Development of ointment with antimicrobial activity from plant materials and its study

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Abstract. Antibiotic resistance and side effects of drugs are the current concerns of mankind. Therefore, studies aimed at finding and creating new drugs with antibiotic activity from plant materials are of high relevance. The authors formulated the composition of an ointment that contains extracts from Cetraria islandica (Icelandic moss), sphagnum and propolis for treatment of purulent wounds, and determined its pH and bioavailability in vivo. The antimicrobial activity of the ointment was studied on experimental animals. To analyze the morphological features of the wound process, three groups of animals were formed: group 1, control without treatment; group 2, treatment of the wound with Levomekol ointment; experimental group 3, treatment of the wound with ointment that consists of Icelandic moss, sphagnum and propolis. Each group included 10 animals. The ointment for treatment of purulent wounds was developed on lipophilic base with optimal pH of 5.3, antibacterial activity against both gram-positive and gram-negative microorganisms, and it can be used in phases 1 and 2 of the wound process. It has a more pronounced therapeutic effect compared with Levomekol ointment, and facilitates earlier regeneration.

1. Introduction

The problem of bacterial resistance to antibacterial drugs is of high relevance worldwide. The development of biotechnologies and the use of pharmaceuticals made from plant materials that exhibit a number of properties (antibacterial, anti-inflammatory, antioxidant and metabolic ones) is a rational way to solve this problem in treatment of purulent wounds. Beekeeping products are also used to expand the range of raw materials and create medicines.

The attention of physicians and biologists is traditionally attracted by lichen, which therapeutic potential is not yet fully understood and therefore remains unused pharmacologically. Substances with antibiotic activity are isolated from lichen. One of these substances is usnic acid (UA), which has anti-inflammatory, antimicrobial, analgesic and wound healing effects [1]. The antimicrobial activity of lichen extracts and isolated compounds, including UA, is of particular interest. It has been proven that UA is active against S. aureus, Bacillus subtilis, P. vulgaris, E. coli and β-hemolytic streptococcus of the group D Enterococcus faecalis [2; 3], it has an effect on methicillin-resistant Staphylococcus aureus strains [4], sensitive and resistant Mycobacterium tuberculosis strains [5].

Among the lichen growing in the North-West region, Icelandic moss occupies a special place. Several preparations based on Icelandic moss have been patented; it is used in cosmetology as regenerating face cream [2; 6; 7].
The mechanism of the antibiotic activity of UA is associated with arrest of oxidative phosphorylation in microorganisms [8].

It was also found that lichen metabolites, including UA, may have inhibitory activity against COX-2 enzyme, and it will be useful in in vivo anti-inflammatory screening of experimental animals, which can contribute to the development of more effective and powerful chemical substances with anti-inflammatory properties [9].

Lichen polysaccharides exhibit immunomodulatory properties; in addition, lichen contains vitamins C and B12, and nicotinic acid [10].

Usnic acid is a component of many types of lichens; however, for drug production, only species with 0.5% concentration of UA or higher are of interest [11].

Propolis is a resinous substance ranging from brown to dark green in color produced by bees that process biologically active substances collected from buds and resins of various plants.

The propolis composition depends on place of its collection and plants species growing in the region [12]; therefore, a different complex of substances affects the biological activity of propolis.

It is known that complex propolis compositions in various samples can contain about 300 compounds, inorganic minerals, and organic substances such as flavonoids, phenylpropanoids, vitamins, amino acids, lipids, terpenes, stilbens, lignans, and coumarins, and their derivatives [13].

The composition of propolis collected in various regions of Russia, Belarus, and Kazakhstan is being studied [14].

The antibacterial activity of propolis against gram-positive bacteria is apparently due to the presence of phenolic compounds, including flavonoids, aromatic acids and esters. Propolis is considered to have a low side effect profile, and it is approved in many countries for treatment of ulcers and abrasions. In vivo studies show that propolis can be effective in treatment of the inflammatory component of burn skin in rats [15].

Propolis inhibits the growth of both gram-positive and gram-negative microorganisms. However, gram-positive flora is more sensitive to propolis than a gram-negative one [16]. Its antioxidant activity is proven for experimental salmonellosis [17].

