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New Trichoderma harzianum Rifai F-180 - L-lysine-α-oxidase antitumor enzyme producer - culture liquid-based substance biotechnology

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ABSTRACT

The biosynthesis of L-lysine-α-oxidase from Trichoderma harzianum Rifai F-180, an antitumor enzyme producer, was carried out on a wheat bran medium.
Hydrophilic bases of lightly crosslinked acrylic polymers and methylcellulose derivatives were selected, and model samples were prepared with a 1% concentration of the culture liquid of the fungus. The wound healing activity of the obtained gel samples was investigated on an experimental model of guinea pig wounds. The results of experimental studies showed a high wound healing activity of the gel with a 1% concentration of the culture liquid of the fungus and can serve as a basis for further preclinical trials of this dosage form of wound healing action.

Keywords: L-Lysine -α- oxidase, Trichoderma, technology of the gel

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INTRODUCTION

Microorganisms are producers of various groups of compounds of great practical importance. Antibiotics, enzymes, growth stimulants, vitamins, amino acids are metabolic products of microorganisms. One of the essential and urgent directions of modern microbiology is the study of physiologically active products of the vital activity of microorganisms and the mechanisms of regulation of their biosynthesis. Currently, one of the most studied fungi is the genus *Trichoderma* [1,2].
L-lysine-α-oxidase produced by the fungus *Trichoderma harzianum* Rifai F-180 was proved to have antitumor and antiviral activity. Acute toxicity of the fungal enzyme L-lysine-α-oxidase was studied in rats. The study showed that the substance, when administered intravenously at 3000 U/kg, does not cause any symptoms of poisoning and, moreover, death of animals [2].
All this offers prospects for the development of new dosage forms based on L-lysine-α-oxidase, which can be recommended for use in various skin infections and mechanical damage to the skin.
The use of the culture liquid of the enzyme producer is also very important in economic terms, as it does not require specific expensive purification [2,3].
The prospect of using the culture liquid of the active producer of L-lysine-α-oxidase from *Trichoderma harzianum* Rifai F-180 (LO) was also shown on objects of plant origin through suppressing phytopathogenic microorganisms by it, causing damage to plants by especially dangerous bacteria and viruses. the studies at the Academician T.T. Berezov Department of Biochemistry, RUDN, proved the LO inhibition effect on phytopathogenic viruses INSV and TRSV and *E. amylovora* and *A. citrulli* [4-8].

Objective: Production of culture liquid from *Trichoderma harzianum* Rifai F-180 (LO), development of the composition and technology of the LO gel, study of the resulting dosage form. To achieve this goal, the following tasks were solved: production of LO, selection of excipients for the manufacture of LO-containing gel, development of an optimal gel technology, study of the stability indicators of LO-containing gel, as well as the specific activity of the gel in experiments on animals.

THE *TRICHODERMA* STRAIN CULTURE LIQUID UNDER STUDY

MEDIA AND METHODS USED.

To choose auxiliary components in the development of the dosage form, we were guided by the following basic principles: the components of the gel should be compatible, not cause irritating effect, promote maximum release of the active component, ensure ease of application and packaging of the gel.
Gel pH was determined using a universal ionometer – Sartorius professional Meter PP 20. Colloidal stability was investigated according to conventional methods. To study the structural and mechanical properties of the dosage form, a rotary viscometer ("Reotest-2" type RV, Brookfield DV-II type RV (Germany) was used [9].
The *Tr. harzianum* Rifai F-180 strain was grown on wort-agar medium for 7 days in a thermostat at 28°C. The resulting culture with medium [1-1.5 cm] was introduced into a 250 ml flask. The medium has the following composition: 7 ml of 11.4% sodium nitrite, 10 g of wheat bran, 10 ml of purified water. Culture was carried out for 14 days at a 28°C. Then, 100 ml of water was added to the flask, shaken for 2 hours and squeezed through cheesecloth. In the resulting aqueous extract (LO), the activity of L-lysine-α-oxidase was determined.
The enzyme activity was determined by the spectrophotometric orthodianisidine micro-method according to the amount of H_2O_2 formed during the enzymatic reaction. The unit of enzyme activity was the amount of the enzyme catalyzing the formation of 1 nmol of H_2O_2 per minute under standard conditions, per 1 ml of culture liquid [3].
LO has a pH optimum for the manifestation of enzymatic activity of 5.9-6.0. This is apparently explained by the fact that the enzyme is in association with other enzymes and waste products of *Trichoderma* and has optimal conditions for life [3].
LO is an opaque liquid of light-yellow color with a weak mushroom-like odor, stable during storage, containing

