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NATURAL DRUGS

ANALYSIS OF THE ANTIMICROBIAL ACTIVITY OF PROPOLIS AND LYSOZYME IN SEMISOLID EMULSION SYSTEMS

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Abstract: Propolis as an active natural substance is attractive due to its antimicrobial and antimycotic properties. Lysozyme was added to semisolid dermatological preparations as a complementary substance capable of potentiating their antimicrobial and antimycotic effect; this substance has been used for several decades as a preservative in food industry. The aim of this study was to model a semisolid emulsion system (o/w) for cutaneous use with moisturizing and antimicrobial properties, where the active substances would be propolis and/or lysozyme. The microbiological examination was performed under aseptic conditions. The microbiological examination was aimed at determining the antimicrobial efficacy of the studied preparation in the solid growth media using the wells technique. The results of the antimicrobial assay showed that the effectiveness of propolis against the growth of *S. aureus* was intensified by the lysozyme introduced into the emulsion systems. In addition to that, the results of examinations showed that the active substance propolis in emulsion systems more efficiently inhibited spore bacteria *(Bacillus cereus)* than lysozyme did, yet lysozyme had a more pronounced antimycotic (against *Candida albicans*) effect, compared to propolis. All studied cream samples inhibited the growth of Gram-negative microorganisms (*Escherichia coli)*. The results of this study suggest that the application of propolis and lysozyme as the active substances may increase the antimycotic and antibacterial effect of the studied preparations.

Keywords: antimicrobial, lysozyme, propolis, semisolid

The growing interest in preparations from natural material and the increasing relevance of such preparations stimulate the search for new or the application of already familiar natural substances in the production of new medicinal products. The modeled product was a topical moisturizing semisolid preparation containing antimicrobial substances.

The product under development should have moisturizing properties. Therefore, the base of the preparation should be aqueous, because water is absorbed into the skin, the skin swells and softens, which increases the permeability of the skin. Moisturizing of the stratum corneum is the major factor increasing the penetration of the active substance in the target site (1). For this reason, the base of the selected emulsion system of the modeled preparation (cream) was oil-in-water (o/w).

Dermatological products are used for five main target sites: the skin surface, the stratum corneum, the viable epidermis and upper dermis, skin glands, and systemic blood circulation. Our modeled preparation – like most cosmetic products – was designed for care or treatment of the skin surface and the stratum corneum, i.e. for strengthening the barrier function of the skin: the antibacterial substance helps to prevent infection in the protective barrier of the skin. Cosmetic products of such type form a protective layer or even destroy bacteria or fungi, at the same time softening the stratum corneum by increasing its water content. For the effective bioavailability of the surface-affecting products, they should release an antimicrobial substance.

Propolis as an active substance is attractive due to its antimicrobial and antimycotic properties and as a natural substance whose effect was proven by biological experiments (2-4). The antibacterial and antiinflammatory effect of European propolis is determined by its flavanone, flavone, and phenolic

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acid content, including esters of phenolic acids; its antioxidant effect depends on its content of flavonoids, phenolic compounds, and their esters (5). Propolis is one of the most potent natural antibiotics characterized by a very wide spectrum of effect. Its therapeutic application does not induce germ resistance and does not destroy useful microflora (6). The antimicrobial effect of propolis includes over 100 species of various bacteria, fungi, and viruses, including the causative agents of tuberculosis, syphilis, diphtheria, and influenza (7, 8). Propolis has a fungicidal effect on a number of species of fungi, including *Candida albicans, Aspergillus niger, Botrytis cinerea, Ascosphaera apis,* and *Plasmopara viticola* (7).

Lysozyme is added as a complementary substance increasing the antimicrobial and antimycotic effect of the preparation. Lysozyme is a natural antimicrobial enzyme that has been used in food industry for several decades as a preservative (9). The application of this enzyme in the production of pharmaceutical and health-promoting preparations clearly has good prospects (9). Studies have shown that lysozyme effectively lyses the walls of Gram-positive bacteria, has an equally good effect on some fungi, and is seen as an "endogenous antibiotic". It has been found that lysozyme is a potentiating factor in antibiotic therapy, and has an analgesic effect. Potential application of lysozyme has been defined scientifically, and includes topical antimicrobial, antifungal, and even antiviral medications. Lysozyme has been found to be effective against *Herpes* virus. Due to these properties, lysozyme may be applied in health-promoting or cosmetic products as an active ingredient or as a natural preservative. This is especially relevant in designing cosmetic products made from natural substances, because the main problem encountered in the manufacturing of such products is preservation, since natural products present good medium for the development of microorganisms.

