ORIGINAL SCIENTIFIC ARTICLE

DEVELOPEMENT AND STABILITY EVALUATION OF NATURAL TOPICAL FORMULATIONS CONTAINING *PINUS SIBIRICA* ESSENTIAL OIL

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ABSTRACT

Pinus sibirica (P. sibirica) is used in folk medicine for centuries in treatment of various diseases due to its anti-inflammatory, antioxidant, and antimicrobial activity. The present study was focused on formulating semi-solid topical cream and gel preparations with P. sibirica essential oil (EOPS) and on evaluation of their stability during three months of storage. After preparation of semi-solid formulations (cream and gel), we followed parameters such as organoleptic characteristics, pH values, electrical conductivity, phase separation, washability, swelling index and microbiological contamination. Tests for physical and microbiological stability were performed considering different storage conditions at temperature (4±2° C, 25±2°C and 40±2°C) for 12 weeks. All test were performed at sampling time points of 7th, 30th, 60th, 90th day after storage. Cream with EOPS was white colour, it is homogenous with pleasant odor characteristic to used EOPS while gel with EOPS was transparent, it is homogenous with specific odor. After application to skin, EOPS cream was leaving non-greasy film while gel with EOPS was leaving sticky film and cooling effect. In all tested samples, we observed no phase separation and pH values were optimal for skin application. During three months of storage at different conditions, all tested formulations were stable and addition of EOPS did not cause any changes in followed parameters. Both cream and gel formulation remained stable since we did not notice any significant changes in all followed parameters during storage at different temperatures. Addition of 0.5% of EOPS did not change visual, sensory and microbial properties of formulations.

Keywords: *Pinus sibirica, stability, topical formulations, essential oil.*

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INTRODUCTION

The continual evolution of pharmaceutical and cosmetic industry has led to the increasing number of topical products for skin diseases or cosmetic purpose (1). In fact, these products are designed to cure symptoms of various skin diseases (eczema, psoriasis, dermatitis etc) or to enhance skin beauty, slow ageing or enable skin cleaning. In recent years, companies produce numerous topical products that are natural and eco-friendly since there is increasing consumer demand for clean, sustainable, and plant-based products. Herbal products are derived from plant materials such as extracts or essential oils which has already confirmed beneficial properties for the skin (1).

Pinus sibirica (*P.sibirica*) is coniferous plant used in folk medicine worldwide for prevention and treatment of various conditions. Both leaves and seeds of *P.sibirica* are utilized to produce extracts, essential oils or oils rich with numerous active ingredients exerting different effects (lowering blood pressure, lipids and glucose as well as to inhibit allergic reactions). Different kind of extractive products (decoction and infusion) are used for curing rheumatism or kidney and liver disorders (3). In addition, wide variety of dermatological disorders (psoriasis, eczema, pimples, and scabies) and wounds are treating with topical applied *P.sibirica* essential oil (EOPS) (4-6). All abovementioned effects inspired scientists to reveal chemical composition of this coniferous plant and to determine if products of this coniferous plant exert antioxidant, antimicrobial and anti-inflammatory activity. The most abundant compounds in EOPS are α-pinene (40.19%), β-pinene (19.64%) and δ -3-carene (16.14%) (5) that are the most responsible for many pharmacological activities (7, 8).

Selection of topical semisolid dosage form (cream, gel, ointment or paste) depends on skin condition or disorder, site and way of application and solubility of ingredients. Nevertheless, designing novel quality dermatological product is demanding process that requires from manufacturer to guarantee safety and stability of final product during distribution and storage process. Instability of topical product is reflected through phase separation, creaming, aggregation or it can cause skin irritation or infections (9). On the other side, the most common process responsible for formulation instability is oxidation of lipid components that consequently lead to a loss of product efficiency (10). In that sense, numerous methods for verification of product stability during storage at different conditions are developed. All relevant information about products physical and microbiological stability should be provided before releasing product to the market. Therefore, the aim of our investigation was to formulate semi-solid topical preparations containing *P.sibirica* essential oil and to determine their stability during three months of storage.

