ANTIBACTERIAL, ANTI-ADHESIVE AND ANTI-BIOFILM-FORMING ACTIVITY OF PLANT COMPLEXES AGAINST PERIODONTOPATHOGENIC BACTERIA *IN VITRO*

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Periodontitis is a problem urgent in Russia and throughout the world in general. Because of the dynamically changing flora causing this diseases, the treatment methods designed against it should be adapted on a regular basis. The classic approach to arresting development of the acute process relies on 0.2–0.12% chlorhexidine, a chemical antiseptic, but after 3 weeks of use, its efficacy drops drastically because pathogenic flora adjusts thereto. In the recent years, plant-based complexes with antiseptic properties have shown their capacity to challenge the classic approach. Obviously, efficacy of active ingredients depends on the form of the final product. The marker of periodontitis in the oral cavity is *Staphylococcus aureus*. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* have virulence markers that are copathogens for periodontitis. This study aimed to find plant-based preparations capable of eliminating the said microbes and *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus salivarius*, *Enterococcus faecalis*. We compared antibacterial, adhesion and biofilm formation preventing properties of Phytodent plant-based products in various forms: water solution, water-alcohol solution, oil solution, gel. Long exposure form — gel — proved to be the most effective in terms of the properties tested. Products with synthetic and plant-based antiseptics, as well as those with plant-based antiseptics in maximum concentration (elixir), had comparable efficacy. Water and oil solutions are less effective because of the lower active ingredient concentration and relatively brief exposure. Our results support the results of clinical studies dedicated to the use of Phytodent products as oral care products in the context of periodontitis prevention and treatment. We recommend conducting further studies comparing compositions, cross- and comparative studies investigating the effect of frequency of application and time of exposure, such studies registering titers of active ingredient concentrations, and with subjects thereof including mixed biofilms.

Keywords: periodontitis, copper derivatives of chlorophyll, gel, aspen bark, dihydroquercetin, biofilm formation preventing properties, antimicrobial properties, adhesion prevention properties, Phytodent

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Compliance with ethical standards: the study was approved by the Samara State Medical University's Ethics Committee (Minutes # ... of January 10, 2022)

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ИССЛЕДОВАНИЕ АНТИБАКТЕРИАЛЬНОЙ, АНТИАДГЕЗИВНОЙ И АНТИБИОПЛЕНКООБРАЗУЮЩЕЙ АКТИВНОСТИ РАСТИТЕЛЬНЫХ КОМПЛЕКСОВ В ОТНОШЕНИИ ПАРОДОНТОПАТОГЕННЫХ БАКТЕРИЙ *IN VITRO*

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Пародонтит — актуальная проблема в России и в мире, требующая регулярной адаптации схем лечения и реабилитации из-за динамично меняющейся пародонтопатогенной флоры. Классическая терапия купирования острого процесса включает использование химического антисептика хлоргексидин 0,2–0,12%, эффективного только до трех недель применения ввиду адаптации патогенной флоры. Растительные комплексы с антисептическим действием в последние годы зарекомендовали себя как способные заместить классическую терапию. Очевидно, что разные формы выпуска имеют разную эффективность. *Staphylococcus aureus* в полости рта служит маркером пародонтита. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* обладают маркерами вирулентности в качестве копатогенов при пародонтите. Целью исследования было выявить растительные препараты для борьбы с перечисленными микробами, а также с *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus salivarius*, *Enterococcus faecalis*. Проводили сравнительную оценку антибактериальной, антиадгезивной и антибиопленкообразующей активности отечественных средств «Фитодент» из растительного сырья: водных, водно-спиртовых и масляных растворов; гелевых форм. Наибольшая антибактериальная, антиадгезивная и антибиопленочная эффективность обнаружена у форм с длительной экспозицией — гелей, сопоставимая — у средств с синтетическими и с растительными антисептиками, а также у форм с максимальной концентрацией растительных антисептиков — эликсира. Водные и масляные формы за счет меньшей концентрации и сравнительно короткого времени контакта имеют меньшую эффективность. Полученные результаты подтверждают результаты клинических наблюдений за применением средств «Фитодент» в качестве ухода за полостью рта при лечении и профилактике пародонтита. Рекомендованы дальнейшие сравнительные исследования композиций, перекрестные и сравнительные исследования в зависимости от частоты применения и времени воздействия и с титрованием концентраций активных компонентов, в том числе на смешанных биопленках.