The aim of this study was to model a semisolid emulsion system (o/w) for cutaneous application, characterized by moisturizing and antimicrobial activity and containing propolis and/or lysozyme as the active ingredients.

EXPERIMENTAL

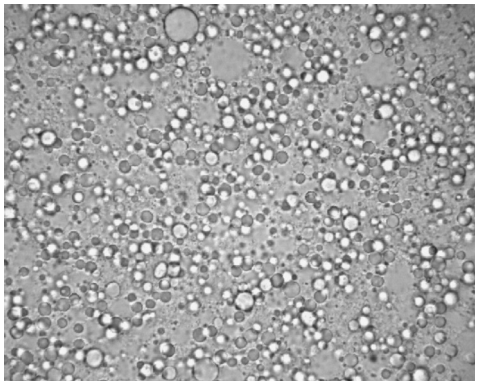
Material and Methods

The modeling of the o/w emulsion system was performed using three different bases (N1, N2, and N3) with progressively increasing amount of the aqueous phase. The compositions of the bases were as follows: N1 – PIONIER®GAHL 19.5%, liquid paraffin 12.5%, water 60.4%, and glycerol 7.6%; N2 – PIONIER®GAHL 14.5%, liquid paraffin 7.5%, water 70.4%, and glycerol 7.6%; N3 – PIONIER®GAHL 10.5%, liquid paraffin 2.5%, water 80.4%, and glycerol 7.6% (Hansen Rozental KG, Germany). The emulsion bases were produced by the close corporation Biok, Lithuania.

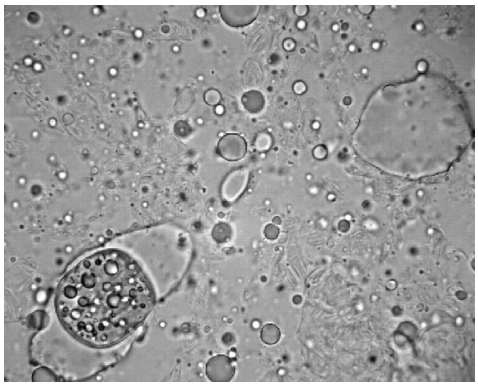
The active substance – propolis – was introduced in the forms of propolis oil (KMU pharmacy, Lithuania), semisolid propolis extract (close corporation Valentis, Lithuania) and liquid propolis extract (close corporation Medicata, Lithuania). The liquid propolis extract contained 30% of propolis, and 70% ethanol was used as an extractant. Propolis oil contained 30% of propolis, and olive oil was used as a diluent. The semisolid extract was obtained by evaporating ethanol from the liquid extract. All propolis preparations introduced into the studied samples as active substances were standardized according to the content of phenolic compounds, using ferulic acid as a standard. The liquid extract used in the study contained 290 mg/mL of phenolic compounds, the oil extract contained 13.97 mg/mL, and the semisolid extract 401 mg/g of phenolic compounds. The second active substance used was lysozyme (Carl Roth GmbH, Germany).

The microstructure of semisolid preparations was determined using a microscope Motic® (Motic Instruments, Inc.), magnification ×100, computer software Motic images 1000, and by photographing with a camera Motic Moticam 1000, with a live image of 1280 × 1024 pixels.