group No.	Dosage form	Number of animals per group	Average wound healing time (days)	t_c	t_{mARS}	t_{MC}
1.	1% carbopol-based gel (974P)	3	6.7±0.4 (6.3-7.1)	-	-1	-2.6
2.	1% mARS gel	3	7.7±0.4 (7.3-8.1)	1	-	-1.6
3.	1% MC-based gel	3	9.3±0.4 (8.9÷9.7)	2.6	6.17	-

protein, unsaturated fatty acids, mono- and disaccharides, starch, fiber, organic acids, vitamin B1, vitamin B2 (gives color), vitamin PP, iron, potassium, calcium, magnesium, sodium, phosphorus, and sodium nitrate.

The choice of the optimal concentration of L-lysine- α -oxidase, providing sufficient anti-inflammatory and wound-healing effects, was based on previous studies, the purpose of which was to determine the activity of the enzyme L-lysine- α -oxidase in animals (laboratory mice and rabbits). To develop the composition and technology of the gel with L-lysine- α -oxidase, model samples with 1% LO concentration were made [2].

The structure-forming components for the manufacture of the gel were chosen based on the therapeutic, technological and consumer requirements for the drugs of this group. According to the literature data, hydrophilic bases of lightly crosslinked acrylic polymers (LCAP) (mARS and Carbopol) and a methylcellulose (MC) derivative were selected as bases for the gel [10].

RESULTS AND DISCUSSION

The first stage of the research involved a screening of the bases capable of providing the maximum wound-healing effect in the dosage form. For this purpose, samples of gel were made on hydrophilic bases: MC and LCAPs. The wound healing activity of the LO gel was investigated on an experimental model of guinea pig wounds.

The studies were carried out on male guinea pigs weighing 320 ± 15 g. The wound was created in animals on a previously depilated back skin area (5x5 cm). Stenciled cuts were made using a 15 mm disposable blade. Treatment was started 18 hours later and was carried out once a day for two weeks until the animals were completely cured. The drug was applied to the wound in a thin layer using a sterile glass rod. The therapeutic effect of 1% gel with LO was studied in comparison with placebo gel (gel base). 1% LO gel on a phospholipid basis, provided by Cand. Chem. N.M. Murashova, Russian University of Chemical Technology (RCTU), served as the control drug. Under the same experimental conditions, a group of untreated animals served as a control. The results of the experiment were considered according to the average time of wound healing (in days). The results were statistically processed by the method of variation statistics. The results are shown in Table 1.

Table 1 - The results of wound healing in animals, depending on the LO gel base.

Note: t_c - time of wound healing with carbopol treatment.
t_{mARS} - time of wound healing with $_{mARS}$ treatment
t_{MC} - time of wound healing with MC treatment

Analysis of the data obtained showed that the base has a significant effect on the anti-inflammatory and wound-healing activity of L-lysine-α-oxidase. Samples made with the use of lightly crosslinked acrylic polymers (mARS and carbopol) showed high therapeutic activity, low activity of the samples on MC, however, the fastest wound healing was observed for carbopol-based samples.

Based on the conducted studies we can advise to use rarely crosslinked acrylic polymers in a gel form as a base, since these bases meet the medico-biological requirements for the dosage form, the intended purpose (sufficient osmotic activity, skin respiration, etc.). In addition, RCAPs are compatible with the enzyme, increase the stability and extend the shelf life of gel compositions, and are technologically advanced in the production process [10,11].