Ключевые слова: пародонтит, медные производные хлорофилла, гель, кора осины, дигидрокверцетин, антибиопленкообразующее действие, антимикробное действие, антиадгезивное действие, Фитодент

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Periodontitis (P) is an extremely urgent oral cavity problem in Russia and the entire world in general [1–4]. Almost all medicines with proven effectiveness against it, those that arrest acute infectious and inflammatory processes, contain a chemical antiseptic, 0.12–0.2% chlorhexidine in most cases, regardless of the form of the product [5-8]. However, bacterial flora can grow resistant to chemical antiseptics, which makes them effective only for a limited period of time. In the recent years, plant-based complexes with antiseptic properties proved they are a viable alternative to the standard antiseptic therapy products, with which these complexes are comparable in terms of the range of action and efficacy [9–12]. Obviously, different forms of products, be they water solutions, water-alcohol solutions, oil solutions, or gels, have different efficacy, which is conditioned by the time the product remains on periodontal tissues, and subsequently, frequency of its use, time needed to arrest the acute process and, ultimately, the time of compensated stable remission of the patient [13–14]. There is evidence confirming antibacterial properties of phytoncides of coniferous plants, extracts from aspen bark, Japanese and sugar kelp. The results of their application have been known for a long time, and various combined forms are used in dentistry in a cosmopolitan manner. However, the evidence base is fragmentary, and its key components are clinical, not fundamental [15–18]. The preferred form of anti-periodontitis drugs is gel, which ensures prolonged exposure of periodontal tissues to active ingredients. The gels currently available to a periodontist contain either an antiseptic or substrates for tissue repair. There is no gel acting in all the directions needed, i.e. eliminating toxins and biological debris, promoting metabolism in periodontal tissues, producing a non–specific immunomodulatory effect, normalizing respiration and trophism in the periodontium, and, as a result, inducting autoregeneration [19].

Oral cavity is a habitat for a large number of different microorganisms. Most of them are commensals. However, a number of bacterial species directly or indirectly drive development of P and other inflammatory processes. For example, Streptococcus sanguinis promotes formation of biofilm on teeth that includes *Fusobacterium nucleatum*, which is one of the bacteria causing P [20], with one of the possible consequences thereof being endocarditis [21]. Many researchers seek for preparations, plant-based or synthesized from substances of natural origin, that would combat cariesogenic and periodontal bacteria *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus salivarius*, *Enterococcus faecalis* [22–25]. Detection of *Staphylococcus aureus* and its metabolites in the oral cavity can indicate a certain stage of P [26]. The "oral cavity — intestine" pathogenetic axis allows considering a number of bacteria, especially those with virulence markers, as copathogens in periodontitis: *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* [27–29]. Therefore, we considered these pathogens in this work. The purpose of this study was to evaluate antibacterial, adhesion and biofilm formation preventing properties of various forms of products combining plant-based and synthetic components factoring in the vectors of their effect on flora causing periodontitis.

METHODS

The study was conducted at the Department of New Technologies of the Pasteur Research Institute in October 2021 – April 2022.

We used the patented gel composition that includes copper derivatives of chlorophyll, aspen bark, sodium alginate and dihydroquercetin (DHQ); this gel meets the requirements for complex therapy of P and has the necessary antibacterial properties [30]. The study has shown positive clinical results of application of gel with copper derivatives of chlorophyll, aspen bark, sodium alginate and DHQ against P [31] and gingivitis concomitant with P [32].

Compositions of active ingredients

The elixir is a water-alcohol concentrate of active ingredients: water, 20% ethyl alcohol, sodium-copper chlorophyllin, aspen bark extract, kelp extract, cocamidopropyl betaine, natural flavor "Mint", polyvinylpyrrolidone.

The mouthwash is a water concentrate of active ingredients: water, sodium-copper chlorophyllin, aspen bark extract, kelp extract, cocamidopropyl betaine, natural flavor "Mint", polyvinylpyrrolidone, sodium benzoate.

Phytolon oil is an oil solution of active ingredients: refined peach pit or olive oil, sodium-copper chlorophyllin.

Provitam oil is an oil solution of active ingredients: refined peach pit or olive oil, spruce concentrate with provitamins.

