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PRACTICALLY VALUABLE METABOLITES OF MARINE MICROORGANISMS

T. P. PIROG[1, 2], *A. O. MARTYNIUK*[1], *O. I. SKROTSKA*[1], *T. A. SHEVCHUK*[2]

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The review considers the modern literature data on the synthesis by fungi, actinobacteria, and bacteria isolated from marine ecosystems (seawater, bottom sediments, flora and fauna, mangrove biomes, glaciers), practically valuable metabolites. Marine microorganisms synthesize a wide range of practically valuable enzymes (cold-active galactosidase, agarase, alginate lyase, fucoidase, chitinase, etc.), surface-active glyco- and lipopeptides with emulsifying, antimicrobial and antiadhesive activity, as well as secondary metabolites with diverse biological activity (antimicrobial, antitumor, cytotoxic). However, the use of marine producers in biotechnological processes is constrained by their low synthesizing capacity and high costs of biosynthesis (complex nutrient media and expensive carbohydrate substrates). In biotechnology, marine microorganisms can be used as sources of genes encoding the synthesis of new biologically active substances with unique properties, including antimicrobial and antitumor.

Key words: marine fungi, bacteria, biologically active substances.

The unexplored marine world, which is characterized by great biodiversity, is a resource for discovering new structures with unique properties. The prolonged evolution of marine life has led to the emergence of species with atypical genes. Marine microorganisms had to adapt to such living conditions as high pressure (up to 1100 atm), anaerobic conditions at great depths at temperatures slightly below 0 °C, high acidity (pH 2.8), and temperatures (above 100 °C) in the area of thermal springs. Also, it was necessary to adapt to high salinity, radiation, light, low concentrations of nutrients [1]. Existence in such conditions, close to the extreme, contributed to the genetic and metabolic diversity of marine microorganisms, which in turn led to the emergence of specific adaptation mechanisms, in particular, the synthesis of unusual protective compounds. It is now found that marine microorganisms can synthesize a huge number of unique metabolites with various biological properties, which are promising for use in the pharmaceutical and cosmetic industries, medicine [2–5]. For example, the hydrolytic enzymes of these microorganisms can function under conditions that lead to deposition or denaturation of most proteins synthesized by mesophilic (terrestrial) microorganisms [6]. Besides, seawater, which is physiologically and chemically close to human blood plasma, can provide the synthesis of biomolecules, which are characterized by lower toxicity and having fewer side effects when used for therapeutic purposes compared to enzymes produced by traditional producers [6].

Therefore, it is not surprising that every year the number of publications devoted to the study of marine microorganisms as producers of various biologically active compounds

increases [7–18]. In the last 5 years, about 400 reviews have been published with the keyword "marine microorganisms". However, such reviews are "narrowly specialized" because they summarize publications related to the formation of antibiotics by marine microorganisms [10, 15], antitumor compounds [13], enzymes [16], polysaccharides [11], or those, which consider the ability of sea fungi [4, 5, 7], actinobacteria [1, 3, 9, 14], algae [17] to the synthesis of certain metabolites. Also, much attention in the most such reviews are paid to the biodiversity of marine microorganisms [1–3, 7], determination of the chemical composition and structure of metabolites [4, 7, 9–12, 14, 15, 17, 18], their biological activity [8–10, 13–17]. Yet the prospects of marine microorganisms using in biotechnology are not considered.

The purpose of this review was to analyze and summarize current literature data on the biotechnological potential of marine fungi and bacteria as producers of a wide range of practically valuable products (surfactant glyco- and lipopeptides, exopolysaccharides, enzymes, metabolites with various biological activity — antimicrobial, antitumor, cytotoxic).

Surfactants

Due to the advantages of microbial surfactants over synthetic analogs (biodegradability, non-toxicity, stability of physicochemical properties in a wide range of temperature and pH), as well as their unique biological properties, interest in these substances is growing every year [19–21]. So, the possibility of using microbial origin surfactants in the oil and mining, chemical, food industries, agriculture, in environmental technologies for environmental cleansing has been already found.

In recent years, a significant number of papers had been published in which it was reported about the isolation of marine microorganisms capable to synthesize surfactants [22–50]. Among marine bacteria, the representatives of *Bacillus, Pseudomonas, Rhodococcus* genera were found [32, 34, 35, 41–43, 45, 46, 49], which were the known producers of surfactants [33]. However, many isolated strains turned out to be the non-traditional producers of surfactants — members of the genera *Buttiauxella* [22], *Serratia* [23], *Staphylococcus* [24, 25], *Vibrio* [28], *Haloimonas* [30], *Nesterenkonia* [37], *Achromobacter* [40].

Analysis of the literature data on the physicochemical and biological properties of surfactants synthesized by marine microorganisms showed that they were virtually indistinguishable from those established for surfactants formed by traditional producers. Thus, by chemical nature, most surfactants of marine microorganisms were glycolipids [22–32] or lipopeptides [34–43]. They were characterized by antimicrobial [22–26, 37–39], antiadhesive [23, 24] and antioxidant [37] activity, as well as the ability to destroy biofilms of pathogenic microorganisms [22–25, 38], emulsification and solubilization of various hydrocarbons [26–30, 32, 34–36, 41, 44].

Notably, that in [22–29, 34–41, 44] the ability to synthesize surfactants was evaluated mainly by the indicators of emulsification index, surface tension, critical concentration of micelle formation, which indicated only the presence of surfactants, not their amount. Therefore, to assess the level of synthesizing ability of marine microorganisms and compare with that of traditional surfactant producers was not possible. Also, in these works, the authors focused on the properties of surfactants to predict the prospects of their practical significance.