MATERIALS AND METHODS

Preparation of semi-solid formulations (gel and cream)

One hundred percent pure *P.sibirica* essential oil was purchased from Alekpharm (Belgrade), and stearic acid, cetyl alcohol, cetearyl alcohol, polysorbate 60, sweet almond oil, glycerin, phenoxyethanol, citric acid, carbomer 940, sodium benzoate, propylene glycol and triethanolamine (TEA) were purchased from Unichempharm (Čačak).

The cream base (F1) was made by precisely measuring and mixing separately oil phase components (stearic acid, cetyl alcohol, cetearyl alcohol, polysorbate 60 and sweet almond oil) and aqueous phase (glycerin, aqua purificata, 10% TEA) heated up to 70-80° C. After heating both phases, oil phase was added to aqueous phase with continuous stirring on a laboratory stirrer RV16 basic (IKA®VERKE, Germany). Emulsion was cooled down and preservative and pH adjuster were added. To create formulation F2 EOPS was added in cooling down stage in abovementioned base by levigation method (11). On the other hand, gel base (F3) was prepared with carbomer 940 by dispersing it in water at room temperature. Produced dispersion was stirred with propylene glycol and gel was formed by adding 10% TEA solution. Gel was preserved with sodium benzoate. Formulation F4 was prepared by incorporating 0.5 g EOPS in gel base. All ingredients of F1-F4 formulations are presented in Table 1.

Physical properties of cream and gel with *Pinus sibirica* essential oil

Organoleptic test

Prepared samples were subjected to a set of organoleptic analysis estimated by visual observation and touch: color, odor, homogeneity and presence of phase separation in creams. Moreover, set of parameters were determined after application of samples to skin (type of smear, application/touch) (12).

pH values determination

The digital pH meter (Mettler Toledo, Columbus, OH, USA) was used to evaluate pH values of all formulations (F1-F4) at room temperature. The pH of each formulation was taken for at least three times, and the average was calculated (13).

Electrical conductivity determination

The conductivity meter (Eutech CON 700, Thermo Fisher Scientific, Shanghai, China) was calibrated and electrical conductivity (σ) of all samples was measured at room temperature. All experiments were done in triplicate and average value is presented (14).

Centrifugation test

Centrifugation test was directly performed after cream and gel preparation in order to evaluate changes such as

phase separation. Samples were centrifuged in laboratory centrifuge (Hettich Mikro 120, Germany) at 3000 rpm at room temperature for 30 min. Samples were considered to be stable if they remained homogenous after centrifugation (15).

Washability

In order to test the level of washing of prepared formulations (F1-F4) with distilled water, creams and gels were applied over the skin (16).

Swelling index

One gram of F1-F4 formulation was added to 10 ml distilled water in Petri dish. After that samples were taken and left in dry place for one and three hours. The mass of formulations was weighed and the degree of swelling was established from the formula (17).

$$Swelling\ Index\ (SW)\% = [(Wt - Wo)/Wo] \times 100$$

(SW) % = Equilibrium percent swelling
Wt = Weight of swollen crem/gel after time t
Wo = Original weight of cream/gel at zero time

Assessing the stability of cream and gel with P.sibirica essential oil

Stability analyses (organoleptic characteristics, pH values, electrical conductivity, and centrifugation test, microbiological test) were performed for topical formulations that are storage in incubator (ICT-R 52 FALC, Italy) at different temperatures such as 4±2° C, 25±2°C and 40±2°C. All parameters were observed at various time intervals during three months. The measurements were done after 7th days, after one month and after two and three months (18).

Both cream and gel formulations are tested according to the following standards (SRPS EN ISO 21149: 2017, SRPS EN ISO 16212: 2017, SRPS EN ISO 18416:2016, SRPS EN ISO 21150 :2016, SRPS EN ISO 22717:2016, SRPS EN ISO 22718:2016) were used to detect and identify the specified microorganisms. We identified presence of noteworthy pathogens like *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Candida albicans.* On the other hand, we have the ability to enumerate the overall aerobic mesophilic bacteria, including both the total aerobic microbial count and the total yeast and mold count (19). Analyses were performed on the day of preparation, after 7, 30, 60 and 90 days.