Gel 1 with chlorhexidine is a gel composition of active ingredients: sorbitol, water, hydrogenated castor oil, hydroxyethyl cellulose, sodium alginate, chlorhexidine hydrochloride, d-panthenol, allantoin, methylparaben, methyl salicylate, flavor "Pectral", menthol, fir extract, sodium-copper chlorophyllin, eugenol, pectin.

Gel 2 with aspen bark and DHQ is a gel composition of active ingredients: sorbitol, water, hydrogenated castor oil, hydroxyethyl cellulose, sodium alginate, dihydroquercetin, d-panthenol, allantoin, aspen bark extract, methylparaben, methyl salicylate, spruce extract complex, menthol, flavor "Pectral", citric acid, sodium-copper chlorophyllin, eugenol.

Gel 3 with chlorhexidine is a gel composition of active ingredients: sorbitol, water, hydrogenated castor oil, hydroxyethyl cellulose, sodium alginate, d-panthenol, chlorhexidine hydrochloride, allantoin, methylparaben, methyl salicylate, Pectral flavor, menthol, fir extract, sodium-copper chlorophyllin, eugenol, pectin; the product was 2 years expired.

Bacterial strains

Reference bacterial strains:
Staphylococcus aureus ATCC № 25923
Enterococcus faecalis ATCC № 29212
Klebsiella pneumoniae ATCC № 13883
Pseudomonas aeruginosa ATCC № 27853
Acinetobacter baumannii ATCC № 19606
Bacterial strains from the laboratory's collection of microorganisms:
Streptococcus sanguinis № 2111
Streptococcus mitis № 2118
Streptococcus oralis № 2114
Streptococcus salivarius № 2107

Research methods

We used the bacteriological method of research to study the effect of plant-based complexes on the bacteria's capability to survive, adhere to surfaces and form biofilms.

Investigation of antibacterial properties of plant-based complexes

We prepared microbial suspensions of 24-hour bacterial cultures in saline, initial content — 1×10^8 CFU/ml. Tenfold dilutions brought the content down to 1×10^5 CFU/ml. For the last