According to the literature, sphagnum mosses are used to treat purulent wounds and to heal more quickly [18]. In addition, Sphagnum fuscum is abundant in nature.

The aim of the study is to formulate the composition and develop an ointment that contains extracts from sphagnum and propolis for treatment of purulent wounds.

The following tasks are set:
1. to formulate the composition and determine the pH of the developed ointment and its bioavailability in vivo
2. to study the antimicrobial activity of the ointment on experimental animals.

2. Materials and methods

Antibiotic activity of the active ingredients of lichen depends on the presence of UA isomers in the raw materials (possibly the presence of D(+) and L (−) isomers and racemate), and the right-handed isomer of UA exhibits higher activity [19]. Therefore, in order to enhance antimicrobial activity, propolis and brown sphagnum were added to the composition of the developed ointment.

Fats and oils in ointment composition prevent penetration of microbes and protect against irritating environmental factors; well absorbed by the skin.

Thalli of Icelandic moss and sphagnum were harvested in July–August in Brest region in an ecologically green area. The collected raw materials of Icelandic moss were in accordance with GOST (GOST 13727-68 Thalli of the Cetraria Islandica (Icelandic moss)) in all indicators. The thalli were crushed to a particle size of 3–5 mm, poured with 70% ethyl alcohol in a ratio of 1:1 and then left to swell for 4 hours. After that, the substance was transferred to a percolator, an extractant was added until a smooth surface appeared, and then percolated after 24 hours of steeping. The extract was settled at 8–10 ºC for 2 days and filtered through a paper filter. The resulting extract was evaporated to thick consistency.
Similar procedure was performed for a thick extract of brown sphagnum.

Propolis samples were prepared in apiaries of Novgorod region, and the propolis physicochemical properties corresponded to the requirements of GOST 28886–90 Propolis. Specifications. Propolis was standardized according to the Provisional Pharmacopeial Article 42-1084-81, the content of flavonoid compounds with regard to rutin was also determined at a wavelength of maximum absorption in the range of 390–420 nm (GOST R 55312-2012). To evaluate antioxidant activity, the total content of polyphenols (GOST R 55488-2013) was determined with regard to gallic acid (trihydroxybenzoic acid) according to the reaction of formation of colored compounds with the Folin-Ciocalteau reagent with maximum absorption of 765 nm. A Shimadzu UV 1800 spectrophotometer was used to measure absorption of solutions in cuvettes with 1 cm layer thickness.

The data obtained for quality characteristics of propolis (n=5) were as follows: the oxidation value was 17.1±1.2 s; the mass fraction of flavonoid and other phenolic compounds was 33.2±1.4%; the rutin content was 1.9±1.4%; the polyphenolic contents content was 12.3±1.8%; the content of mechanical impurities was 16.8±1.0%; the wax content was 21.1±1.2%, and the iodine number was 49.5±1.5%.

The pH level was determined in accordance with the requirements of the general pharmacopeial article Ointments according to the procedure: a portion of the ointment 1.0 was placed in a flask, 50 ml of purified water heated to 50–60 °C was added, and the mixture was shaken for 30 minutes in a vibrator. The extract was filtered through a cotton swab. After that, the pH of the resulting solution was determined potentiometrically.

The samples were dialyzed through a semipermeable cellophane membrane to determine the bioavailability of the ointments prepared in vitro. Dialysis was carried out in purified water in a thermostat at 37±1 °C for 24 hours. The samples were taken after 2, 3, 4, 5, and 24 hours [20]. The UA concentration was determined by the validated method published earlier using a spectrophotometer at a wavelength of 290 nm [21].

The effectiveness of the developed ointment was experimentally tested on 30 white Wistar male rats weighing 180±20 g. The animals were kept under laboratory vivarium conditions on a standard diet in compliance with the International Recommendations of the European Convention for Protection of Vertebrate Animals used in experimental studies, and in line with the laboratory practice of preclinical studies in the Russian Federation (GOST ISO/IEC 17025-2019) and Order of the Ministry of Health of Russia No. 199n of 04/01/2016 On the Approval of Good Laboratory Practice (GLP) Regulations.