Statistical analysis

The results of the determinations were subjected to analysis of variance (ANOVA) ($p \leq 0.05$). The analysis was performed using the IBM SPSS 21.0 *for Windows.*

Table 1. Composition and concentration of the cream-based and gel-based formulations

Ingredients %	Cream base (F1)	EOPS cream (F2)	Ingredients%	Gel base (F3)	EOPS gel (F4)
Stearic acid	10	10	Carbomer 940	0.5	0.5
Cetyl alcohol	2	2	Propylene glycol	10	10
Cetearyl alcohol	2	2	TEA 10%	q.s	q.s
Polysorbate 60	2	2	Sodium benzoate	0.2	0.2
Sweet almond oil	4	4	Citric acid	q.s.	q.s.
Glycerin	3	3	EOPS	/	0.5
Phenoxyethanol	0.8	0.8	Aqua purificata	ad 100	ad 100
TEA 10%	q.s.	q.s.			
Citric acid	q.s.	q.s.			
EOPS	/	0.5			
Aqua purificata	ad 100	ad 100			

EOPS-*Pinus sibirica* essential oil; TEA- Triethanolamine

RESULTS

Organoleptic properties of the formulations

The results obtained for the organoleptic characteristics of both creams and gels are presented in Table 2. Cream formulation F2 has white color, it is homogenous with pleasant odor characteristic to used essential oil. After application to skin, creams spread easily, leaving non-greasy film on the skin. On the other hand, gel formulations F3 and F4 were transparent, homogenous and spread easily after application to skin with leaving sticky film and cooling effect. F4 formulation had specific odor due to addition of EOPS. Analyzing creams during three months at different temperatures, we didn't noticed changes in all observed parameters. During period of three months at different storage conditions, both gel and cream formulations were stable showing good visual and sensory properties. Addition of EOPS did not affect all abovementioned parameters.

Table 2. Organoleptic characteristics and physical appearance of gel and cream base and with EOPS after 7, 30 , 60 and 90 days of preparation and storage at different temperatures

		7th day				30th day				60th day				90th day			
Characteristics		F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
Color	4±2°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	25±2°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	40±2°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Odor	4±2°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	25±2°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	40±2°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Homogenity	4±2°C	E	E	E	E	E	E	E	E	E	E	G	G	E	E	G	G
	25±2°C	E	E	E	E	E	E	E	E	E	E	G	G	E	E	G	G
	40±2°C	E	E	E	E	E	E	E	E	E	E	G	G	E	E	G	G
Phase separation	4±2°C	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	25±2°C	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	40±2°C	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Application/ touch	4±2°C	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
	25±2°C	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
	40±2°C	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
Type of smear	4±2°C	E	E	E	E	E	E	E	E	E	E	G	G	E	E	G	G
	25±2°C	E	E	E	E	E	E	E	E	E	E	G	G	E	E	G	G
	40±2°C	E	E	E	E	E	E	E	E	E	E	G	G	E	E	G	G

Legend: Color, Odor: N - Normal; M – Modified; IM – Intensely Modified; Homogenity/Type of smear: G-good, E-excellent, A-average, Phase separation: No or Yes; Application /Touch: P– Pleasant touch, easy skin application (spreadability); D – Unpleasant touch, sticky, relative difficulty to apply on skin; MD – Very unpleasant touch, very sticky, compromises skin application;

pH determination

The pH values of base formulations (F1 and F3) as well as EOPS loaded formulations (F2 and F4) are shown in Figure 1A and 1B respectively. pH values of cream base were in 5.14-5.55 while that range for EOPS cream were 5.11-5.30 during 3 months of storage at different temperatures. On the other hand, pH values for gel samples (F3 and F4) were slightly higher, 6.29-6.59 for gel base and 6.49-6.68 for EOPS gel. Importantly, during 12 weeks of storage at different temperatures, pH values of the examined samples did not change remarkably. Addition of EOPS in formulations did not cause changes in pH values.

Conductivity

Figures 2A and 2B displayed the values of electrical conductivity of creams and gels after storage for up to 90 days, respectively. The values of conductivity for cream base and EOPS cream were in range from 40.1-49.1 μS and 43.6-41.2 μS respectively. Electrical conductivity values of gel base and EOPS gel were higher in comparison to cream formulations (78.5-84.0 μS/ for gel base and 72.5-80.4 μS for EOPS gel. Storage of all formulations during period of three months did not cause significant changes in electrical conductivity values although EOPS formulations (F2 and F4) had lower values relative to cream and gel bases (F1 and F3).