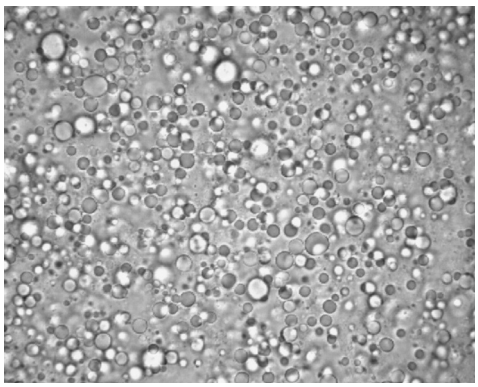
The microbiological examination was performed in aseptic conditions. During the microbiological study we determined the antimicrobial activity of the studied preparation, using solid growth media and the well technique. Resistance to preparations from natural material was examined in Mueller-Hinton agar (Mueller-Hinton Agar II, BBL, Cockeysville, USA) with standard cultures of *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 33499, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 8035, and *Candida albicans* ATCC 60193. Agar was poured into sterile Petri dishes 85 mm in diameter (20 mL in each dish). Agar wells were 7 mm in diameter and 8 mm deep. The density of microorganism suspensions applied in the test was 0.5 Mac Farland standard scale (5.10^7 – 1.10^8 CFU/mL). The density of the bacterial suspension in the Mueller-Hinton agar was 10^6 CFU/mL. After the agar solidified, wells were formed in the medium and were filled with the stud-



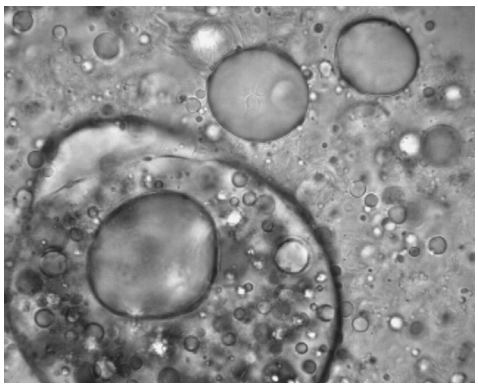
a) base No. 1, liquid propolis extract 4%



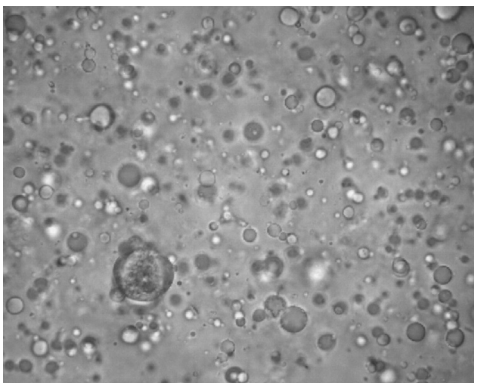
c) base No. 2, liquid propolis extract 4%



b) base No. 1, propolis oil 15 %



d) base No. 3, liquid propolis extract 4%



e) base No. 2, semisolid propolis extract 2%

Figure 1. Examination of the microstructure of the modeled semisolid emulsion systems

ied preparation. The number of the preparations (Table 1) indicates their succession during the examination on Petri dishes. The cultures were incubated for 24 h in a thermostat at 37°C, and then the microorganism growth in the whole agar volume was evaluated.

Statistical analysis was performed using statistical software package Statistica 5.5, Student's *t* test, and $p < 0.05$ was used as the level of significance. All samples were prepared in triplicate.

RESULTS AND DISCUSSION

We produced cream samples whose bases were o/w emulsion systems with different concentrations of the oil phase (No. 1 > No. 2 > No. 3). During the

Table 1. Composition of the modeled emulsion creams

Numbers of the specimens	Composition of the specimens	
	Propolis	Lysozyme
2	0.25% liquid extract	-
5	0.5% liquid extract	-
3	1% liquid extract	-
6	2% liquid extract	-
13	4% liquid extract	-
10	5% propolis oil	-
1	15% propolis oil	-
8	-	0.02%
9	-	0.04%
4	0.25% liquid extract	0.02%
12	0.25% liquid extract	0.04%

Table 2. Antimicrobial activity of the modeled creams containing propolis and lyzosyme

