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PHARMACEUTICAL TECHNOLOGY

MODELING AND BIOPHARMACEUTICAL EVALUATION OF SEMISOLID SYSTEMS WITH ROSEMARY EXTRACT

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Abstract: Scientific literature provides a great deal of studies supporting antioxidant effects of rosemary, protecting the body's cells against reactive oxygen species and their negative impact. Ethanol rosemary extracts were produced by maceration method. To assess biological activity of rosemary extracts, antioxidant and antimicrobial activity tests were performed. Antimicrobial activity tests revealed that G+ microorganisms are most sensitive to liquid rosemary extract, while G- microorganisms are most resistant to it. For the purposes of experimenting, five types of semisolid systems were modeled: hydrogel, oleogel, absorption-hydrophobic ointment, oil-in-water-type cream and water-in-oil-type cream, which contained rosemary extract as an active ingredient. Study results show that liquid rosemary extract was distributed evenly in the aqueous phase of water-in-oil-type system, forming the stable emulsion systems. The following research aim was chosen to evaluate the semisolid systems with rosemary exctract: to model semisolid preparations with liquid rosemary extract and determine the influence of excipients on their quality, and perform *in vitro* study of the release of active ingredients and antimicrobial activity. It was found that oil-in-water type gel-cream has antimicrobial activity against *Staphylococcus epidermidis* bacteria and *Candida albicans* fungus, while hydrogel affected only *Candida albicans*. According to the results of biopharmaceutical study, modeled semisolid systems with rosemary extract can be arranged in an ascending order of the release of phenolic compounds from the forms: water-in-oil-type cream < absorption-hydrophobic ointment < Pionier PLW oleogel < oil-in-water-type eucerin cream < hydrogel < oil-in-water-type gel-cream. Study results showed that oil-in-water-type gel-cream is the most suitable vehicle for liquid rosemary extract used as an active ingredient.

Keywords: rosemary, phenolic compounds, semisolid, antimicrobial activity, biopharmaceutical, release *in vitro*

When modeling a semisolid preparation with protective effect, it is highly important to choose appropriate base substances, because they determine physicochemical properties and therapeutic effects of the end product. The base of protective preparation has to soften and moisturize the skin and give it elasticity; therefore, substances acting on the surface layer of the skin are used. Equally important criterion is proper consistency and acceptable appearance of the modeled preparation (1). Emulsion systems or hydrophilic gels are often used as bases due to their positive sensory properties. Proper base ensures stability of semisolid preparations during storage and good distribution on the skin, efficient release of a drug substance from the preparation (2).

Scientific literature provides a great deal of studies supporting antioxidant effects of rosemary, protecting the body's cells against reactive oxygen species and their negative impact (3, 4). It is argued that rosemary leaf extract has antioxidant properties due to several main compounds, which are: phenolic terpenes, rosemary acid and caffeic acid esters. It has been noticed that antioxidant activity of these compounds is higher than that of α-tocopherol or butylated hydroxytoluene (BHT) (5). Examination of effects of ethanol rosemary extract on Gram-positive and Gram-negative bacteria revealed antimi-

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crobial activity against methicillin-resistant *Staphylococcus aureus* and *Bacilus subtilis* strains; however, the effect on Gram-negative bacteria was weak. Studies of A. Hamed revealed antibacterial effects of rosemary essential oil on *E. coli* (MIC = 5 µL/mL; MBC = 25 µL/mL) and *Staphylococcus aureus* (MIC = 1.25 µL/mL; MBC = 2.5 µL/mL) (6). Other studies confirmed the hypothesis that aqueous extract of rosemary has inhibitory effects on HSV-1 virus (7). Due to their antimicrobial and antifungal properties, preparations containing rosemary extract destroy bacteria and fungi on the skin surface, thereby reducing the likelihood of inflammatory diseases and mycoses caused by microorganisms. Meanwhile, phenolic diterpenes and phenolic acids ensuring antioxidant activity terminate chain reactions, thus inhibiting the formation of free radicals and their destructive effects on skin cells (4, 8). Studies of Steinmetz and other scholars revealed that rosemary essential oil inhibits the growth of *Candida albicans in vitro* and *in vivo* (5, 9). Data presented in the literature suggest that rosemary can be used as an active ingredient in the production of semisolid preparations used on the skin and possessing antimicrobial and protective effects (10-12). Semisolid preparations with rosemary extract can also be used as protective preparations that protect the skin from adverse environmental factors, such as microbial pollution, UV radiation and free radicals (13). It is relevant to use rosemary extract as an active ingredient in the production of semisolid preparations characterized by antimicrobial and antioxidant effects. After an evaluation of applicability of rosemary in semisolid systems, the following research aim was chosen: to model semisolid preparations with liquid rosemary extract and determine the influence of excipients on their quality, and perform *in vitro* study of the release of active ingredients and antimicrobial activity.