Glycolipids of marine microorganisms

It was found that the marine halotolerant strain of enterobacteria *Buttiauxella* sp. M44 synthesized a glycolipid consisting of glucopyranose, associated with fatty acids C14, C16, and C18, including octadecanoic acid [22]. Glycolipid was characterized by antimicrobial activity against some pathogens (*Escherichia coli* ATCC25922, *Bacillus subtilis* ATCC465, *Bacillus cereus* ATCC11778, *Candida albicans* ATCC10231, *Aspergillus niger* (strain number was not given), *Salmonella enterica* in concentrations of 100–300 μg/ml.

Dusane et al. [23] reported a glycolipid synthesized by another member of the marine enterobacteria *Serratia marcescens* CFS, which had antimicrobial and antiadhesive activity. Glycolipid contained glucose and palmitic acid. The minimum inhibitory concentration (MIC) of this surfactant against *C. albicans* BH and *Pseudomonas aeruginosa* PAO1 were 25 μg/ml, against *Bacillus pumilus* TiO1 — 12.5 μg/ml. Glycolipid of the CFS strain at a concentration of 50–100 μg/ml reduced the adhesion of these test cultures to polystyrene by 75–94% and destroyed their biofilm by 55–80%.

The report [24] concerning the surfactant synthesis increase in case of co-cultivation

of the producer *Staphylococcus lentus* SZ2 isolated from a sea snail surface with *Vibrio harveyi* aquaculture pathogen is rather interesting. At the end of co-cultivation of both strains, the growth of *V. harveyi* was completely inhibited, and the surfactant formed under such conditions (called BS-SLSZ2) was characterized by the highest antiadhesive activity and ability to destroy biofilms compared to the drug formed by *S. lentus* SZ2 monoculture. By chemical nature, BS-SLSZ2 was a glycolipid preparation. It contained triose (a four-carbon carbohydrate) as well as hexadecanoic and octadecanoic acids. The glycolipid BS-SLSZ2 at a concentration of 20 μg/ml destroyed the biofilms of *V. harveyi* and *P. aeruginosa* by 78.7 and 81.7%, respectively. The disadvantage of strain SZ2 as a surfactant producer was low concentration of the target product, which even in case of co-cultivation with *V. harveyi* did not exceed 70 mg/l [24].

Another *Staphylococcus* genus, which synthesized glycolipid with similar biological properties, was a strain of *Staphylococcus saprophyticus* SBPS-15 isolated from oil-contaminated coastal waters [25]. The surfactant strain SBPS-15, which contained mannose and oleic acid, was named staphylosan. At concentrations of staphylosan 200–400 μg/ml, complete destruction of biofilms of *P. aeruginosa* BHKH-19 and *Serratia liquefaciens* BHKH-23 was observed. Destruction of biofilms of *Acinetobacter beijerinckii* BHKH-11, *Micrococcus luteus* BHKH-39, *B. subtilis* BHKH-7 and *Marinobacter lipolyticus* BHKH-31 by 93, 91, 90 and 85%, respectively, was achieved at a surfactant concentration of 400 μg/ml.

In addition to antimicrobial activity, glycolipids of marine microorganisms have emulsifying properties, due to which they can be promising for the degradation of oil pollution and polycyclic aromatic hydrocarbons [27–32].

It was found that *Dietzia maris* As-13-3 strain isolated from deep-sea thermal waters had synthesized dirhamnolipids during cultivation on hydrocarbons, olive oil, glycerol, and glucose [27]. Under conditions of strain As-13-3 growth on tetradecane, hexadecane, and pier, the surface tension decreased to 33–35 mN/m. Rhamnolipids formed stable emulsions with toluene, hexane, cyclohexane, hexadecane, pier, and diesel, so the emulsification index was 54–64%. The authors of [27] noted that the advantage of *D. maris* As-13-3 as a producer of rhamnolipids for environmental bioremediation was the non-pathogenicity of the strain.

Halomonas sp. MV-30, isolated from a sea sponge, synthesized glycolipids under conditions of growth on both glucose and hydrocarbons (reduction of surface tension to 30 mN/m) [30]. The emulsification index of glycolipids using crude oil as a substrate was 93.1%, kerosene — 86.6%. The emulsions remained stable for a month and were formed at temperatures above 80 °C, pH ≥ 7.0, and NaCl concentrations up to 10%. In the presence of partially purified *Halomonas* sp. MV-30 surfactant, degree of oil washing from sand was 62%. The authors of [30] noted that the glycolipids of strain MV-30 were promising for increasing oil production and bioremediation of hydrocarbons in extreme conditions.

It has been reported in [29] about isolation from sea water of *Nocardiopsis* sp. VITSISB strain, which synthesized rhamnolipids on an oil-containing medium. On a model system that simulated the spillage of engine oil in an ocean, the researchers found the possibility to use the cells immobilized in Ca^{2+} -alginate balls of the VITSISB strain for bioremediation of this xenobiotic. However, engine oil destruction occurred in the presence of sugar cane juice and soybean meal in an aquatic environment as sources of carbon and nitrogen, respectively. Rhamnolipids of *Nocardiopsis* sp. VITSISB strain proved to be stable in the temperature range 5–100 °C, pH 2–12, and sodium chloride concentrations 3–10%. The authors of [29] considered rhamnolipid-producing strain VITSISB as promising for the elimination of oil spills in the ocean.

Summarized information concerning glycolipids synthesized by marine microorganisms is given in Table 1. These data indicate that marine microorganisms synthesize glycolipids on various substrates, though mainly on fairly high-value (carbohydrates, hexadecane, purified glycerol). Lack of transparency on the synthesis of the target product enables to assess the prospects to use the strains as potential producers of surfactants. However, the stability of surfactants and their emulsions in a wide range of temperature, pH, and salinity make them attractive for practical application in environmental technologies for bioremediation of the environment.