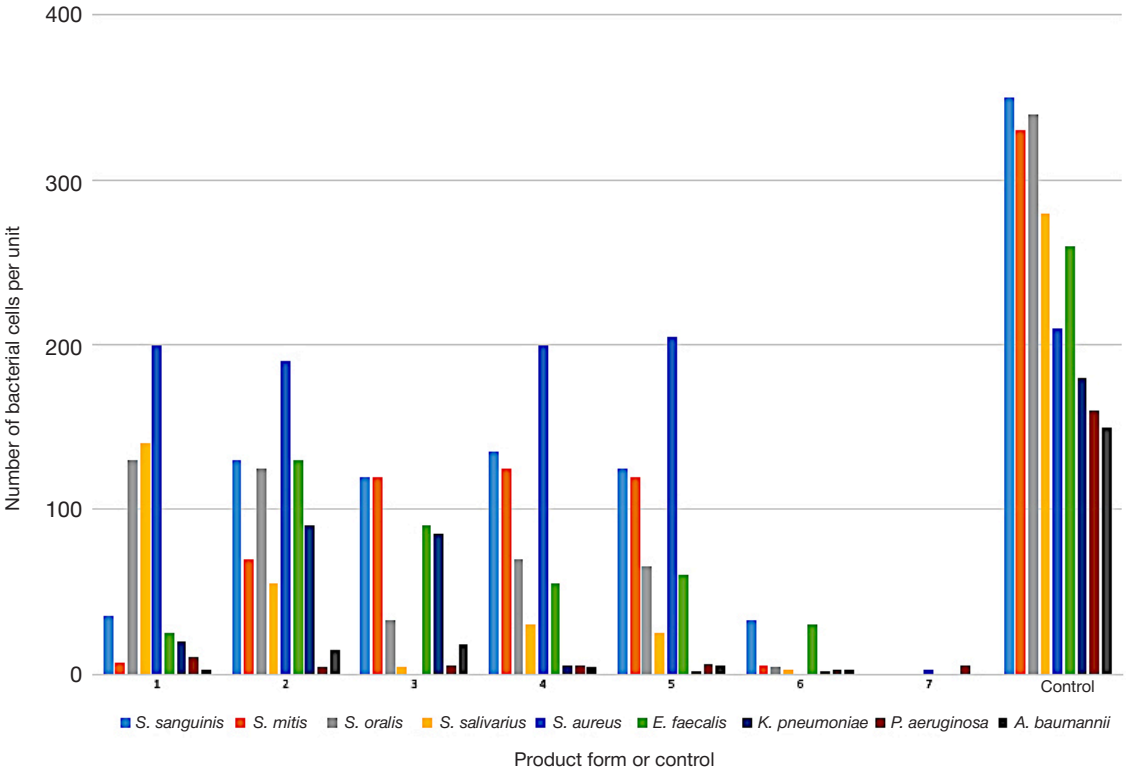

Fig. 1. Investigation of antibacterial properties of plant-based complexes. 1 — mouthwash; 2 — gel with chlorophyll, aspen bark and DHQ (Gel 2); 3 — gel with chlorophyll and chlorhexidine (Gel 1); 4 — oil with chlorophyll (Phytolone oil); 5 — oil with carotenoids from fir needles (Provitam oil); 6 — gel with chlorophyll and chlorhexidine (Gel 3); 7 — dental elixir with aspen bark extract and chlorophyll (Elixir)

dilution, we used meat peptone broth. Microbial suspensions were transferred to twenty four 1 ml tubes. One ml of each plant-based complex was added to 3 test tubes containing 1 ml of microbial suspension (final concentration), and the remaining 2 test tubes received 1 ml of saline. All test tubes were kept in the thermostat at +37 °C for 30 minutes. Then, we plated 10 mcl from each tube onto nutrient agar: blood agar for streptococci, meat peptone agar for other bacteria. Plating followed the lawn pattern. Petri dishes were incubated in a thermostat at +37 °C for 24 hours. Next, we counted colonies on dish and calculated simple mean for each plant ingredient sample.

Investigation of adhesion preventing properties of plant-based complexes

For this investigation, we applied A.S. Blagonravova's method [33] and used cells of buccal epithelium. The cells were washed three times from the indigenous microflora in buffered saline solution at pH 7.2–7.4, speed of 35 g, for 10 minutes. Then the cells were put into test tubes, 0.5 ml of bacterial suspension containing 3×10^8 CFU/ml of S.sanguinis and 0.5 ml of each plant-based complex into each tube. One test tube contained only buccal epithelium and was used as control (natural colonization). We did three cycles of all experiments and controls. Next, the tubes with all the components were intensively shaken and put into a thermostat to incubate at +37 °C for 30 minutes. After that, we washed out non-attached microorganisms, prepared smears on slides, fixed and Gram stained the bacteria. The adhesion index was calculated by the formula:

$$AI = AB50/50E, (1)$$

where AI is the adhesion index, AB50 is the number of bacterial cells that attached to 50 epithelial cells, and 50E is the 50 studied epithelial cells.

Investigation of biofilm formation preventing properties of plant-based complexes

In the context of this investigation, we registered microcolonies of bacteria forming on a dense nutrient medium. First, we applied the 0.5 McFarland turbidity standard to the daily bacterial culture of the studied strains. Then we reduced concentration of cells to 1×10^6 CFU/ml by serial dilutions, added each plant-based complex to each resulting solution at a ratio of 1 : 10, and spread the resulting mixture on a sterile slide. As a control, we used an inoculum of bacteria in a nutrient broth without the drug. Next, the samples were placed in a thermostat to incubate at +37 °C for 3 hours. After that, the slides were examined under an Axio Scope A1 microscope (Zeiss; Germany) at a magnification of x400. The photos were taken with a professional stationary digital camera AxioCam HRc Rev3 (Zeiss; Germany). We noted the number of bacterial microcolonies that had grown on control and experimental slides on several camera coverages. Whenever the number of microcolonies was twofold or more smaller on experimental slides than on control slides, the respective plant-based complex, in the given form, was considered to produce strong biofilm growth preventing effect.

For statistical analysis of the data, we used MS Excel 2010 (Microsoft; USA), and applied Student's t-test to the results. The results were considered significant at $p < 0.05$.

RESULTS

Investigation of antibacterial properties

Table 1 and Figure 1 present the results of investigation of antibacterial properties.

