

The mere presence of fluorine as an active ingredient in a mouthwash does not significantly influence the reduction of *S. mutans* strains, when compared with a mouthwash containing chlorhexidine and fluorine. This was revealed in evaluating influence of mouthwashes with various ingredients on recently extracted maxillary premolars. In this case, the synergistic antibacterial activity of chlorhexidine and fluorine against *S. mutans* was also confirmed [14].

Greater antibacterial activity of a mouthwash containing sodium fluoride and cetylpyridinium chloride (Oral B®) against *S. mutans*, as compared to a mouthwash involving sodium fluoride (Listerine®) was found out in saliva after the use of the mentioned mouthwashes in the study by Cardoso *et al.* Therein, the inhibiting influence of both products against *S. mutans* was demonstrated. In this study, the antibacterial activity of Oral B® mouthwash was higher, and a reduction of *S. mutans* quantity from 6.75 CFU/ml to 6.00 CFU/ml in saliva was observed after the use of this product. After the use of Listerine®, however, insignificant reduction of *S. mutans* quantity from 6.81 CFU/ml to 6.79 CFU/ml was noticed [15]. In our studies, the significant inhibiting impact of Oral B® mouthwash on *S. mutans* ATCC 25175 was confirmed, as well as lack of this impact in the case of Listerine® mouthwash. Similarly, the inhibiting influence of Oral B® mouthwash on reference *Streptococcus* bacteria strains was confirmed in previous studies [16].

Evans *et al.* compared the antibacterial influence of mouthwashes containing individual active ingredients – sodium fluoride in concentration of 0.05% (Colgate Neutrafluor 220®) and cetylpyridinium chloride in the concentration of 0.05% (Cepacol®) on reference *S. mutans* and *L. acidophilus* strains.

They saw that inhibition zone was, respectively, equal to 7.5 mm for *S. mutans* and 12 mm for *L. acidophilus* in the case of mouthwashes with sodium fluoride, and, respectively, 8.6 mm and 11.1 mm – with cetylpyridinium chloride. Each of the active ingredients separately showed similar antibacterial properties, however, both ingredients together (present in Colgate Plax Cool Mint, Oral B Pro-Expert, Smile 3D Protection “Multi Care”) showed significantly weaker influence on reference streptococci strains than in the case of own studies – which was confirmed by almost twice bigger inhibition zones [17].

In another study, a comparison of the antibacterial influence of Colgate Plax® and Listerine® mouthwashes on reference *S. mutans* and *L. acidophilus* strains, both of which play significant roles in the etiology of dental caries, showed the impact of both mouthwashes on the reduction of streptococci colonies. After the use of Colgate Plax® mouthwash, a *S. mutans* inhibition zone with diameter of 22±2 mm was observed, while after the use of Listerine®, this was 23±1.73 mm. Furthermore, the inhibition zone of lactobacilli growth after the use of Colgate Plax® was equal to 20±2 mm, while after the use of Listerine® the figure was 11±1 mm [18]. In our work, the inhibition zone of *S. mutans* growth by Colgate Plax Cool Mint® mouthwash amounted to 23-24 mm, whereas Listerine® mouthwash did not show any inhibiting activity against *S. mutans*. Moreover, weaker activity against *L. acidophilus* and *L. rhamnosus* was shown by Colgate Plax Cool Mint® mouthwash – in both cases, the inhibition zone was equal to 20-21 mm, whilst Listerine® mouthwash showed greater antibacterial properties against lactic acid bacteria – from 12-13 to 16-17 mm, than was in the case of the above mentioned results of other authors.

Interesting results were obtained by Ximenes *et al.*, who studied the influence of active ingredients of mouthwashes on reference *S. mutans*, *L. acidophilus*, and *E. faecalis* strains. They saw greater antibacterial impact of a mouthwash containing NaF and chlorhexidine on *L. acidophilus* than a mouthwash involving only sodium fluoride. What is more, greater reduction of *S. mutans* quantity was stated with mere sodium fluoride than with the use of a mouthwash including chlorhexidine and sodium fluoride [19].

Our studies indicated the similar influence of Colgate Plax Cool Mint®, Smile 3D Protection®, and Oral B Pro-Expert® mouthwashes, therefore those containing cetylpyridinium chloride and sodium fluoride, on inhibition of reference *C. albicans* growth. However, no inhibiting activity was found in Listerine® and Mint Perfekt Sensitiv®, i.e., mouthwashes with sodium fluoride and amine fluoride as active ingredients. Similar results were obtained by the Polish researchers [20] who evaluated an influence of, among others, Colgate Plax®, Oral B Pro-Expert® and Listerine® mouthwashes against reference *Candida* yeasts strains, and revealed that Colgate Plax® mouth rinse inhibited growth of *Candida* yeasts in a zone with a diameter of 19.14 mm. A similar inhibition zone was achieved in our study, wherein it was equal to 20 mm. An approximately similar impact of Oral B® mouthwash on reference *C. albicans* strains was also indicated in other

studies, in which the inhibition zone of *Candida* yeasts after the use of this mouth rinse amounted to 23 mm [21].

As with our own study, Brazilian researchers did not find a positive impact of Listerine® mouthwash on reduction of the quantity of *Candida* yeasts colonies, whereas Oral B® and Colgate Plax® mouthwashes demonstrated a reduction of *C. albicans* strains [13].





Paulone *et al.* also achieved a reduction of the number of reference *C. albicans* strains after the use of a mouthwash containing both fluorine and chlorhexidine. The studies confirmed that both compounds showed synergistic activity. This enabled the achievement of better effects in reducing of the number of *C. albicans* yeasts [22]. In our study conducted on the reference fungi strains, the greatest inhibiting influence on the growth of selected *Candida* strains was confirmed by mouthwashes containing sodium fluoride and cetylpyridinium chloride, while mouthwashes including sodium fluoride as an active ingredient did not inhibit the growth or inhibited it in a very limited range. Among the latter, only Elmex® mouthwash showed inhibition zone of growth of selected *Candida* strains (from 13-14 mm to 17-18 mm).

Our work and research by other authors confirm that mouthwashes containing multiple ingredients with different antimicrobial mechanisms have a broader spectrum of activity against the bacterial and fungal microflora responsible for the accumulation of dental plaque than do mouthwashes with single active ingredients [11-15, 20-22]. This knowledge is extremely important and useful for dentists in their everyday clinical practice, and provides a guide for their recommendations for choice of appropriate mouthwashes for individual patients, both for prophylactic and therapeutic purposes, according to the clinical condition and composition of the patient's oral microbiome.

CONCLUSIONS

The varied antimicrobial activity of mouthwashes was confirmed. Mouthwashes containing sodium fluoride and cetylpyridinium chloride showed an inhibitory effect against a greater number of reference strains than did mouthwashes which contained only sodium fluoride or sodium fluoride and aminofluoride as active ingredients. From the clinical perspective, it is necessary to choose mouthwashes containing different active ingredients, according to the prophylactic and therapeutic needs of patients.

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