Lipopeptides of marine microorganisms

Lipopeptides consist of a lipid moiety coupled to a short linear or cyclic oligopeptide [33]. The data presented in [34–41] show

that most lipopeptide surfactants of marine microorganisms are considered promising for use in bioremediation and environmental problems. It is known that the synthesis of lipopeptides by traditional producers (representatives of the genera *Bacillus* and *Pseudomonas*) is carried out mainly using carbohydrate substrates [33]. Similarly, marine microorganisms can synthesize lipopeptides under conditions of growth on carbohydrates [38, 39, 42]. However, many of them can metabolize agro-industrial waste and waste from other industries [34, 36, 37].

Mani et al. [34] isolated from marine sediments a *Bacillus simplex* SBN19 strain, which synthesized surfactants of lipopeptide nature in various waste oils.

Maximum concentration of surfactants (908 mg/l) was achieved under conditions of the strain SBN19 growth in fried sunflower oil. It was found that in the presence of purified lipopeptide (100 mg/l) after 24 h, the degree of washing of oil from contaminated sand (5 ml of oil per 100 g of sand) in the range of salinity 0–30% was 80–85% (with maximum salinity — 84.7%).

It was found [35] that *Bacillus stratosphericus* FLU5, isolated from oil-contaminated seawater, synthesized surfactants on a wide range of carbon substrates (crude oil, diesel fuel, engine oil, spent engine oil, corn and olive oil, refried oil, glycerol). Surfactant synthesis rates were the highest (1.88–2.25 g/l) in a process of growing the strain on oil-containing substrates. By chemical nature, *B. stratosphericus* FLU5 lipopeptides are a complex of surfactin and pumilacidin. The lipopeptide complex showed stability in a wide range of pH (2–12), temperature (4–121 °C), and NaCl concentration (0–25%). In the presence of supernatant after culturing the FLU5 strain on fried oil, the remobilization of motor oil hydrocarbons (20%) from contaminated soil was several times higher compared to the use of synthetic surfactants (Tween 20, Tween 80, Triton X-100 and SDS).

Vilela et al. [36] isolated from marine invertebrates the *Brevibacterium luteolum* AC189a strain, which synthesized surfactants of lipopeptide nature under conditions of mineral oil growth. The emulsification index of surfactants with different hydrocarbons was 60–79%. The lipopeptide showed the ability to clean sand from oil. In the presence of 0.1% surfactant, the degree of washing of oil (10%) from contaminated sand after 6 h was 83%.

It was reported [37] that the strain *Nesterenkonia* sp. isolated from the *Fasciospongia cavernosa* MSA31 sea sponge, which synthesized a lipopeptide when grown in olive oil, was characterized not only by emulsifying but antioxidant and antimicrobial activity as well. Thus, the level of neutralization of 2,2-diphenyl-1-picrylhydrazyl radical (DFPG) at a surfactant concentration of 6 mg/ml was 65%. At a surfactant concentration of 125 μg/ml, the destruction of *Staphylococcus aureus* biofilm was noticeable. Also, the authors used a lipopeptide of strain MSA31 as an emulsifier in production of muffins with a protective effect against *S. aureus*. The addition of lipopeptide to the dough at a concentration of 0.5–1% improved the organoleptic characteristics of the finished product [37].

Cyclic lipopeptide surfactant pseudofactin II, synthesized by the arctic strain *Pseudomonas fluorescens* BD5 [42] can activate the apoptosis of melanoma A375 cells as a result of the effect of surfactant micelles on the permeability of the cell membrane, accompanied by the release of lactate dehydrogenase and Ca^{2+} [38]. This lipopeptide reduced the adhesion of pathogenic microorganisms *Escherichia coli*, *Enterococcus faecalis, Enterococcus hirae, Staphylococcus epidermidis, Proteus mirabilis* and *Candida albicans* on glass, polystyrene and silicone, as well as prevented the formation of biofilms on medical materials. Pre-treatment of polystyrene with a solution of pseudofactin II at a concentration of 0.5 mg/ml reduced the adhesion of bacterial test cultures by 36–90%, and *C. albicans* — by 92–99%. Thus, pseudofactin II could be used as an agent against microbial colonization of various surfaces, such as implants or urethral catheters [43].

The *Aneurinibacillus aneurinilyticus* SBP-11 strain [39] under the conditions of growth on glucose synthesized lipopeptide surfactant aneurinifactin with high antimicrobial activity against pathogenic bacteria. Thus, the minimum inhibitory concentrations of aneurinifactin were (μg/ml) as follows: *Klebsiella pneumoniae* — 4, *E. coli* — 8, *S. aureus* — 8, *P. aeruginosa* — 16, *B. subtilis* — 16, *Vibrio cholerae* — 16. Also, at a concentration of 200 mg/l aneurinifactin, the degree of washing of oil from contaminated sand (5 ml of oil per 100 g of sand) after 24 h was 81% [39].