Specimens	*Staphylococcus aureus* ATCC 25923	*Escherichia coli* ATCC 25922	*Bacillus cereus* ATCC 8035	*Candida albicans* ATCC 60193
	Clear zone, mm (the mean ± SD)			
2	24.0 ± 1.45	27.62 ± 0.75	36.24 ± 0.10	18.80 ± 0.75
5	26.65 ± 0.21	29.75 ± 0.95	39.55 ± 1.55	21.60 ± 1.45
3	27.86 ± 1.20	30.85 ± 0.84	40.10 ± 1.62	25.96 ± 1.20
6	30.53 ± 0.62	31.46 ± 0.60	43.20 ± 0.24	28.86 ± 0.74
13	33.45 ± 1.02	32.50 ± 0.48	45.10 ± 0.60	28.26 ± 0.23
10	33.50 ± 0.25	28.75 ± 0.85	44.35 ± 1.46	30.35 ± 1.40
1	37.25 ± 0.55	31.85 ± 0.25	45.30 ± 0.27	43.30 ± 0.18
8	24.10 ± 0.32	26.46 ± 0.23	38.52 ± 0.17	38.52 ± 0.91
9	29.10 ± 0.44	32.50 ± 1.23	40.20 ± 1.28	40.98 ± 0.31
4	29.66 ± 0.25	34.63 ± 0.54	40.45 ± 1.06	39.46 ± 0.28
12	43.0 2± 0.79	38.33 ± 0.61	43.91 ± 0.33	48.12 ± 0.12

production process and examinations of the microstructure, we determined which form of propolis should be introduced onto the o/w emulsion base, and which concentration of the oil phase promotes even distribution of propolis in the base. Pictures clearly show the integrity of the structure when the base was No. 1 – this base allows for even distribution of propolis in the forms of liquid extract (Fig. 1a) and oil (Fig. 1b). Semisolid extract of propolis failed to distribute in any of the three bases (Fig. 1e).

On the basis of the results of examination, we modeled cream compositions using base No. 1 and propolis oil, liquid extract, and/or lysozyme as the active ingredient (Table 1).

We investigated the antimicrobial activity of these specimens (Table 1) to determine the effectiveness of propolis, lysozyme, and propolis/lysozyme combination against certain microorganisms.

According to literature data, the antimicrobial activity of propolis depends on its content of flavonoids, benzoic and caffeic acid, and other chemical compounds, as well as on the quantitative composition and synergistic effect of these compounds (10). Propolis is a non-toxic antimicrobial preparation affecting Gram-positive and Gram-negative bacteria (6).

Active substances in propolis have an antimicrobial and antibacterial effect. For this reason we

evaluated antimicrobial activity of propolis oil using experimental studies *in vitro,* and determined minimal concentration of phenolic compounds that inhibited respective microorganisms. The results (Fig. 2) showed that the Gram-negative bacterium *Escherichia coli* was most resistant to the effect of propolis preparations, the minimal inhibitory concentration (MIC) of phenolic compounds of propolis oil was 0.130 ± 0.0081 mg/mL, and the MIC of phenolic compounds of propolis extract was 0.08 ± 0.0320 mg/mL ($p = 0.0586$). Other Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Proteus mirabilis* were more sensitive, yet the determined MIC of propolis oil (0.043 ± 0.0032 and 0.043 ± 0.0062 mg/mL, respectively) and propolis extract (0.076 ± 0.0029 ($p = 0.0002$) and 0.068 ± 0.0042 mg/mL ($p = 0.0012$), respectively) indicate that these bacteria were also more resistant to the studied propolis preparations, compared to Gram-positive bacteria (Fig. 2). The MIC of propolis oil for the growth of *Staphylococcus aureus* was 0.024 ± 0.0054 mg/mL, and for *Enterococcus faecalis* – 0.034 ± 0.0043 mg/mL, and the MIC of propolis extract was 0.019 ± 0.0029 ($p = 0.2306$) and 0.030 ± 0.0051 mg/mL ($p = 0.3577$), respectively. The most sensitive microorganisms to the studied preparations were those that had eucaryotic cell structure – *Candida albicans* that belongs to fungi, and also a spore procaryotic bacterium *Bacillus cereu*s – the MIC of propolis oil was 0.022 ± 0.0036, and the MIC of propolis extract was 0.034 ± 0.0022 mg/mL ($p = 0.0079$). The results of this study confirm the findings presented in the literature, indicating that spore bacteria – like *Bacillus cereus* – are more sensitive to propolis, compared to bacteria that do not form spores (3, 4, 10). The MIC of phenolic comopunds of propolis oil for the growth of *Klebsella pneumoniae* was 0.055 ± 0.0042 mg/mL, and the MIC of propolis extract was 0.059 ± 0.005 mg/mL ($p = 0.3485$). This may be explained by the fact that *Klebsiella pneumoniae* forms a capsule, which is one of the factors of bacterial pathogenicity (11), increasing bacterial resistance to antimicrobial substances such as propolis (8). The results of the study showed that the studied microorganisms (*Pseudomonas aeruginosa*, *Proteus mirabilis* and *Bacillus cereu*s) were statistically significantly ($p < 0.05$) more sensitive to propolis liquid extract than to propolis oil. The effect of propolis oil and propolis liquid extract on other microorganisms – *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis,* and *Klebsiella pneumoniae* – was slight with statistically non-significant differences ($p > 0.05$).