The morphological study was carried out using an AxioscopeA1 light-optical binocular microscope (CarlZeiss, Germany). Biopsy specimens were fixed in 10% neutral formalin and, according to the generally accepted technique, embedded in paraffin blocks. Histological sections made of paraffin blocks 4–5 μm thick were stained using standard histological techniques. The preparations were studied and photographed using DMLB microscope (Leica, Germany) – JVC video camera (USA) – Pentium IV computer. To analyze the morphological features of local dynamics, animals were divided into three groups: group 1, control without treatment; group 2, treatment of the wound with Levomekol ointment; experimental group 3, treatment of the wound with ointment that consists of Icelandic moss, sphagnum and propolis. Each group included 10 animals.

Modeling of surgical wounds was performed in animals under anesthesia in aseptic conditions. Anesthesia was induced using 3 mg zoletil, 0.8 mg xylazine, and 0.1% atropine solution, 0.02 ml per 100 g of animal weight.

After suppression of the corneal reflex and the lack of response to pain stimuli, hair in the interscapular region of rats was cut and shaved, after preliminary treatment of the surgical area with 5% iodine solution followed by 70% ethyl alcohol. Next, a skin flap with a subcutaneous fat layer was cut.

In animals kept in a cage, a wound infection developed spontaneously. On day 2 after the operation, skin crust was found to cover the wound skin defect, which releases purulent effluent when pressed.

The average amount of the healing agent (ointment) applied was on average 0.2–0.3 g per 1 cm² of the wound surface. In groups I and III, the wound surface was treated with 3% solution of hydrogen peroxide, and then a gauze impregnated with the developed ointment or with Levomekol ointment was
applied to the wound. All rats of control group II and experimental group III had debridement of wounds daily throughout the experiment.

3. Results
To formulate the ointment composition, 3 ointment bases of various types were used: hydrophilic (composition: 2% agar-agar, 2% sodium carboxymethyl cellulose, 3% glycerin, 0.5% citric acid, 1% dimethyl sulfoxide (DMSO), water purified to 100); lipophilic (50% coconut oil, 30% cocoa butter, 1% alpha-tocopherol acetate, 1% DMSO) and diphilic (15% olive oil, 10% emulsion wax, 15% apricot oil, 0.1% rose oil, 0.5 % citric acid, 1% DMSO, water purified to 100).

Purified water was poured into sodium carboxymethyl cellulose (NaCMC); the mixture was then allowed to swell for 60 minutes and heated in a water bath until NaCMC was completely dissolved. Agar-agar was dissolved in water upon heating and added to the solution, glycerin was then added and the mixture was stirred. Citric acid was added to the resulting mixture as a preservative.

To prepare a lipophilic base, coconut oil and cocoa butter were fused in a water bath, and tocopherol was added to the molten base.

To prepare a diphilic base, emulsion wax was added to olive and apricot oil and melted in a water bath and, after cooling, rose oil was added. Citric acid was dissolved in purified water, added to the mixture and emulsified until the mass was homogeneous.

The thick extracts of Icelandic moss (5%) and sphagnum (3%) were mixed with DMSO and propolis (10%) dissolved by heating in 96% ethanol was added to the composition. Then, everything was thoroughly emulsified with the base in a water bath until the ethanol was completely removed.

The acidity (pH) of the hydrophilic ointment was 5.5, that of the lipophilic ointment attained 5.3 and that of the diphilic ointment was 4.2.

The concentration of UA (μg/ml) released over different periods of time was the highest in composition No. 2. Thus, after 2 hours, it was 0.383±0.024 μg/ml in composition No. 1, 0.600±0.128 (μg/ml) in composition No. 2, and 0.373±0.019 (μg/ml) in composition No. 3. After 24 hours, the UA concentration was similar and equaled 0.537±0.080 μg/ml, 2.077±0.152 μg/ml, and 0.933±0.230 μg/ml, respectively.

The lipophilic ointment, which exhibited the highest UA bioavailability, was used for treatment of experimental animals.