In [41], the authors investigated the possibility of intensifying lipopeptide

Table 1. **Synthesis of glycolipids by marine microorganisms**

Producer	Source of selection	Cultivation temperature	Source of carbon, g/l	Physico-chemical properties		Prospects for practical use	Literature
				chemical composition	stability		
Vibrio sp. 3B-2	Marine bottom sediments	28 °C	Lactose, 5	–	–	Bioremediation, increase of oil production	[28]
Buttiauxella sp. M44	Coastal waters	33 °C	Molasses, 10	glucopyranose, octadecanoic acid	20–60 °C, pH 7–8, salinity to 3%	Antimicrobial activity	[22]
Dietzia maris As-13-3	Deep-water hydrothermal vents	28 °C	Hexadecane, 20	dirhamnolipid	–	Emulsifier, bioremediation, hydrocarbon degradation	[27]
Staphylococcus lentus SZ2	The surface of the sea snail	30 °C	Casein hydrolysate, 10	triose, hexadecanoic, octadecanoic acids	–	Destruction of biofilms, antimicrobial and antiadhesive activity	[24]
Serratia marcescens CFS	Sea coral *Symphyllia* sp.	30 °C	Peptone, 5	glucose, palmitic acid	–	Destruction of biofilms, antimicrobial and antiadhesive activity	[23]
Staphylococcus saprophyticus SBPS-15	Oil-contaminated coastal areas	37 °C	Glucose, 20	mannose, oleic acid	4–80 °C, pH 3–9	Destruction of biofilms, antimicrobial and antiadhesive activity	[25]
Nocardiopsis sp.VITSISB	Seawater	37 °C	Oil, 0,5% (volume fraction)	rhamnolipid	5–100 °C, pH 2–12, salinity to 3–10%	Bioremediation, decomposition of oil and engine oil	[29]
Halomonas sp. MB-30	Sea sponge	30 °C	Oil, 2% (volume fraction)	–	5–100 °C, pH 2–12, salinity to 3–10%	Bioremediation, increase oil production	[30]
Streptomyces sp. MAB36	Marine bottom sediments	–	Starch, 15,8 Oil, 16 ml/l	glycolipid	30–50 °C, pH 5–9, salinity 1,5%	Bioremediation, antimicrobial activity	[26]
Rhodococcus sp. PML026	Seawater	30 °C	Sunflower oil, 2 % (volume fraction)	trehalose lipid	20–100 °C, pH 2–10, salinity to 25%	Bioremediation	[32]
Aureobasidium pullulans YTP6-14	Seawater	30 °C	Glucose, 50 Glycerol, 2,5%	Lactone 5-hydroxy-2-decanoic acid	4–100 °C, pH 2–12 salinity to 12%	Emulsifier	[31]

Note: «–» — no data.

synthesis by a strain of *Bacillus licheniformis* NIOTAMKV06 isolated from a sea sponge. Under conditions of growth of strain NIOTAMKV06 on glucose, the surfactant concentration was 1.8 g/l. After optimizing the composition of the nutrient medium it increased to 3 g/l. The use of a mixture of 20 g/l of glucose and 2.5% of oil as a carbon source made it possible to increase the concentration of surfactants to 6 g/l. The authors obtained a recombinant strain of *E. coli*, which synthesized 11.78 g/l of lipopeptide [41]. Surfactant *B. licheniformis* NIOTAMKV06 emulsified crude oil, kerosene, and diesel. This work was the first to report a highly active marine lipopeptide-producing strain.

Deng et al. [40] isolated from oil-contaminated seawater a strain of *Achromobacter* sp. HZ01, which on a medium with glycerol synthesized a new cyclic lipopeptide at a concentration of 6 g/l. This surfactant formed emulsions with coconut, peanut, sesame, soybean, sunflower, olive, corn oil, kerosene, and diesel fuel, and the emulsions were characterized by high stability in the temperature range of 40–100 °C, pH 6–12, and salinity of 0–3%.

It was found [44] that the *Marinobacter* sp. M22.20 strain isolated from marine sediments under conditions of growth in the medium with 2% (volume fraction) of soybean oil synthesized phospholipopeptides (concentration was not specified) with high emulsifying activity. The emulsions were stable when stored for 30 months at a NaCl concentration of 300 g/l, temperature of 4 °C and after heat treatment 120 °C, 20 min).

Summarized data on lipopeptides synthesized by marine microorganisms are given in Table 2. Lipopeptides are characterized by the same physicochemical and biological properties as glycolipids (Table 1). However, a significant advantage of lipopeptides is the possibility to obtain them on cheap and available industrial waste in large quantities.

During 2018–2019, several papers were published [45–49] on the synthesis of surfactant glyco- and lipopeptides by marine microorganisms, in which the authors studied in detail the chemical composition and/or biological (mainly antimicrobial) activity of surfactants, but did not provide conditions of cultivation and synthesis of surfactants. The review [50] provided information on the chemical composition and prospects of practical use of surfactants of marine microorganisms in medicine, perfumery, cosmetics, food industry, bioremediation, and noted that marine microorganisms were not currently promising biological agents for biotechnological processes, as they needed improvement methods of genetic and/or metabolic engineering.

Enzymes

Enzymes synthesized by marine microorganisms are characterized by high activity in a wide range of pH, temperature, light, pressure, salt concentrations, and are resistant to organic solvents, metal ions, detergents [16].

Recently, the number of publications on the synthesis of β-galactosidases by microorganisms, which show high activity at low temperatures (so-called cold-active β-galactosidases), has increased in the literature [51–57]. This is due to the following reasons. Firstly, it is the possibility to use these enzymes in the production of lactose-free dairy products, as well as to create biosensors that control the lactose content in their production. It is known that biosensors based on immobilized β-galactosidase can be used to analyze lactose in milk [51]. Interest in the production of lactose-free dairy products (milk, including condensed milk, ice cream) is because today a third of the world's population suffers from lactose intolerance [52, 53]. Secondly, the hydrolysis of lactose by cold-active β-galactosidase reduces the hygroscopicity of milk, thereby preventing its crystallization in ice cream and condensed milk. Also, the use of such enzymes at low temperatures minimizes the risk of microbial contamination of finished products [53]. Thirdly, the use of β-galactosidase for the destruction of lactose in serum can reduce wastewater contamination of dairy plants [52]. Most currently used β-galactosidases in the food industry (Maxilact, Lactozym, and neutral yeast lactase (DYL)) are mesophilic and have an optimum temperature of approximately 35 to 50 °C and are characterized by low activity at 20 °C.

Also, cold-active β-galactosidases can be used to produce water-soluble oligosaccharides that are less sweet than mono- and disaccharides. The interest in oligosaccharides is because they are prebiotics that activate the development of bifidobacteria in the intestine [56].