RCAP-based gels have a number of advantages over lipophilic ointments, since when applied to the skin, they form the thinnest smooth films, providing a prolonged effect of the preparations; more fully and evenly release medicinal substances, absorb skin excretory and secretory products, are well distributed over the skin surface; have a cooling effect, do not violate the physiological functions of the skin, do not cause allergic reactions and irritating effects, do not contaminate clothes, have a pleasant appearance and consistency [4].

In order to select the optimal structure-forming agent for LO gel, the effect of polymer concentration on the effective viscosity of carbopol and mARS gel compositions were neutralized with sodium hydroxide solution (pH 5.5-6.5) was studied. The viscosity of the structurant (Fig. 1) was measured on a Reotest -2 device at 9 rpm, close to the rate of application of the dosage form when applied to the affected skin areas [9].

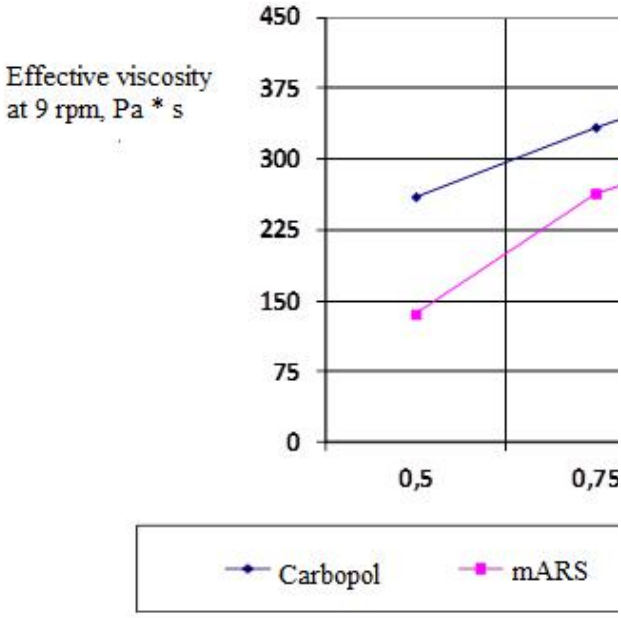

Figure 1 - Relationship of the effective viscosity of gel bases (carbopol and mARSa) to the polymer concentration

Samples of bases, which include the structure-forming component in a concentration of up to 0.25% for carbopol and up to 0.5% for mARS, are fluid systems and can be classified according to liniment. A comparative study of the rheological parameters of RCAPs showed that 1% carbopol and 1.5% mARS are the most acceptable. Viscosity of 1% carbopol-398 Pa*s gel and 1% mARS-401 Pa*s gel. Carbopol (974 R) was chosen for further research due to its significantly lower amount at comparable viscosity values, and significantly shorter time of polymer swelling.

To study the thixotropic properties, the curves of the deformation kinetics of the gel with L-lysine-alpha-oxidase were plotted in the coordinates: shear rate - shear stress in the range of flow rate gradients from low to high and from high to low (Figs. 2 and 3).

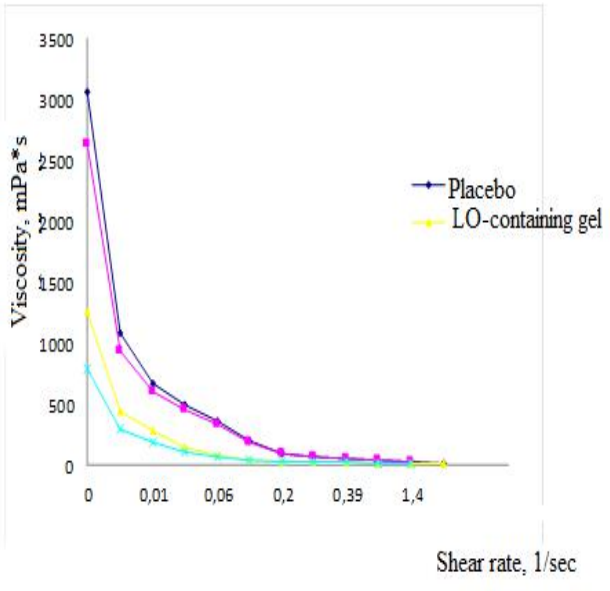

Figure 2 - Rheogram of effective viscosity of 1% carbopol and LO gel.