Centrifugation test

There were no signs of samples (F1-F4) physical instability such phase separation by conducting centrifugation test during three months of storage at different storage temperatures. Results are displayed in Table 2.

Washability

The performed washing experiments revealed that cream and gel formulations (F1-F4) can be removed easily by washing with distilled water.

Swelling index

The swelling index of all formulations is represented in Figure 3. Gel formulations (F3 and F4) with carbomer 940 showed higher swelling index in comparison to cream formulations (F1 and F2). The highest swelling index was noticed in cream with EOPS after half an hour.

Microbiological stability test

Since *E.coli, P. aeruginosa, S. aureus* and *C. albicans* are main skin pathogens we aimed to detect them as well as the numbers of aerobic mesophilic bacteria, yeast and molds (Table 3). All four formulations (F1-F4) meet all the requirements of ISO microbiological standards. There was absence of *E.coli, P. aeruginosa, S. aureus* and *C. albicans* while number of aerobic mesophilic bacteria, yeast and molds were <1 cfu/1g. Elevated value of cream aerobic mesophilic bacteria was noticed in cream base group (F1) that is stored at high temperature but still in permitted range.

Figure 1. A: pH of the cream formulations with and without *P.sibirica* essential oil during the stability testing period; B: pH of the gel formulations with and without *P.sibirica* essential oil during the stability testing period. F1-cream base; F2-EOPS cream; F3-gel base; F4-EOPS gel

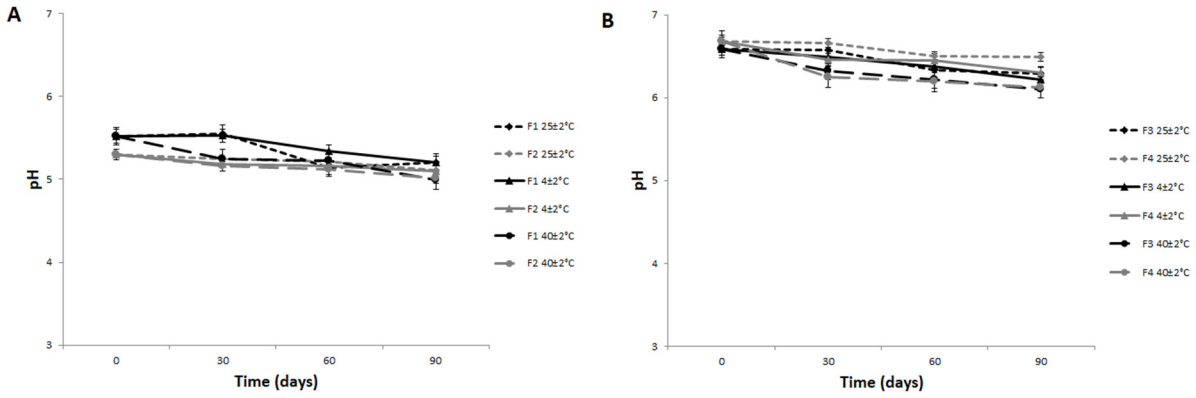


Figure 2. A: Conductivity of the cream formulations with and without *P.sibirica* essential oil during the stability testing period; B: Conductivity of the gel formulations with and without *P.sibirica* essential oil during the stability testing period. F1-cream base; F2-EOPS cream; F3-gel base; F4-EOPS gel