The largest number of producers of cold-active β-galactosidases is found among marine microorganisms. For example, the marine arctic bacteria *Enterobacter ludwigii* KS92 synthesizes

Table 2. **Synthesis of lipopeptides by marine microorganisms**

Producer	Source of selection	Cultivation temperature	Carbon source, g/l	Physico-chemical properties	Prospects for use	Literature
Bacillus stratosphericus FLU5	Seawater contaminated with oil	37 °C	Fried oil (1%, volume fraction)	4–121 °C, pH 2–12, salinity (0–25%)	Bioremediation, destruction of motor oil	[35]
Aneurinibacillus aneurinilyticus SBP-11	Marine bottom sediments	37 °C	Glucose, 15	4–80 °C, pH 2–9	Antimicrobial activity, increase oil production	[39]
Bacillus simplex SBN19	Marine bottom sediments	37 °C	Spent sunflower oil (2%, volume fraction)	salinity to 30%	Bioremediation, increase oil production	[34]
Pseudomonas fluorescens BD5	Arctic strain	37 °C	Glucose, 20	–	Destruction of biofilms, antimicrobial, antiadhesive and antitumor activity	[38, 42, 43]
Brevibacterium luteolum AC189a	Marine invertebrates	30 °C	Marine invertebrates (2%, volume fraction)	4–100 °C, pH 2–12, salinity (0–12%)	Bioremediation, increase oil production	[36]
Bacillus licheniformis NIOT-AMKV06	Sea sponge	38 °C	Glucose, 20 Oil 2,5%	20–70 °C, pH 5–10	Emulsifier. Bioremediation, increase oil production	[41]
Achromobacter sp. HZ01	Seawater contaminated with oil	28 °C	Glycerol, 40	40–100 °C, pH 6–12, salinity (0–3%)	Bioremediation. Emulsifier	[40]
Nesterenkoia sp. MSA31	Sea sponge *Fasciospongia cavernosa*	28 °C	Olive oil, 10	4–121 °C, pH 6–9 salinity (0–10%)	Antioxidant and antimicrobial activity. Emulsifier for the food industry	[37]

Note: «–» — no data.

β-galactosidase [52], which is characterized by high activity at a temperature of 15–25 °C and a pH of 5–10. At the same time, enzymes formed by deep-sea marine bacteria *Thalassospira* sp. 3SC-21 and *Pseudoalteromonas* sp. KNOUC808 showed maximum activity in the range of lower temperatures (from 4 to 20 °C), but at different pH values [53, 54]. The optimum pH of β-Galactosidase strain 3SC-21 was 6.5, and strain KNOUC808 — 7–8. β-galactosidase synthesized by another deep-sea bacterium *Alteromonas* sp. ML52 [55], was also characterized by high activity at a temperature of 4–25 °C. Thus, marine microorganisms existing in the polar and other regions, where the average annual temperature does not exceed 5 °C, are a source of virtually valuable cold-active β-galactosidases.

Commercial α-amylases are inactivated at low pH and high temperatures, but these conditions must be maintained at the stage of starch saccharification during molasses production. In turn, α-amylase synthesized by the marine bacterium *Geobacillus* sp. 4j

was active in the pH range of 4.5–7.0 and at a temperature of 55–90 °C [58]. In this work, the authors investigated not only the properties of the enzyme but also optimized the conditions for culturing strain 4j in flasks and a bioreactor. This made it possible to increase significantly its activity. Thus, in the process of *Geobacillus* sp. 4j growing in flasks on an optimized medium with starch as a carbon source, the amylase activity was 6.4 U/ml, which is 5 times higher than before optimization. Cultivation of the producer in a 15 l bioreactor at a temperature of 60 °C, an initial pH value of 6.0 and maintaining the dissolved oxygen concentration at 20% of air saturation allowed to increase the activity of the enzyme to 79 U/ml [58].

Currently, proteases account for 60% of the world market for enzymes, and what is more, proteases that are highly active in a wide range of pH and temperature, in the presence of metal ions and organic solvents are in great demand [59]. These requirements are met by an alkaline protease synthesized by the *Staphylococcus saprophyticus* BUU1 strain, which is isolated from marine sediments. Protease was characterized by stability at temperatures from 10 to 80 °C, pH from 3 to 12, in the presence of dodecyl sulfate, hydrogen peroxide, bleaching agents (zeolite), hydrophobic solvents (benzene, hexane, hexadecane) [59].

At present, alginatliases, especially endolytic ones, are widely used in the production of alginate oligosaccharides and the production of protoplasts from red and brown algae [60–63]. Alginate oligosaccharides are attracting more and more attention due to their wide application in the food and pharmaceutical industries, as they are characterized by antioxidant, antiproliferative, antitumor activity. Also, they are anticoagulants, stimulate the production of cytokines, exhibit anti-allergic properties. Moreover, alginatliase is promising for use in the treatment of cystic fibrosis due to the decomposition of the polysaccharide biofilm of the pathogen [60–62]. However, most known alginatliases are unstable at elevated temperatures.

Zhu et al. [61] obtained a genetically engineered strain of *Escherichia coli*, which transferred the genes of thermostable alginate lyase from the marine strain of *Flammeovirga* sp. NJ-04. Purified recombinant alginate lyase FsAlgA showed the highest activity (3 343.7 U/mg) at 50 °C and pH 7.0. In addition, this enzyme was characterized by broad substrate specificity and degraded not only sodium alginate but also polymanuronates and polyguluronates.

Alginate lyase AlgC-PL7, synthesized by the halophilic strain *Cobetia* sp. NAP1 appered to be heat-resisting [60]. At 90 °C, the enzyme activity was decreased after 15 min by only 20%, as well as in the presence of 2.0 M NaCl and KCl, the enzyme retained up to 80% of activity.

Not only polymanuronates and polyguluronates were characterized by hydrolytic properties but alginate lyases synthesized by marine bacteria *Vibrio* sp. NJU-03 [62] and *Vibrio furnissii* H1 [63] were as well. The enzyme of strain NJU-03 showed maximum activity at 30 °C and pH 7.0, and strain H1 — 40 °C and pH 7.5.