Description of the results:

1. The most effective product against bacterial flora was

Table 1. Investigation of antibacterial properties of plant-based complexes

Studied object (microorganism)	Number of colonies grown in samples with plant-based complexes (CFU/ml), (M + *m*)							Number of colonies grown in control samples (CFU/ml)
	1	2	3	4	5	6	7	
S. sanguinis	35 ± 5	130 ± 13	120 ± 11	135 ± 15	125 ± 14	33 ± 5	0 ± 1	350 ± 28
S. mitis	7 ± 2	70 ± 6	120 ± 14	125 ± 11	120 ± 9	5 ± 2	0 ± 1	330 ± 31
S. oralis	130 ± 13	125 ± 11	33 ± 5	70 ± 6	65 ± 6	4 ± 1	0 ± 1	340 ± 24
S. salivarius	140 ± 8	55 ± 5	4 ± 2	30 ± 4	25 ± 3	3 ± 1	0 ± 1	280 ± 18
S. aureus	200 ± 18	190 ± 15	0 ± 1	200 ± 17	205 ± 20	0 ± 1	3 ± 1	210 ± 15
E.faecalis	25 ± 4	130 ± 15	90 ± 8	55 ± 6	60 ± 5	30 ± 3	0 ± 1	260 ± 25
K. pneumoniae	20 ± 3	90 ± 6	85 ± 7	5 ± 2	2 ± 1	2 ± 1	0 ± 1	180 ± 15
P. aeruginosa	10 ± 2	4 ± 2	5 ± 2	5 ± 1	6 ± 2	3 ± 1	5 ± 2	160 ± 14
A. baumannii	3 ± 1	15 ± 2	18 ± 3	4 ± 1	5 ± 2	3 ± 1	0 ± 1	150 ± 17

Note: 1 — mouthwash; 2 — gel with chlorophyll, aspen bark and DHQ (Gel 2); 3 — gel with chlorophyll and chlorhexidine (Gel 1); 4 — oil with chlorophyll (Phytolone oil); 5 — oil with carotenoids from fir needles (Provitam oil); 6 — gel with chlorophyll and chlorhexidine (Gel 3); 7 — dental elixir with aspen bark extract and chlorophyll (Elixir)

elixir with 20% of ethyl alcohol (by weight) and maximum concentrations of active ingredients: aspen bark extract, sodium alginate and sodium-copper chlorophyllin.

2. Gel 1 had comparable antibacterial efficacy; its composition includes a chemical antibacterial agent (chlorhexidine) in a bactericidal concentration of 0.12%, and plant-based ingredients (sodium alginate, D-panthenol, allantoin, methyl salicylate, menthol, fir extract, sodium-copper chlorophyllin, eugenol).

3. Gel 3, which had similar but expired 2 years ago, showed average antibacterial activity.

4. Mouthwash and Gel 2 with aspen bark and DHQ were not highly effective against pathogenic flora of periodontium.

5. Oil solutions had poor eliminating effect on the pathogenic flora of periodontium.

Investigation of adhesion preventing properties

Table 2 shows the results of investigation of adhesion preventing properties.

Description of the results:

Elixir and gel with DHQ (Gel 2) showed comparable maximum adhesion preventing effect.

2. Gel with chlorhexidine and mouthwash had an average effect.

3. Oil solutions had poor adhesion preventing effect.

Investigation of biofilm formation preventing properties of plant-based complexes

Figures 2–4 reflect biofilm preventing effect of various forms of products.

Description of the results:

All the products proved to be highly effective in preventing formation of biofilm. Gels (1 and 2) were the most effective.

DISCUSSION

Biofilm tends to change its composition and qualities [1, 8, 20, 21, 23], therefore, it is necessary to evaluate its physical parameters in a particular P patient before starting treatment to more accurately assess the involvement of systemic and general somatic problems, the level of hygienic habits of the patient, personalize treatment plan, factor in phenotypic indicators and achieve the predicted positive clinical result that persists in the long term.