The pattern of wound healing corresponds to the classical description of the wound healing process and is provided in three phases. The first phase of inflammation is followed by regeneration and formation of granulation tissue and neoplasm of blood vessels. The wound healing process results in the scar with the intercellular substance reconstructed. The above phases can occur simultaneously in different areas of the wound surface [22, 23].

In the control group of male Wistar rats (group I), histological analysis showed cell death in the form of necrosis and dystrophy for five days in the epidermis and dermis. Edema of the extracellular matrix, swelling and disorganization of collagen fibers, vascular congestion, microcirculatory disorders (erythrocyte stasis, margination of leukocytes, thrombosis) were observed. In this group, wounds were showed no signs of purulent effluent on day 9.7±0.3, edema of the surrounding tissues stopped on day 8.7±0.7, and activation of proliferative processes, that is the formation of granulation tissue was observed on day 8.9 ± 0.3. In Levomekol treated rats from group II, purulent effluent was no longer secreted on day 8.4 ± 0.2, hyperemia edema of the surrounding tissues disappeared on day 7.8±0.2, and areas of growing granulation tissue with neoangiogenesis appeared in the connective tissue on day 7.9±0.2. In the experimental group of rats treated with the developed ointment, the wounds were free of purulent effluent on day 6.0±0.5, inflammation in the tissues surrounding the wound subsided on day 5.8±0.5. On day 5.2±0.2, young granulation tissue formed, which consisted of newly formed vessels, fibroblasts and macrophages with collagen fibers located between them.

The comparison of the results in experimental groups II and III obtained by the planimetric method showed that during treatment with the developed ointment the total area of wounds reduced faster and
the healing rate was 1.9±0.2 times higher than that observed during standard treatment with Levomekol ointment.

Compared with the two control groups, rats treated with ointment containing extracts from Icelandic moss, sphagnum and propolis, the rate of wound area reduction and the healing rate were consistently high from day 1 to day 10 (these are phases I and II of the wound process).

The analysis of histological preparations in the dynamics of the study showed the best results in experimental rats from group III treated with ointment containing extracts from Icelandic moss, sphagnum and the first two phases of the wound process.

Microflora of the wound consisted of several pathogens with prevalence of gram-positive S. aureus (32.5%), Streptococcus spp. (25%) and gram-negative P. vulgaris (12.5%). The maximum growth of colony forming units was observed for coagulopositive S. aureus and Streptococcus spp. and amounted to $10^{10} – 10^{12}$ CFU/ml.

Wound treatment with propolis and lichen based ointments sharply decreased the rates of wound contamination by microbial flora in group II by 5% and in group III – by 21% (5–7 days of the wound healing process).

4. Discussion

In purulent surgery, one of the main methods for treatment of purulent wounds is local treatment with various ointments [23, 24]. A stable pH level of the skin is one of the main requirements for ointment bases. The optimal pH level of the skin ranges from 3.5 to 5.9. In addition, variation of this indicator indicates a change in physicochemical properties of the ointment components. All the developed samples of ointments satisfied this requirement. Experimental data showed that the best bioavailability of UA was exhibited by ointment on the lipophilic base of coconut and cocoa butter, which was tested on animals. The fabrication of ointments on this base is relevant because coconut oil is known for its anti-inflammatory activity due to inhibition of cytokines [25].

We found that rats from group III treated with the ointment prepared using extracts from Icelandic moss, sphagnum and propolis significantly (p <0.05) accelerated termination of inflammatory phenomena, and showed the predominance of regeneration processes, which indicated the transition from phase I of the wound process to phase II. The data obtained do not contradict the studies of the propolis paste, which showed a significant decrease in the area of the wound surface after 14 and 21 days compared with the control group [26].

5. Conclusion

Preliminary data obtained in the studies have shown that the lipophilic-based ointment developed by the authors for treatment of purulent wounds has an optimal 5.3 pH level, exhibits a wide range of antibacterial activity against gram-positive and some gram-negative microorganisms, and can be used in phases I and II of the wound process. The ointment shows a more pronounced therapeutic effect compared with Levomokol and facilitates earlier regeneration.

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