EXPERIMENTAL

Materials and Methods

Raw material obtained from Alvas, UAB, was used to produce rosemary extract. A solvent, i.e., ethanol of Stumbras, AB (Kaunas, Lithuania) was used. Ethanol rosemary extracts were produced by maceration method (14) using 40% ethanol, raw material and extractant ratio 1 : 1.

Production of semisolid systems with rosemary extract

For the purposes of experimenting, five types of semisolid systems were modeled: hydrogel (N1), oleogel (N2), absorption-hydrophobic ointment (N3), oil-in-water-type cream (N4, N5) and water-in-oil-type cream (N6, N7), which contained rosemary extract as an active ingredient. Experimentative semisolid systems with rosemary extract (ointments, oil-in-water and water-in-oil emulsion creams) were produced at room temperature using a mixing system Unguator® 2100 (GAKO® International GmbH, Munich, Germany). Gels were produced using a "cold" method (2).

The microstructure of semisolid systems 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.

pH of semisolid systems was analyzed using pH meter HD 2105.1 (Delta OHM, Italy). Five percent solution was prepared to determine pH levels. The appropriate amount of semisolid formula was topped with purified water and stirred for 30 min on IKAMAG® C-MAG HS7 magnetic stirrer (IKA-Werke GmbH & Co. KG, Staufen, Germany) at a temperature of 50°C. Then, the solution was cooled and filtered through a paper filter.

The viscosity (Pa×s) of semisolid systems were assessed using a rotary viscometer ST-2010.

In vitro release studies of phenolic compounds from the experimental semisolid rosemary formulas were conducted (n = 3) using modified Franz-type diffusion cells (15, 16). Donor phase (infinite dose: ~0.85 g) was added to a cell with regenerated cellulose dialysis membrane Cuprophan® (Medicell International Ltd., London, Great Britain). Before the experiment, the membranes were stored in purified water (~30°C) for at least 12 h. The diffusion area was 1.77 cm^2. Aqueous acceptor medium that provided sink conditions was mixed using IKAMAG® C-MAG HS7 magnetic stirrer with heating plate, while maintaining a temperature of 32 ± 0.1°C. Acceptor medium samples (1 mL) were taken after 1, 2, 4 and 6 h, whilst adding an equal volume of fresh acceptor medium. The quantity of phenolic compounds was determined using Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Inc., Santa Clara, USA) according to p-coumaric acid equivalents after reaction with Folin-Ciocalteu phenol reagent (17).

To assess antimicrobial activity, two most common cultures of microorganisms causing skin infections were chosen: *Staphylococcus epidermidis* bacteria culture and *Candida albicans* fungus culture. The study was conducted under aseptic conditions, using agar diffusion well method (18). Each semisolid system was tested five times. Results are

presented as the mean values and expressed as the width of area in which reproduction of microorganisms is inhibited (mm). It is argued that semisolid system has antimicrobial activity, when the width of area in which reproduction of microorganisms is inhibited, exceeds 7 mm (19).

Statistical analysis

All tests were repeated three times. The mean values and standard deviations of the results were calculated using IBM SPSS statistics 20 and Microsoft Office Excel 2007 programs. The significance of differences in study results was evaluated using Student's t-test. The differences were statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

According to the scientific literature (6, 20, 21), 40, 70 and 96% ethanol (v/v) was selected as a solvent for the production of rosemary extracts. Ethanol rosemary extracts were produced by maceration method (14). The following forms of extracts were chosen: tincture and extract with a ratio of raw material and extractant 1 : 5 and 1 : 1, respectively. Experimental studies have shown that higher quantities of active compounds were present in extracts, where the solvent used was 40% ethanol (Table 1). The study data suggest that higher concentration of active ingredients was in rosemary extracts based on a ratio of 1 : 1.

To assess biological activity of rosemary extracts, antioxidant and antimicrobial activity tests were performed. Antioxidant activity tests revealed that stronger antiradical activity is characteristic of rosemary extract prepared using 40% ethanol with effective concentration that binds 50% of DPPH radicals (EC_{50}) amounting to 0.096 ± 0.003 mg/mL, compared with the extraction prepared using 70% ethanol as the extractant. Given the fact that the

Table 1. The content of active ingredients in rosemary extracts.