The antibacterial activity of plant-based complexes against pathogenic bacteria of periodontium includes three components: adhesion preventing effect, antibacterial action proper and biolfilm formation preventing effect [2, 8]. Any infectious process of bacterial etiology begins with the adhesion and colonization of the site by microorganisms. Therefore, identification of adhesion preventing capabilities of products used in dental practice translates into the possibility of early prevention, i.e., disallowing adhesion and arrest of colonization. Thus, bacterial invasion is undermined at the first phase of the infectious process, and no biofilm is formed. The studied plant-based complexes were revealed to be highly active against biofilm, such activity significantly reducing the likelihood of appearance of a chronic infection locus in the periodontal area. In turn, direct antibacterial capabilities of the complexes decrease the number of bacteria and renders initiation of the infectious process unlikely [6, 12].

Allegedly, the reasons behind high antiseptic effectiveness of the elixir are 20% of ethyl alcohol in its content (by weight) and high concentrations of plant-based antibacterial components.

Table 2. Investigation of adhesion preventing properties of plant-based complexes

Studied composition	Adhesion index (M + *m*)	
	Control	*S. sanguinis*
1 — Mouthwash	75 + 6	33 + 5
2 — Gel with chlorophyll, aspen bark and DHQ (Gel 2)		24 + 4
3 — Gel with chlorophyll and chlorhexidine (Gel 1)		30 + 5
4 — Oil with chlorophyll (Phytolone oil)		42 + 6
5 — Oil with carotenoids from fir needles (Provitam oil)		37 + 4
6 — Gel with chlorophyll and chlorhexidine (Gel 3)		48 + 5
7 — Elixir with aspen bark extract and chlorophyll (Elixir)		21 + 4

Note: 1 — mouthwash; 2 — gel with chlorophyll, aspen bark and DHQ (Gel 2); 3 — gel with chlorophyll and chlorhexidine (Gel 1); 4 — oil with chlorophyll (Phytolone oil); 5 — oil with carotenoids from fir needles (Provitam oil); 6 — gel with chlorophyll and chlorhexidine (Gel 3); 7 — dental elixir with aspen bark extract and chlorophyll (Elixir).

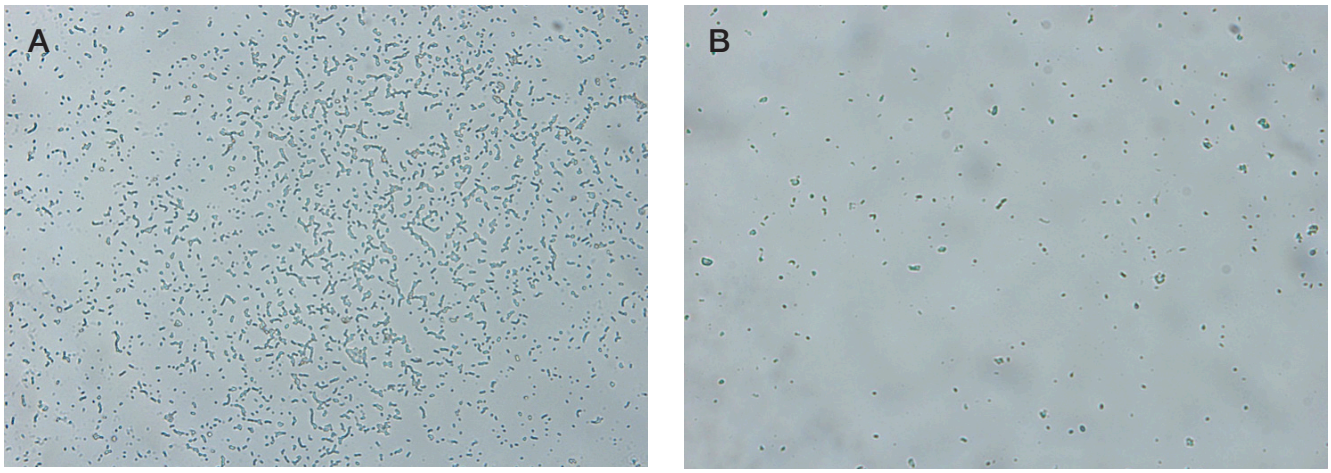

Fig. 2. Biofilm formation preventing properties: Gel 1 vs. *S. sanguinis*. **A**. Colonies of *S. sanguinis* before application of gel with chlorophyll and chlorhexidine. **B**. Colonies of *S. sanguinis* after application of gel with chlorophyll and chlorhexidine (magnification ×400)

The efficacy of Gel 1 is most likely associated with the prolonged release of active ingredients ensured by its unique bioadhesive film-forming base.