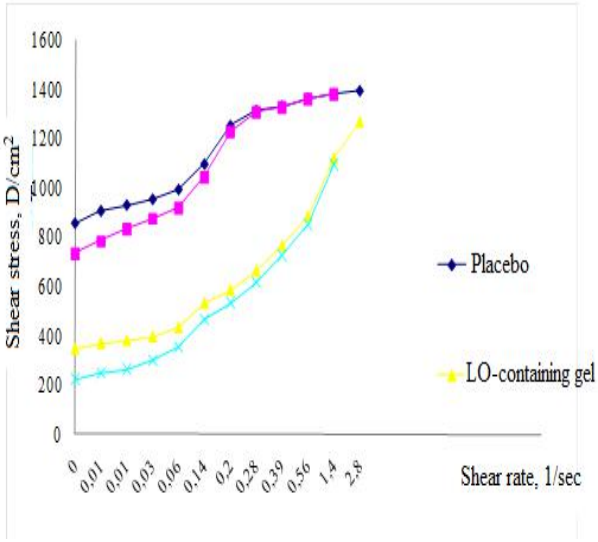

Figure 3 - Rheogram of flow of 1% carbopol and LO gel.

Analysis of the data presented in Figs. 2 and 3 shows the presence of ascending and descending curves of the "hysteresis loop" indicating that the sample under study has weak thixotropic properties which help assume good spreadability, squeezability out from tubes and the stability of the developed LO gel.

As Figures 2 and 3 show, at a given polymer concentration, an increase in hysteresis loops occurs (an ascending curve - system failure, a descending curve - system recovery), which indicates an increasing depth of structure formation in the carbopol: water system

Dosage form	Number of animals per group	Average wound healing time (days)	t_1	t_2	t_p	t_c
1% LO carbopol-based gel (1)	5	6.60±0.48 (6.12÷7.08)	-	-2	-4.2	-5.4
1% LO phospholipid-based gel (2)	5	8.60±0.48 (8.12÷9.02)	2	-	-2.2	-3.4

confirms the thixotropic properties of the resulting gel [11].

Thus, as a result of a series of physicochemical, biological and rheological studies, the following composition of the LO gel was developed.

LO gel ingredients:

Carbopol (974 P NF)

European Pharmacopoeia 2001, "Carbomers" 1.0 0.7 - 1.1

Culture liquid (LO) 0.9-1.1 1.0 (5.4 U/ml.)

Sodium hydroxide solution (30%) (GOST 4328-77) to pH 6.0-6.5

Purified water (FS 42-2619-97) up to 100.0

The gel has a pleasant appearance, without mechanical impurities, colorless, slightly yellowish, odorless, gel-like consistency, easily applied, tightly adhering to the surface of the skin, dries quickly, forming a film, not sticky, does not contaminate clothes, stable during storage

The process flow includes a preparatory stage and the actual production process of a LO-containing gel.

Preparatory stage: preparation of disinfectant solutions, preparation of production facilities, equipment, personnel, as well as packaging.

The process flow consists of the following stages: gel production, packaging, packaging and quality assessment of the dosage form. The preparation of the gel begins with the preparation of the drug substance and base. The base preparation operation includes the process of dissolution and swelling, as well as regulation of the pH value with sodium hydroxide.