	E1	**E2**	**E3**	**E4**
Ratio of raw materials and extractant	1 : 1	1 : 1	1 : 5	1 : 5
Solvent	40% ethanol (v/v)	70% ethanol (v/v)	40% ethanol (v/v)	70% ethanol (v/v)
Concentration of phenoliccompounds, mg/mL	14.139 ± 0.025	7.625 ± 0.037	9.597 ± 0.0411	8.117 ± 0.011

Table 2. MIC (minimum inhibitory concentration) of ethanol rosemary extracts expressed as the concentration of phenolic compounds (mg/mL) in accordance with p-coumaric acid equivalents.

Tested extract	Antimicrobial activity								
	Gram positive bacteria cultures				Gram negative bacteria cultures				Fungal culture
	Staphylococcus aureus	*Staphylococcus epidermidis*	*Enterococcus faecalis*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Escherichia coli*	*Proteus mirabilis*	*Candida albicans*
1 : 1 40% ethanol	0.071	0.071	0.707	0.094	0.141	0.071	0.707	0.141	1.414
1 : 1 70% ethanol	0.191	0.071	0.763	0.191	0.508	0.381	0.763	0.381	0.763

strongest antioxidant activity was found with rosemary extracts that had the highest content of phenolic compounds, this confirms information presented in the literature that phenolic compounds are responsible for the antioxidant acitivity of plant extracts (22). The obtained results confirmed once again that rosemary preparations have antioxidant activity (23, 24) and liquid rosemary extract can be used in the production of dermatological preparations with protective effects.

In antimicrobial activity study of ethanol rosemary extracts it was found that rosemary extracts have inherent antimicrobial activity. Data in Table 2 show that rosemary extracts produced with 40% ethanol as the extractant have stronger antibacterial activity, while extracts produced with 70% ethanol have stronger antifungal activity. There are a number of scientific studies where it was found antifungal effects of rosemary essential oil on *Candida albicans* (5, 9). Our study results revealed that liquid rosemary extract also inhibits the growth of *Candida albicans* fungus. The assessment of the effects of extracts on Gram positive and Gram negative bacteria confirmed the information presented in the scientific literature that rosemary extracts have stronger antimicrobial activity against Gram positive bacteria cultures (25, 26). Antimicrobial activity studies confirmed the suitability of liquid extract for being used as an active ingredient in the production of semisolid preparations with protective effects. Therefore, due to greater content of phenolic compounds and stronger biological effects, liquid rosemary extract (1 : 1) produced using 40% ethanol as the extractant was chosen as an active ingredient for further studies. There are study data that rosemary extracts are made with 50% ethanol (21); however, when modeling semisolid formulas, it is expedient to use