As for Gel 3, the expired subject of the experiment, it retained antiseptic properties but its efficacy had decreased compared to the tested gel that had not expired. Likely, the decrease was caused by aging of the components of the base and its rapid degradation, deterioration of the cumulative activity of plant-based complexes, while chlorhexidine remained active as the antiseptic component. This also gives reason to assume the studied composition has a multidirectional combined effect.

Elixir's high adhesion prevention activity is most likely formed by sodium alginate, aspen bark extract and alcohol content, while Gel 2's similar capability stems from sodium alginate, dihydroquercetin and aspen bark extract.

The comparatively lower antiseptic activity of water and oil forms is associated with a low concentration of antibacterial agents (aspen bark extract in the first place) and a short time on the tissues.

The form of the product has a significant effect on the time of exposure of tissues to the active ingredients of plant-based complexes. Gels are the most effective because of the slow and uniform release of the active substances, bioadhesion and development of a film on the gum and mucosa [13–14]. High concentration of active ingredients in water-alcohol solutions also delivers a persistent and long-lasting antibacterial effect, but the time of exposure of periodontal tissues thereto is shorter. The expired gel loses its effect because of the aging of the base due to moisture loss. It is expedient to produce gels in small batches or, in some cases, as a pharmacy-made compounded drug. Base of gels acts in a special way: it adheres to the dried surface of mucosa or gum, remains in the exposure locus for a long time, slowly releases the active ingredients and allows reduction of dosage in the composition; this set of peculiarities calls for additional research.

Thus, the three-stage antibacterial action of plant-based complexes reduces the risk of periodontitis, even in the presence of periodontal pathogenic bacteria in the locus. The use of plant-based complexes in the acute period after professional hygiene, especially if such complexes are part of gels, boosts restoration of the structure and condition of periodontium, normalization of trophism and nutrition, respiration and metabolic processes in the tissues. Used after the arrest of the acute process, plant-based complexes prolong the remission and significantly reduce the risk of repeated exacerbations, their course and repeated tissue damage by infectious agents.

In the acute period, up to days 14 through 21, it is advisable to use a combination of elixir + gel with 0.12% chlorhexidine, and after arrest of the acute process — mouthwash and gel with aspen bark and DHQ [11, 13]. Mouthwash and gel with aspen bark and DHQ can be used to prevent exacerbations in patients with chronic generalized periodontitis since they contain no chemical components bacteria grow resistant to

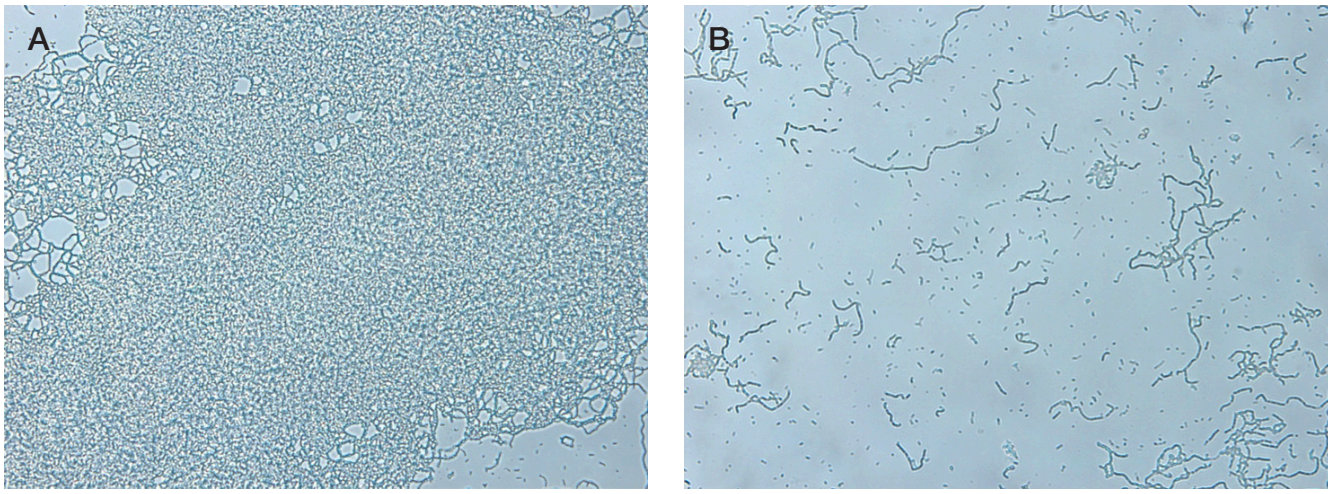

Fig. 3. Biofilm formation preventing properties: mouthwash vs. *S. mitis*. **A**. Colonies of *S. mitis* before use of mouthwash. **B**. Colonies of *S. mitis* after use of mouthwash