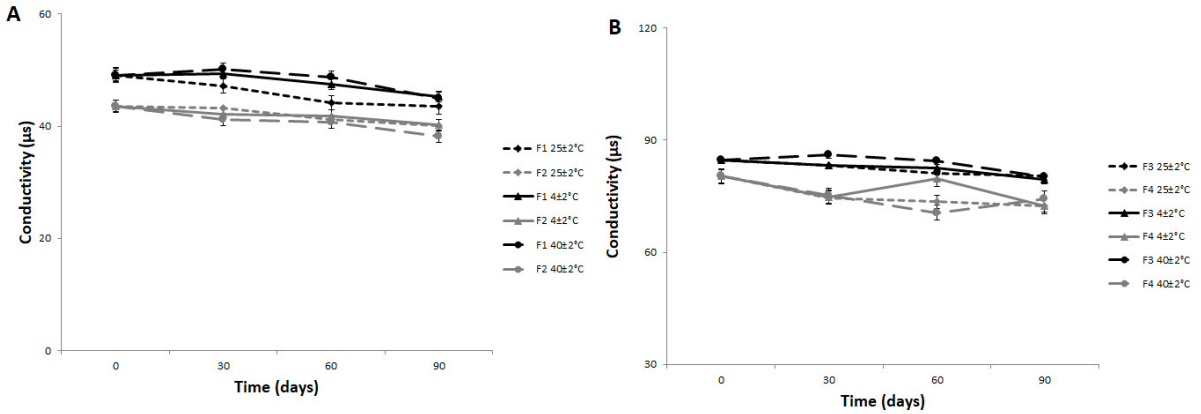


Figure 3. Swelling index of the formulations (F1-F4).
F1-cream base; F2-EOPS cream; F3-gel base; F4-EOPS gel

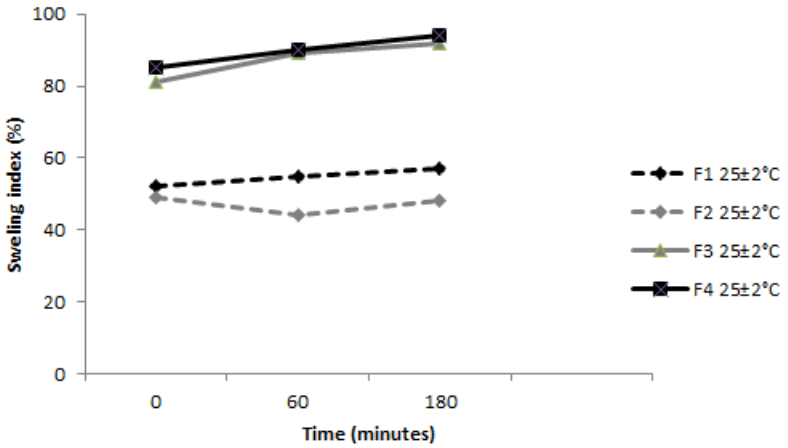


Table 3. List of tested formulations according to microbial contamination during three months of storages at different temperatures

		Day 7				Day 30				Day 60				Day 90			
Parameters		F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
Number of AMB(cfu/g)	8±2°C	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
	25±2°C	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
	40±2°C	10	<1	<1	<1	40	<1	<1	<1	60	<1	<1	<1	80	<1	<1	<1
Number of yeast and mould (cfu/g)	8±2°C	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
	25±2°C	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
	40±2°C	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Escherichia coli (per g)	8±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	25±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	40±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Staphylococcus aureus (per g)	8±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	25±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	40±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pseudomonas aeruginosa (per g)	8±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	25±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	40±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Candida albicans (per g)	8±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	25±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	40±2°C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Legend: nd-not detected; AMB: aerobic mesophilic bacteria

DISCUSSION

Nowadays, herbal ingredients present very attractive components of numerous topical semi-solid formulations exerting auspicious effects on skin. Before these products find their place on market, they had to be stable during manufacturing, transport and storage period. Stability studies play a crucial role in the development and marketing of pharmaceutical and cosmetic semi-solid topical products, including creams and gels. In that sense, we aimed to formulate new topical semisolid (cream and gel) products with *Pinus sibirica* essential oil as well as to determine stability properties while storage them at different temperatures during period of three months.

In first part of our investigation, all preparations (F1-F4) showed satisfactory organoleptic properties such as color, odor and appearance. Additionally, application of all samples was pleasant and easy without leaving sticky and greasy film. As we expected, creams were emollient and cause skin hydration while gels cause cool feeling after application on skin. Moreover, results of centrifugation tests confirmed

physical stability of all examined samples, showing absence of phase separation. Importantly, addition of EOPS to both cream and gel samples, did not cause different properties except the smell (characteristic for *Pinus sibirica* essential oil). All aforementioned properties of cream and gel formulations (F1-F4) are of great importance for commercial products and may influence on consumer decision significantly (20).