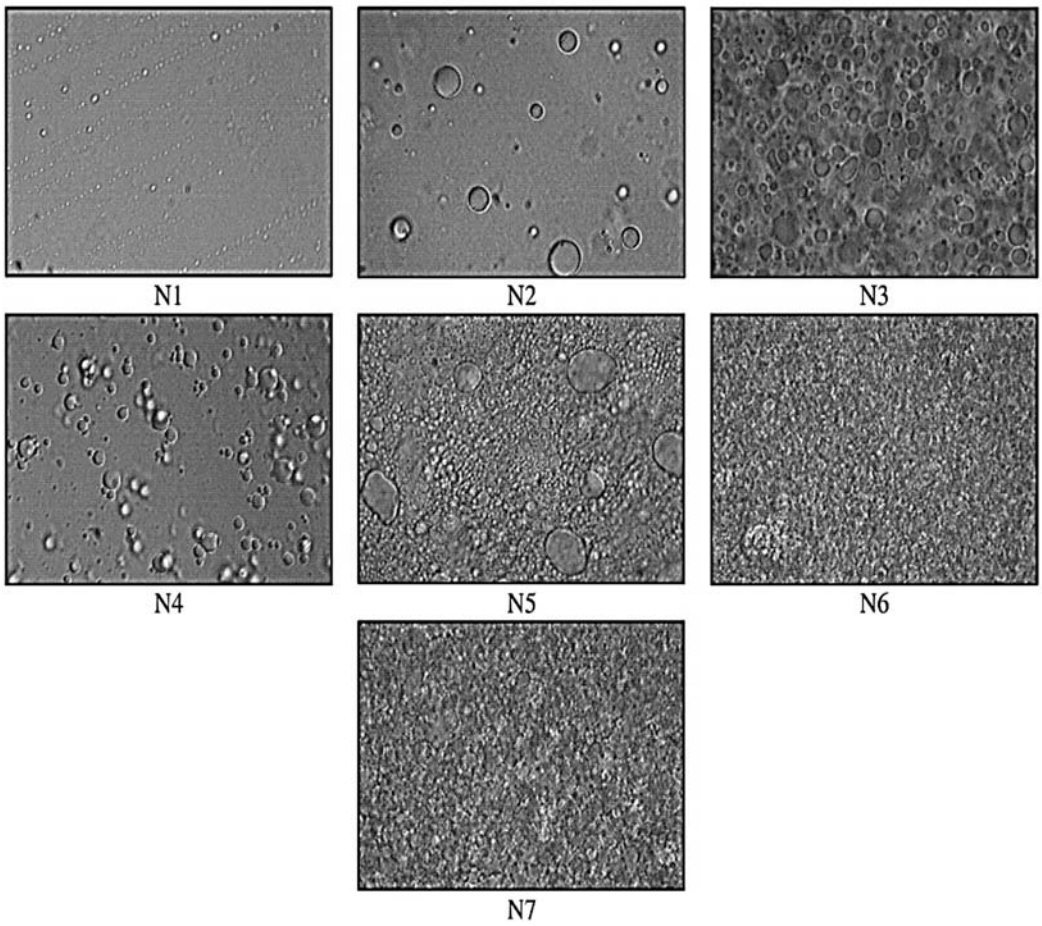

Figure 1. Microstructure of semisolid systems with rosemary extract

Table 3. Compositions of the modeled semisolid systems (100 g).

Composition	Semisolid systems						
	Hydrogel	Oleogel	Hydrophobic ointment	Oil-in-water-type cream		Water-in-oil-type cream	
	N1	N2	N3	N4	N5	N6	N7
Carbomer	934	0.77					
Purified water	85					30	55
10% NaOH	1.23						
Pionier PLW		90				53	
Pionier KWH							30
White vaseline			61.4				
Anhydrous lanolin			13.6				
Glycerol			15				5
Sorbitan oleate						7	
Gel-cream oil-in-water base				90			
Eucerin oil-in-water base					90		
1 : 1 rosemary extract in 40% ethanol	10	10	10	10	10	10	10
Viscosity, Pa×s	1.9 ± 0.06	4.9 ± 0.0.08	> 10 *	0.85 ± 0.06	3.36 ± 0.05	3.61 ± 0.07	> 10 *

*reached the limit of measurement instrument

extract prepared with ethanol of lower concentration (27).

In the course of studies, different semisolid systems (Table 3) were produced with active ingredient added in the form of liquid extract. During the first study stage, microstructure of produced semisolid preparations was assessed.

Pictures of microstructure presented in Figure 1 show that active ingredient (liquid rosemary extract) is evenly distributed in hydrogel (N1), forming a homogeneous structure. Meanwhile, in oleogel and absorption-hydrophobic base (N2, N3), it forms a heterogeneous structure (Fig. 1). In terms of microstructure of oil-in-water-type systems (N4, N5), we can see that liquid rosemary extract is distributed in an aqueous medium. Moreover, study results show that in water-in-oil-type systems (N6, N7), active ingredient, i.e., liquid rosemary extract, is distributed in the aqueous phase, forming stable emulsion systems.

Study data presented in Figure 2 show that pH of all the tested semisolid systems is weakly acidic (4.98-5.91). pH value of all the modeled semisolid preparations corresponds to or is very close to the physiological pH value of the skin; therefore, it can be argued that the modeled semisolid systems are suitable for use on the skin.

Drug release testing from semisolid systems is considered as necessary prerequisite in development of efficient formulations. *In vitro* release testing assessed the influence of the base on the release of phenolic compounds from the semisolid systems. Application of Higuchi plots permitted comparison of release rates (corresponding to the slopes of Higuchi plots) of phenolic compounds from semisolid systems, demonstrating up to 3-fold higher release process from gel-cream based formulation (N4) if compared to other semisolid systems (Fig. 3). Linearity of Higuchi plots was confirmed by coefficients of determination (R^2) ranging from

0.9096 to 0.9849 for the formulated systems, and indicated that diffusion of phenolic compounds from tested gels and creams was a rate limiting step in the release of active compounds. Also it confirmed that applied membrane was not affecting diffusion of phenolic compounds.