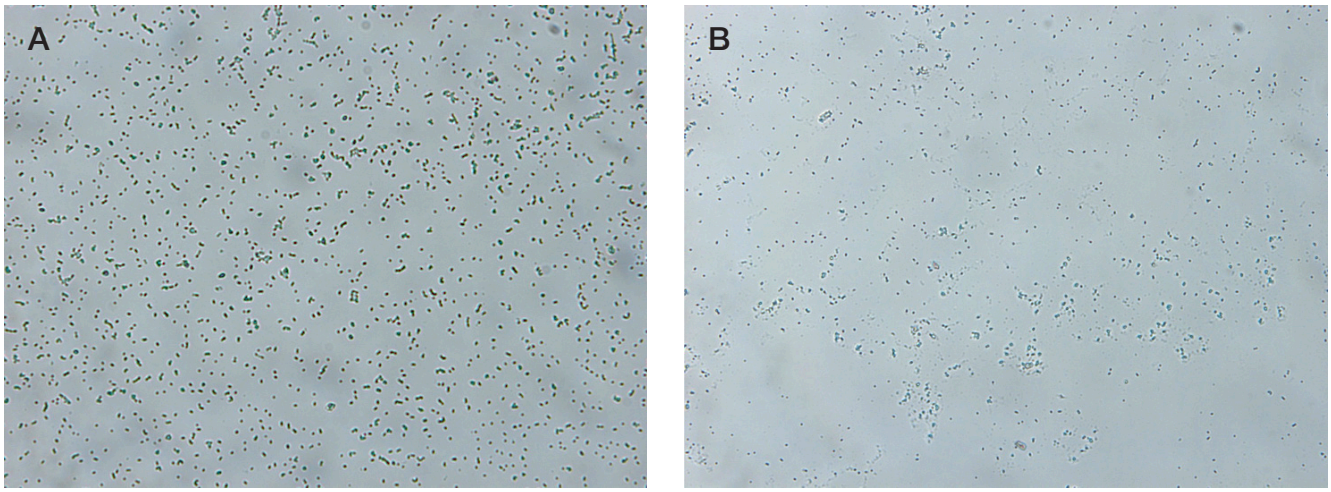

Fig. 4. Biofilm formation preventing properties: Provitam oil vs. *S. oralis*. **A**. Colonies of *S. oralis* before application of oil with fir needle carotenoids. **B**. Colonies of *S. oralis* after application of oil with fir needle carotenoids

while having a non-specific antibacterial effect based on the induced changes in the permeability of bacterial cell wall.

CONCLUSIONS

The studied plant-based complexes in the form of gel have proved to have high antiseptic, adhesion and biofilm formation preventing capabilities. Oil solutions are advisable as part of a complex therapy aimed at diseases of the oral mucosa; elixir and gel with 0.12% chlorophyll and chlorhexidine — as part of a complex therapy of periodontitis; gel with aspen bark and DHQ plus mouthwash — as means of prevention of periodontitis and other gum lesions. It is necessary to continue studying capabilities of plant-based complexes in various forms with titration of concentrations in elixirs compared to mouthwashes, comparison of frequency-dependent effect of application of gels, and determination of when the bacteria become resistant in case of use of gel with a chemical antiseptic. It is important to continue work aimed at *in vitro* evaluation of the biological properties of pathogenic bacteria of periodontium using experimental models of mixed (multi-species) biofilms. Flora of the oral cavity has changed a lot over the past 20 years, with specifics of the regions of residence contributing to the changes. For research activities, it is recommended to first evaluate the actual composition of periodontal pathogenic bacteria in plaque and on the surface of the tooth root, as well as in periodontal fluid.

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