As seen from data presented in Table 2, all the studied cream specimens inhibited the growth of the Gram-positive *S. aureus in vitro*.

The results of the study showed that the strongest effect against *S. aureus* was demonstrated by cream No. 12 – the inhibition zone was 43.02 ± 0.79 mm, while the least potent effect was seen in cream No. 2, where the inhibition zone was 24.0 ± 0.45 mm. The evaluation of the examination results showed that the effectiveness of the specimens against *S. aureus* may be explained by the fact that the effect of propolis was statistically significantly potentiated by the introduction of lysozyme into the investigated emulsion systems No. 4 and No. 12 ($p < 0.05$). The results of the investigations also showed that the effect of the cream containing 0.02% of lysozyme (No. 8.) was similar to that of cream No. 2, which contained 0.25% of propolis liquid extract. The comparison of creams containing lysozyme alone (No. 8 and No.9) with creams containing propolis liquid extract and lysozyme (No. 4 and No. 12) showed that creams containing lysozyme and propolis had a statistically significantly ($p < 0.05$) more potent effect on the studied microorganisms; the findings also showed that increasing lysozyme concentration statistically significantly ($p < 0.05$) increased the potency of the preparation. The most potent effect was observed in cream No. 1, containing 15% of propolis in the form of oil (Table 2). It is noteworthy that all the investigated cream specimens inhibited the growth of standard cultures of Gram-negative microorganisms: *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. For instance, the growth of *Escherichia coli* was most strongly inhibited by cream No. 12, and the weakest inhibition was observed in cream No. 8 (the inhibition zone was 26.46 ± 0.23 mm, Table 2), which contained 0.02% of lysozyme. The growth of *Bacillus cereus* was more efficiently inhibited by preparations in which the active ingredient propolis was introduced in the form of liquid extract and oil (Table 2). The weakest effect was exhibited by preparations that contained the liquid extract and lysozyme (No. 8 and No. 9, Table 2). The results presented in Table 2 show that cream containing propolis more efficiently ($p = 0.0059$) inhibited spore bacteria, compared to creams where the active ingredient was lysozyme. The results of this study confirmed the literature data indicating that spore bacteria, like *Bacillus cereus,* are more sensitive to propolis, compared to bacteria that do not form spores (10, 12, 13).

The experiment showed that creams containing lysozyme were most effective ($p = 0.0124$) against