Results demonstrated that 1 h after the beginning of testing, from 13.03% to 14.53% of phenolic compounds were released from hydrogel (N1), oleogel (N2) and oil-in-water-type cream (N5), and no statistically significant difference was observed ($p > 0.05$). The lowest amount of phenolic compounds after 1 h of testing was released from absorption-hydrophobic ointment (N3); and the highest amount was released from oil-in-water-type cream (N4). Over 1 h, 7.5% and 30.33% of phenolic compounds, respectively, were released from these systems. After 6 h of testing, the amount of released total phenolic compounds was 15.35% from absorption-hydrophobic ointment (N3), 19.92% from oleogel (N2), 22.21% from oil-in-water-type cream (N5), 34.41% from hydrogel (N1) and 78.35% from oil-in-water-type cream (N4), which was the highest amount of phenolic compounds released. The lowest released amount was observed with absorption-hydrophobic ointment (N3). No release of phenolic

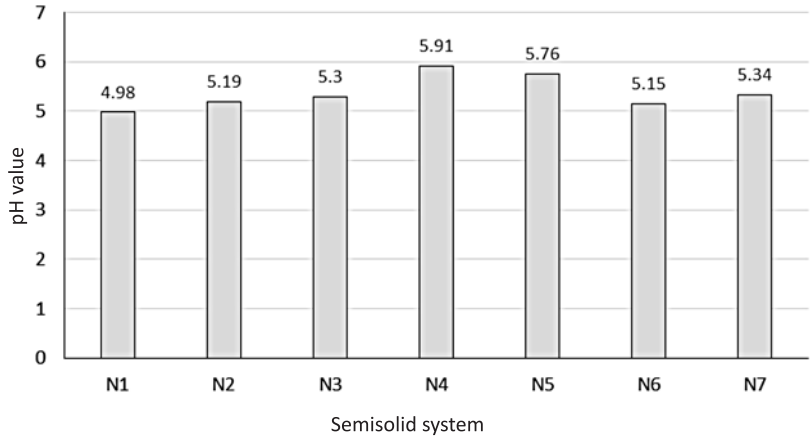

Figure 2. pH value of semisolid systems with rosemary extract

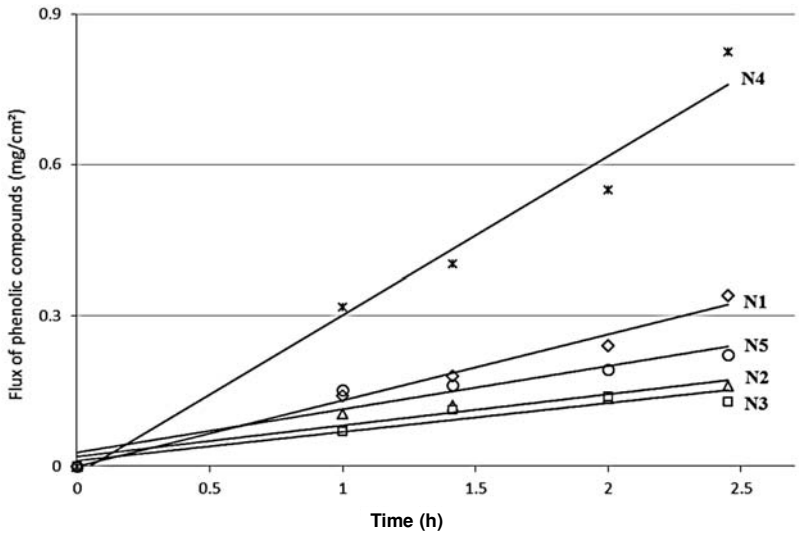

Figure 3. Release of phenolic compounds from semisolid systems *in vitro*

Table 4. Evaluation of antimicrobial activity of semisolid systems.