RCAP-water base production: The calculated amount of purified water is measured into the production container. RCAP powder (carbopol) is layered on the water surface and left to swell for 1 hour, then the system is stirred with a mechanical stirrer (EKROS-8100) at 100-120 rpm until a homogeneous gel is obtained. After that, with constant stirring, a calculated amount of sodium hydroxide is added to obtain a given pH value of the medium (5.5-6.5). The introduction of a neutralizing agent thickens the system and increases viscosity. The ingredients are thoroughly mixed using a high-speed mixer (EKROS-8100). The resulting gel is left to complete the structure formation for 4-6 hours.

LO gel production:

The culture liquid is introduced into the resulting gel base and homogenized on a paddle mixer (EKROS-8100, 120 rpm) for 10 minutes.

The manufacturing technology of the developed LO-containing gel is sufficiently reproducible.

The study of the stability of the LO gel during storage was carried out using physicochemical, chemical, and microbiological methods of analysis. For this purpose, three series of LO gel were produced according to the developed and experimentally studied composition, packaged in 10 g glass jars (TU 9463-015-07609129-2003). The samples were stored in a refrigerator (8 ± 2°C). At the time of manufacture and then after 6 months, they were monitored visually, determined the authenticity, the activity of L-lysine- α -oxidase, the pH value and microbiological purity, the viscosity of the gel.

To determine the activity of L-lysine- α -oxidase, the method of spectrophotometric analysis was used. Measurement of the optical density of the solution was carried out at a wavelength of λ = 540 nm +3 nm (maximum absorption of a hydrogen peroxide solution). The reference solution was the placebo solution of the dosage form [5].

Indicators such as the appearance, odor, pH value and uniformity during storage of the gel did not change. No separation of the aqueous phase was observed during the tests. The authenticity, the activity of L-lysine-α-oxidase in the gel, the viscosity of the gel and the microbiological purity were in accordance with the FSP draft "L-lysine-α-oxidase gel, 1% concentration".

One of the mandatory stages of the preclinical study of a newly developed drug is a set of experiments, including the study of specific activity in laboratory animals.

An experiment was carried out on 20 male guinea pigs weighing 320 ± 15 g (Table 2). The gel with 1% LO showed a wound healing effect, reducing the average time of wound healing in animals by 5.4 days compared with the control group (without treatment); and by 4.2 days compared to placebo gel, respectively.

As Table 2 shows, the difference in the healing times for animals belonging to different experimental groups is reliable.

Table 2 - Timing of wound healing in guinea pigs using different dosage forms.

Carbopol gel (LO-free) (p)	5	10.80±0.32(10.48÷11.12)	4.2	2.2	-	-1.2
Control (untreated) (c)	5	12.0±0.4 (11.6÷12.4)	5.4	3.4	1.2	-

Note: t_1 is the wound healing time using 1% L-lysine-α-oxidase carbopol-based gel.
t_2 is the wound healing time using 1% L-lysine-α-oxidase phospholipid-based gel.
t_p is the wound healing time using carbopol without L-lysine-α-oxidase
t_c is the wound healing time (control) without treatment

SUMMARY
As the result, the researchers chose auxiliary substances, developed the composition and technology of obtaining a gel with a 1% concentration of the culture liquid *Trichoderma harzianum* Rifai F-180, and established the stability of the developed drug substance within 6 months of storage.
The results of experimental studies showed a high wound healing activity of the gel with a 1% concentration of the *Trichoderma* culture liquid and can serve as a basis for further preclinical trials of this dosage form of wound healing action.

CONCLUSION

The researchers have cultured *Trichoderma harzianum* Rifai F-180 - an antitumor enzyme producer on a wheat bran medium.
Gel model samples with 1% culture liquid of the fungus were made. The possibility of the obtained substances in the experiment on guinea pigs as wound healing agents has been shown, which can serve as the basis for further preclinical trials of this dosage form of wound healing action. The proposed dosage form can be used in veterinary medicine: as a wound-healing drug, for skin viral infections and ophthalmic herpes. In health care: in medical cosmetology (after plastic surgery), in gynecology, for skin oncological diseases.

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CONFLICT OF INTERESTS
The authors declare that the provided information has no conflicts of interest.

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