The ability of the fungus strain *Dendryphiella arenaria* Nicot isolated from red algae to synthesize alginate lyase and fucoidanase was found in a process of growing on a medium with fucoidan and alginate extracted from brown macroalgae *Sargassum latifolium* [64]. Using mathematical methods of experiment planning, the authors determined the optimal composition of nutrient medium for the synthesis of fucoidanase (fucoidan content was 1.5%, NaCl — 1.5%, urea — 0.3%) and alginate lyase (alginate content was 1.5%, NaCl — 4%, NH_4Cl — 0.3%), providing the maximum activity of enzymes in the culture fluid (4 and 24 U/ml, respectively). Subsequent experiments showed that the degree of release of free carbohydrates from polysaccharides under the action of fucoidanase and alginate lyase was 365 mg/g of fucoidan and 439 mg/g of alginate, respectively [64]. The authors noted that these enzymes were promising for use in the production of biofuels from algae.

As a result of agar hydrolysis by agarase, monomers (*D*-galactose, 3,6-anhydro-L-galactose, and L-galactose-6-sulfate) were formed, which could be used for biofuel production [65–67]. Promising is the use of agarase in the production of biologically active agar-oligosaccharides, which are characterized by prebiotic potential, exhibited antitumor, anti-inflammatory, and antioxidant activity, as well as moisturizing and whitening effect in cosmetics. Also, agarase can be used to produce red algae protoplasts, process DNA and RNA from agarose gels, and extract biological substances (carotenoids, vitamins, and fatty acids) [65–67]. Most often agarase is active in the temperature range from 30 to 40 °C. Few enzymes are characterized by high activity at weakly alkaline pH. The high activity of these

enzymes at temperatures above 40 °C is an advantage for the industrial production of agar-oligosaccharides because such temperatures exceed the gelation temperature of agar.

It has been informed of the isolation from marine sediments of the *Acinetobacter junii* PS12B strain, which in the process of cultivation on medium with 0.5% agar at 35 °C and pH 7.0 synthesized agarase with an activity of 0.17 U/ml of culture fluid [65]. In the presence of simple carbohydrates (glucose) in the medium with agar, the enzyme activity was doubled. Later [66], the same authors investigated the physicochemical properties of the purified enzyme and found that agarase synthesized by strain PS12B was thermostable and was characterized by high activity at 50 °C, and maximum activity was observed at 40 °C and pH 8.0.

Not only marine bacteria but also fungi can synthesize agarase. So, the strain *Dendryphiella arenaria* Nicot. under conditions of semi-solid cultivation on a medium containing as a substrate 7.5% biomass of red algae *Palisada perforata*, 0.25% sucrose and 0.08% glucose synthesized agarase with an activity of 7.69 U/ml of culture fluid [67]. Agarase was characterized by high activity in the temperature range from 40 to 80 °C. When heated to 40–50 °C after 30 min, the activity decreased by 25%, and after incubation at 80 °C for 60 min — by 60%. The authors of [67] believed that agarase *D. arenaria* Nicot. could be used with high efficiency to produce biofuels from seaweed.

Enzyme of a wide range of applications is xylanase. In baking, it is used to break down xylan flour with a predominant effect on arabinoxylans, characteristic of endosperm and aleurone layer of the grain, in the production of paper for bleaching cellulose, in animal husbandry to improve the digestibility of animal feed [68, 69].

As a result of screening 493 strains of sea fungi, Dos Santos et al. [68] selected a strain identified as *Aspergillus* cf. *tubingensis* LAMAI31, which was the most active producer of xylanase. Under conditions of growth on medium with xylan xylanase activity was 49.41 U/ml. In the next step, the authors optimized the composition of the nutrient medium, which allowed to increase the xylanase activity to 561.59 U/ml. Xylanase of the strain LAMAI31 was stable in the temperature range of 40–50 °C and pH from 3.6 to 7.0 with an optimum of 55 °C and 5.0, respectively.

The review [69] summarizes the literature data on the potential of sea fungi as an alternative to terrestrial ones for obtaining extracellular enzymes by bioconversion of plant and macroalgae polymer substrates. The authors noted that these biotechnologies might be integrated, because the decomposition of such substrates could produce not only hydrolytic enzymes but plant protein-enriched fungal biomass as well, which in turn was a source of virtually valuable biologically active substances.

Generalized information about enzymes synthesized by marine microorganisms is given in Table 3. Microorganisms isolated from marine habitats capable of synthesizing enzymes that are stable over a wide range of pH and temperature and maintaining high specific activity in contrast to the enzymes synthesized by their traditional terrestrial producers.

Exopolysaccharides

In marine habitats, most microbial cells are surrounded by a layer of extracellular carbohydrate polymers, which are usually exopolysaccharides (EPS). They help microorganisms to survive in adverse conditions by affecting the physicochemical environment near the microbial cell. Some extreme marine environments, such as deep-sea hydrothermal vents, shallow underwater thermal springs, and polar marine habitats, are considered as new sources of EPS-producing bacteria [11, 72].

In 2018, we have published a review [73] on non-traditional producers of microbial EPS, including marine microorganisms (thermo-, psychro-, halophiles and bacteria isolated from deep-water hydrothermal vents). Hereunder we summarized information that appeared or was not published after the publication of the review [73]. In 2018, a review [11] was published on the EPS of marine microorganisms. However, it summarized mostly outdated literature (from the 90s of the twentieth century to 2010–2012), which related mainly to the study of their chemical composition, structure and environmental role, whereas practically valuable properties were considered very briefly.