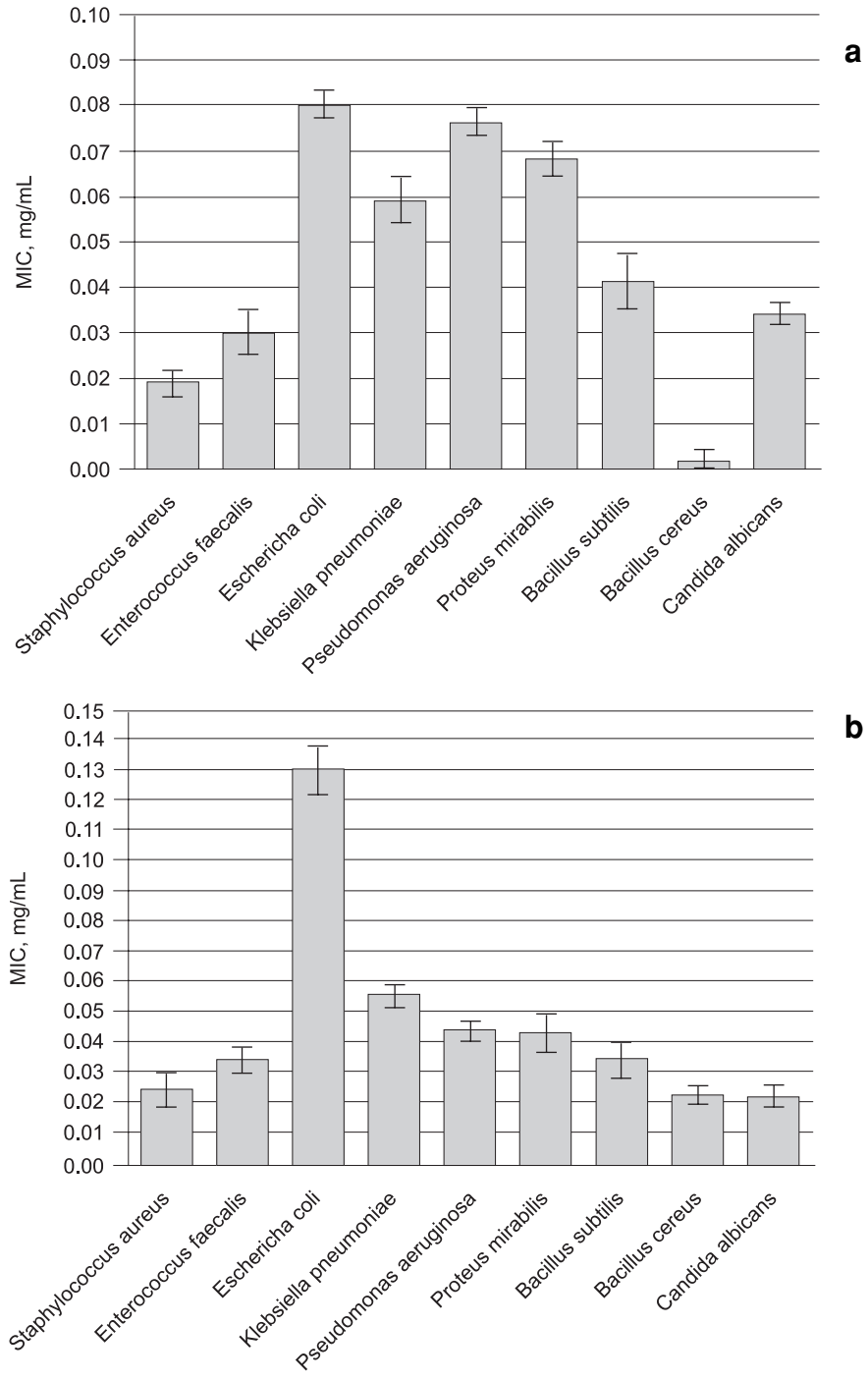


Figure 2. Minimal inhibitory concentration (MIC) of the liquid extract of propolis (a) and propolis oil (b)

fungi with eucaryotic cell structure – *Candida albicans* (the inhibition zone of specimen No. 8 was 38.52 ± 0.91 mm, and the inhibition zone of specimen No. 9 – 40.98 ± 0.31 mm). The strongest effect was observed in preparation No. 1 (Table 2), which contained 15% of propolis oil. The weakest activity against *Candida albicans* was observed in preparations No. 2, 3, 5, 6, and 13, where the active ingredient was introduced in the form of a liquid extract (Table 1). Creams containing lysozyme exhibited good activity against *Candida albicans*: cream No. 8 was active in the inhibition zone of 38.52 ± 0.91