After confirming similar visual and sensory features of manufactured creams and gels, we decided to determine if those formulations are physically stabile during period of three months by storage at different temperatures. Since our novel formulations, creams and gels were produced with large amount of water, measuring pH and electrical conductivity values were inevitable. There are some reasons why the pH of cream and gel formulations might decrease such as chemical interaction among acidic or alkaline substances present in the formulation, exposure to air leading to oxidation processes, microbial contamination, absorption of carbon dioxide from the air, temperature changes etc. All those factors are crucial and may lead to pH misbalance of the formulation and consequently to skin irritation, dryness, redness, inflammation and damage (21, 22). Storing of prepared formulations (F1-F4) at 4±2°C, 25±2°C and 40±2°C did not lead to significant change of pH values during 12 weeks, and pH range was from 4.99-6.58 which is suitable for topical products according to Regulations of cosmetic products in our country (23). Interestingly, we noticed linear reduction of pH values during 90 days but it was not significant change.

Another method we used to determine physical stability of creams and gels is conductrometry, which is sensitive to small changes in structure of formulations. Our results indicate that prepared cream formulations are O/W emulsions. Given the range of values observed in this investigation, the electrical conductivity of creams falls within a low to moderate range. The conductivity of a cream is influenced by its composition, encompassing components such as water, emollients, thickeners, and other ingredients (24). We did not notice significant changes of this parameter in cream formulations (F1, F2) during storage of three months. On the other side, values of electrical conductivity of prepared gels (F3-F4) was higher in comparison to cream formulations (F1-F2) due to several factors related to their composition and structure. Gels and creams are both semisolid formulations, but their specific ingredients and properties can lead to differences in electrical conductivity. Gels often have higher content of water compared to creams. Water is a polar solvent that can enhance the ionization of certain substances within the formulation, leading to increased electrical conductivity. Furthermore, presence of electrolytes or ionic species that can facilitate the flow of electrical current through the gel matrix (25, 26). Moreover, three months of storage did not have significant influence on electrical conductivity of all gel formulations. Adding the essential oil to formulations (F2, F4) electrical conductivity values remained constant, which can be explained by hydrophobic properties, low water content and nonpolar compounds, found in EOPS (6, 27). It was noticed that gel formulations showed high swelling index due to its porous nature and big surface area. Higher values of swelling index in gels relative to creams can be due to polymer nature, degree of cross-linking, ionic strength and presence of water. This effect is very important if semi-solid formulation is applied to wounds (28).

Beside physical stability, semi-solid aqueous formulations should maintain good microbiological properties during storage time. Microbes, such as bacteria, mold, and yeast, can significantly influence the stability of topical creams and gels (29). Microbial growth may cause changes in the appearance, texture, and odor of the cream, leading to spoilage of product. Main reason for spoilage of topical product by microbes is production of metabolic by-products or acids leading to pH fluctuations, aggregation or separation of phases (30, 31). Moreover, certain microbes can produce toxins that may pose health risks. Contaminated creams can become unsafe for use, leading to potential adverse effects on the skin. In that sense, topical semi-solid products should include preservatives (synthetic or natural origin), should be packed in airtight and light-resistant containers and produced according to Good Manufacturing Practices (GMP) (32, 33). Considering all this, microbial testing is necessary and crucial to detect microbiological contamination promptly. We investigated main skin pathogens and proved absence of *E.coli, P. aeruginosa, S. aureus* and *C. albicans* in all formulations. Moreover, number of aerobic mesophilic bacteria, yeast and molds also meets ISO standards for microbiological safety. We noticed slight increase of aerobic mesophilic bacteria number in cream base (F1) but still within the standard values ($<10^3$). In that sense, we believe that presence of preservatives (phenoxiethanol/sodium benzoate) as well as essential oil in our formulations led to good microbiological stability during 12 weeks. Antimicrobial effect of Siberian pine essential oil was proven earlier by Russian scientist (7).

CONCLUSION

According to the results obtained from this study, we may conclude that all cream and gel formulations showed good pH value, conductivity and physical and microbial stability during three months of storage at different temperatures. Furthermore, addition of 0.5% *P. sibirica* essential oil did not affect pH values, conductivity, sensory and stability properties of the cream and gel formulations. Further investigations of safety and efficacy of these formulations will be performed.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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