In our review, we tried to focus on new non-traditional areas of application of EPS of marine microorganisms. This is due to the following reasons. Most currently the known microbial EPSs are characterized by similar functional properties that determine their practical significance. Therefore, it is not surprising that of a large number of isolated, described and studied polysaccharides of

Table 3. **Synthesis of enzymes by marine microorganisms**

Producer	Source of selection	Enzyme	Activity	Properties	Prospects for use	Literature
Enterobacter ludwigii KS92	Siege of the Arctic Fjord	Galactosidase	–	15–35 °C, pH 5–10	Manufacture of lactose-free products at low temperatures	[52]
Geobacillus sp. 4j	Depths of the sea	Amylase	79 U/ml	55–90 °C, pH 4,5–7	Hydrolysis of starch for molasses production	[58]
Flammeovirga sp. NJ-04	Depths of the sea	Alginatliase FsAlgA	3 343,7 U/ml	Optimum 50 °C, pH 7	Treatment of cystic fibrosis. Obtaining alginate oligosaccharides with low molecular weight. Obtaining protoplasts of algae	[61]
Cobetia sp. NAP1	Brown algae	Alginatliase AlgC-PL7	–	Optimum 45 °C, pH 7–8	Obtaining alginate monosaccharides. Biofuel production	[60]
Dendryphiella arenaria Nicot.	Red algae	Alginatliase	24 U/ml	Optimum 40 °C, saccharification 439 mg/g fucoidan	Biofuel production from brown algae	[64]
		Fucoidanase	4 U/ml	Optimum 40 °C, saccharification 365 g/g Fucoidanase	Biofuel production from brown algae	[64]
		Agarase	7,69 U/ml	40–80 °C	Biofuel production from red algae	[67]
Acinetobacter sp. PS12B	Sea sediments	Agarase	45,76 U/ml	Optimum 40 °C, pH 8	Obtaining of agar oligosaccharides, algal hydrolyzate, red algae protoplasts. Biofuel production	[66]
Aspergillus cf. *Tubingensis* LAMAI 31	Sea sponge	Xylanase	561,59 U/ml	30–70 °C, pH 3–7	Pharmaceutical — drugs to improve digestion. Food Industry. Paper and textile industry	[68]
Nigrospora sp. CBMAI 1328	Sea sponge	Laccase	25,2 U/ml	–	Bioremediation, organic catalysis and degradation of xenobiotics. Lignin cleavage. Biosensor systems. Antimicrobial Activity	[70]
Aeromonas caviae CH129	Zooplankton	Chitinase	–	Optimum 37 °C, pH 5	Formation of N-acetylglucosamine, antifungal activity	[71]

Note: «–» — no data.

microbial origin, only some of them (xanthan, gelan, alginate, dextran) are obtained on an industrial scale. Today, in order to enter the market, new polysaccharides must have certain unique properties, thanks to which they can be used in the "free" spheres of rapidly developing industries such as medical, pharmaceutical, cosmetic, environmental.

Most exopolysaccharides synthesized by marine microorganisms are promising for use in medicine and pharmaceuticals [72, 74–78, 80–86], as they show a wide range of biological activity such as follows antitumor [74, 75], antioxidant [72, 75, 78–80], anti-inflammatory [74, 75], anti-adhesive [81, 83], immunomodulatory and antiviral [84, 85], and cryoprotective [76, 77, 82]. Both psychrophilic, mesophilic, and thermophilic strains have been identified among EPS producers.

Most psychrophilic strains were isolated from the polar marine regions of the Arctic and Antarctica. Exopolysaccharides synthesized by microorganisms from polar media are often characterized by unique physicochemical properties and functions. Usually, cold habitats are characterized by frequent temperature changes (freeze-thaw cycles, etc.) [73]. Under these conditions, EPS can act as a cryoprotectant. This property of psychrophiles' EPS makes it possible to consider them as alternative cryoprotectants for long-term storage of suspension cultures [82].

It was found [76] that EPS, synthesized by the psychrophilic γ-proteobacteria *Colwellia psychrerythraea* 34H isolated from Antarctic ice, has a unique structure that mimics antifreeze and prevents recrystallization of ice. Moreover, when cells are frozen to −80 °C, this EPS is a better cryoprotectant than a 10% solution of glycerol. In turn, in [82] it was found that in the presence of 10% of EPS *Pseudomonas* sp. ID1 survival of *Escherichia coli* ATCC 10536 cells after freezing and keeping for 7 days at a temperature of −20 and −80 °C was 36 and 64%, respectively.

The authors [77] showed that the addition of polysaccharide of psychrotolerant Arctic bacteria *Flavobacterium* sp. ASB 3-3 at a concentration of 50 mg/ml increased the number of living cells of the strain *Flavobacterium* sp. ASB 3-3 and *E. coli* DH5α after 2 freeze-thaw cycles 4 times compared to those without EPS. Polysaccharides of psychrophilic and psychrotolerant bacteria had likewise the ability to retain moisture [72], emulsification [77, 82], flocculation [77], and adsorption of metals [79].

During the EPS study of the bacterial strain *Zunongwangia profunda* SM-A87 [72] it was found that after 72 h of dehydration in a chamber with silica gel (relative humidity 43%) the moisture-holding capacity of the polysaccharide strain SM-A87 reached 76%, which was higher than using hyaluronic acid, sodium alginate. According to the authors, such results were due not only to the presence in the EPS of a large amount of glucuronic acid and N-acetylglucosamine, but as was also fucose, which had moisturizing properties. This polysaccharide had antioxidant activity as well [72].

To reduce the cost of the target product the authors of [72] optimized the composition of the nutrient medium (whey — 60.9%, soy flour — 10 g/l and NaCl — 2.9%), and the implementation of the feed process allowed to increase the concentration of EPS of strain Z. *profunda* SM-A87 up to 17 g/l, which was 1.93 times higher than the original technology.