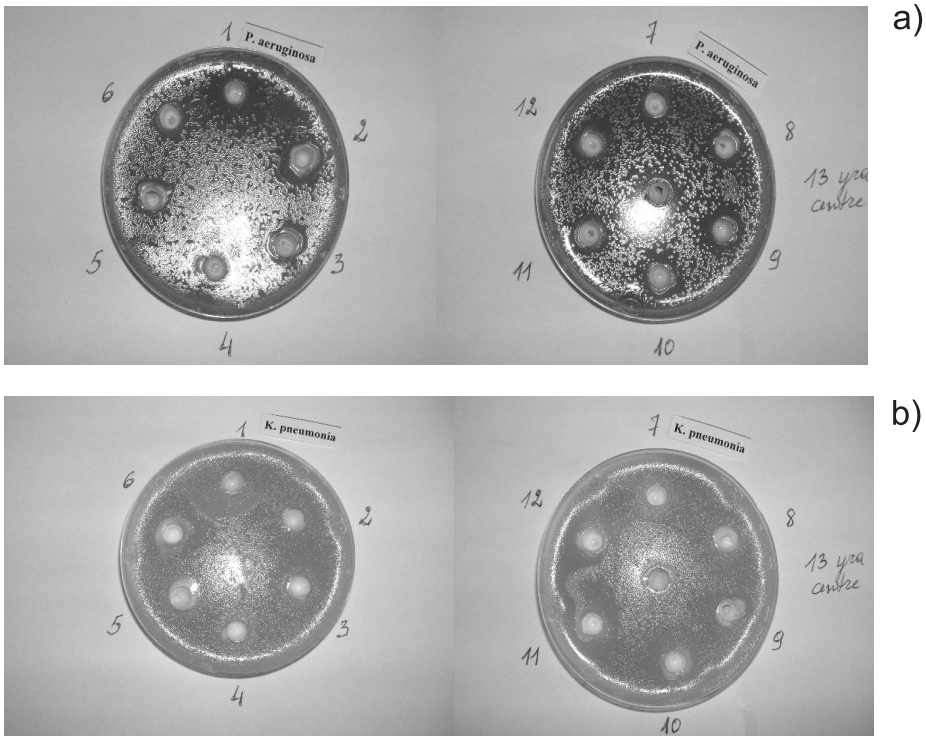


Figure 3. Preparations did not inhibit the growth of *Pseudomonas aeruginosa* (a) or *Klebsiella pneumoniae* (b)

mm, and cream No. 9 – in the inhibition zone of 40.98 ± 0.31 mm, while the effect of cream No. 4 containing propolis and lysozyme (Table 1) was similar to that of cream No. 8, which contained only lysozyme. The results of the investigation showed that lysozyme had a stronger effect against *Candida albicans*, compared to propolis ($p < 0.05$).

Although, according to literature data (3), propolis is effective against *Pseudomonas aeruginosa*, our studied semisolid propolis- and lysozyme-containing (concentration: 0.02-0.04%) preparations did not inhibit the growth of *Pseudomonas aeruginosa* (Fig. 3a). A probable cause of failure of the tests with *P. aeruginosa* was the layer of polysaccharides around cell wall. The concentrations of propolis selected for our study also failed to inhibit the growth of *Klebsiella pneumoniae* (Fig. 3b). *Klebsiella pneumoniae* creates a capsule, which is one of the factors of bacterial pathogenicity (11, 14) and increases bacterial resistance to antimicrobials such as propolis (3, 6). We did not find any literature data on the effect of lysozyme on the growth of *Klebsiella pneumoniae*. The results of the performed examinations showed that lysozyme at the concentration of 0.02 – 0.04 was ineffective against *Klebsiella pneumoniae* (Fig. 3b).

The results of the experiments showed that homogenous semisolid emulsion systems may be produced when the active ingredient propolis is introduced in the form of a liquid extract or oil. The comparison of the antimicrobial evaluation of propolis liquid extract, propolis oil, and propolis cream showed that the emulsion system affected (decreased) antimicrobial activity of propolis; differently from propolis extract and propolis oil, propolis cream had no effect on *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*. The results of the testing of antimicrobial activity showed that the effect of propolis against the growth of *S. aureus* was potentiated by the introduction of lysozyme into the emulsion systems. These results prompted further examination of lysozyme as a potential antimicrobial agent or even as the active ingredient of dermatological products. For this reason we continue experimental studies on the antimicrobial activity of lysozyme in emulsion systems. All the studied cream samples inhibited the growth of the Gram-negative microorganism – *Escherichia coli*. In addition to that, the results of the investigation showed that the active ingredient propolis in emulsion systems better inhibited spore bacteria, compared to lysozyme, but lysozyme exhibited better efficacy

against *Candida albicans*, compared to propolis. The results of the study suggest that propolis and lysozyme used as active ingredients in semisolid emulsion systems may potentiate the antimicrobial effect of such preparations against *Candida albicans*.

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