Semisolid system	Width of area in which reproduction of microorganisms is inhibited (mm)	
	Staphylococcus epidermidis	*Candida albicans*
N1	11.6 ± 0.55	0
N2	0	0
N3	0	0
N4	13.0 ± 1.0	10.6 ± 2.07
N5	0	0
N6	0	0
N7	0	0

compounds was determined when water-in-oil-type creams (N6, N7) were applied for 6 h. Data analysis showed that statistically significant difference ($p < 0.05$) existed among all amounts of phenolic compounds released within 6 h from the experimental semisolid systems with rosemary extract. Lower release rates of phenolic compounds from semisolid systems were determined for lipophilic bases that could be due to lipophilic properties of the active compounds. Active compounds of lipophilic nature do not diffuse or diffuse only to a small extent into the aqueous acceptor phase (28). Studies have shown that viscosity of the base slows down the diffusion of drug substance through a membrane (29). In view of the fact that excipients with high viscosity (vaseline, anhydrous lanolin) were used in the production of preparation N3 (Table 3), this may have influenced slow release of phenolic compounds from the tested system due to its high viscosity lipophilic medium. Meanwhile, larger amounts of active compounds were released from oleogel (N2) than those released from absorption ointment. This could have been influenced by the viscosity of the base (Table 3). Pionier PLW base is often used as a substitute for vaseline in the production of cosmetic and pharmaceutical products due to lower viscosity and more acceptable sensory properties. Study results supported data provided in the literature that drug substances are released from oleogel of this composition more quickly than from vaseline bases. It is worth noting that N3 base contains vaseline and N6 and N7 contains vaseline oil (Table 3) that form a film on the surface of the skin (30), and this may create additional barrier to penetration of phenolic compounds. One can predict that the diffusion of phenolic compounds from the aqueous phase of preparations N6 and N7 was inhibited by a lipophilic medium. The test results showed that water-in-oil-type emulsion bases are not suitable for introduction of liquid rosemary extract, because during the period of 6 h phenolic compounds were not released from the tested bases. With gel-cream base (N4), where less viscous excipients were used in the production (Table 3), larger amount of the compounds is released than that released from eucerin base (N5) that contains ingredients with higher viscosity. The test results confirmed the data presented in the literature that the diffusion of drug substances from emulsion dispersion system can be influenced by the complex nature of the system itself, because it enables drug substances, vehicles and excipients to form a variety of physical structures (31). Thus, test results revealed that the amount of phenolic compounds released from semisolid preparations depends not only on the properties of the active ingredient, but also on the chosen base (32). Evaluation of the quality of semisolid preparations requires the analysis of their homogeneity, viscosity, particle size, amount of active compounds; it is also recommended to perform release test. Results of this study showed that *in vitro* release test is necessary to assess the suitability of the base for the introduction of the active ingredient, which would allow determining therapeutic effectiveness in advance.

The next stage was devoted to the analysis of antimicrobial activity of semisolid preparations.

The antimicrobial activity test revealed effects of semisolid systems N1 and N4 on the tested microorganisms (Table 4). Data in Table 4 show that preparation N4 inhibits the growth of *Staphylococcus epidermidis* bacteria and *Candida albicans* fungal cultures (the width of sterile area is, respectively, 13.0 ± 1.0 and 10.6 ± 2.07 mm).

Meanwhile, it was found that hydrogel N1 had antimicrobial activity only against *Staphylococcus epidermidis* bacteria culture (the width of sterile area: 11.6 ± 0.55 mm). The study results confirmed data presented in the literature that rosemary preparations have antifungal effects on *Candida ablicans* (5, 9).

Differences in antimicrobial activity of tested semisolid systems could have occurred due to different diffusion of active compounds from the modeled semisolid systems into the microorganism growth medium used during the testing. The test results showed that preparations N4 and N1 have antimicrobial effects and can be used on the skin as preparations with protective effects.

CONCLUSIONS

Antimicrobial activity tests revealed that G+ microorganisms are most sensitive to liquid rosemary extract, while G- microorganisms are most resistant to it. Due to its antimicrobial properties, liquid rosemary extract can be used as an active ingredient in the production of semisolid preparations. Studies have shown that chosen bases affect antimicrobial activity of formulas. It was found that oil-in-water-type gel-cream has antimicrobial activity against *Staphylococcus epidermidis* bacteria and *Candida albicans* fungus, while hydrogel affected only *Candida albicans*. Based on the study results, it can be argued that the activity of semisolid dosage forms is influenced not only by the amount of active compounds, but also by the base type. According to the results of biopharmaceutical study, semisolid forms can be arranged in an ascending order of the release of phenolic compounds from the forms: water-in-oil-type cream < absorption-hydrophobic ointment < Pionier PLW oleogel < oil-in-water-type Eucerin cream < hydrogel < oil-in-water-type gel-cream. Study results showed that oil-in-water-type gel-cream is the most suitable vehicle for liquid rosemary extract used as an active ingredient.

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