EPS of the psychrotolerant strain *Flavobacterium* sp. ASB 3-3, in addition to cryoprotective, has flocculating and emulsifying properties [77]. Thus, the flocculation activity in a suspension of kaolinite (0.5%) at an EPS concentration of 40 mg/l reached 91.3%, respectively. EPS of *Flavobacterium* sp. ASB 3-3 also emulsified n-hexane (emulsification index 66.3%) and *n*-hexadecane (64.3%) with the same efficiency as sodium dodecyl sulfate.

As for psychrophilic bacteria, the synthesis of exopolysaccharides by thermophilic marine microorganisms is an adaptation mechanism that ensures their survival in adverse environmental conditions.

A representative of thermophilic marine bacteria is the strain *Bacillus licheniformis* T14, isolated from the hydrothermal vein of the island of Panarea (Italy) [81]. This polysaccharide has a wide range of biological activity. Thus, treatment of human peripheral blood mononuclear cells with a solution of polysaccharide (300 μg/ml) led to stimulation of the production of type I cytokines and, as a consequence, inhibition of 77% replication of herpes simplex virus type II [85]. Scientists have also found that this EPS has anticytostatic activity. Thus, fraction 1 EPS of *B. licheniformis* T14, consisting of fructose, fucose, and glucose (1: 0.75: 0.28), at a concentration of 500 ppm increased the LD50 of avarol (cytostatic) from 0.18 to 0.99 mg/ml [84].

It was found that polysaccharides synthesized by *Bacillus licheniformis* T14 [81] and *Pseudomonas stutzeri* 273 [83]

had a unique ability to destroy biofilms of multidrug-resistant strains of pathogenic bacteria *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococus aureus*. Notably that such properties had not been previously detected in exopolysaccharides formed by traditional producers.

Due to the high content of sulfated and uronic groups, polysaccharides synthesized by mesophilic members of the genus *Bacillus* showed antitumor and anti-inflammatory activity [74, 75].

Thus, polysaccharides synthesized by mesophilic bacteria *Bacillus velezensis* MHM3 [74] at a concentration of 5–80 μg/ml reduced the proliferation of transplanted breast cancer cells (MCF-7) by increasing the expression of genes encoding proapoptotic proteins and reducing the activity of the *Bcl-2* gene, which inhibited the development of apoptosis. The authors suggested that EPS MHM3 might also activate apoptosis by increasing the permeability of the mitochondrial membrane and stimulate the release of cytochrome *c* from mitochondria to the cytosol. The advantage of the strain *B. velezensis* MHM3 was that it synthesized about 6 g/l of polysaccharide, which was many times more than other marine mesophiles.

Selective inhibition of cyclooxygenase (COX) plays a key role in the treatment of inflammatory diseases [75]. Therefore, the development of potent COX inhibitors is an urgent problem. Cyclooxygenases are also involved in lipid metabolism. In particular, they catalyze the oxygenation of polyunsaturated fatty acids, especially arachidonic with the formation of prostaglandin, which is a regulator of inflammatory processes. Prostaglandin can stimulate the growth of tumor cells and suppress the immune response. Also, COX activates carcinogens. In [75] it was found that the exopolysaccharide synthesized by *Bacillus amyloliquefaciens* 3MS inhibited the cyclooxygenase COX-1 and COX-2.

Inhibition of NO formation with selective inhibitory properties of COX-2 activity is considered a promising approach to the treatment of various diseases associated with inflammation, including cancer. EPS of strain 3MS showed antitumor activity against Ehrlich carcinoma [75].

Polysaccharides synthesized by mesophilic members of the *Bacillus* genus had antioxidant activity as well [74, 75]. Thus, the level of neutralization of 2,2-diphenyl-1-picrylhydrazyl radical (DFPG·), hydroxyl radical (·OH) and superoxide anion ($O_2^{-\cdot}$) at the concentration of EPS strains MHM3 and 3MS 500 and 1 000 μg/ml was 52.1–84.4 and 91.44–99.39%, respectively.

Antioxidant activity is also shown by EPS strains of *Halolactibacillus miurensis* T7 (SEEN MKU3) [80].

Also, the EPS of mesophilic bacteria has emulsifying and flocculating properties. Wang et al. [78] isolated a bacterial strain *Aerococcus uriaeequi* HZ, which synthesized EPS with high flocculation activity. With the introduction of 0.2 g of EPS in 100 ml of wastewater, the flocculation index reached 79.90%. The level of neutralization of hydroxyl radical (· OH) and superoxide anion ($O_2^{-\cdot}$) at concentrations of this EPS of 100 and 250 μg/ml, was 45.65 and 67.31%, respectively.

In [79] it was shown that the EPS of the mesophilic bacterium *Alteromonas* sp. JL2810 due to its ability to bind metals, could be used in the processes of biosorption of heavy metals Cu^{2+}, Ni^{2+} and Cr^{6+} in the treatment of mining and industrial waste.

Summarized information about the exopolysaccharides of marine microorganisms is given in table. 4. It is worthwhile to note that, despite the synthesis by marine microorganisms of exopolysaccharides with unique properties, as substrates for their production, carbohydrate raw materials are used. In such case the concentration of the target product is not high enough. Also, such producers require complex mediums with high salt content.

Metabolites with antimicrobial activity

Every year the number of publications on the synthesis of antimicrobial compounds by marine microorganisms increases [87–104]. The largest number of such studies are dated to 2014–2015 [88]. Producers of metabolites with antimicrobial activity are sea fungi of the genus *Beauveria* [89], *Aspergillus* [90, 97, 99], *Penicillium* [91], *Stachybotrys* [93], *Trichoderma* [94], *Engyodontium* [96] and actinobacteria of the genus *Streptomyces* [101–103]. These microorganisms synthesize antimicrobial compounds of different chemical nature, in particular xanthones polyketides [96, 97], terpenoid derivatives [91], butyrolactone [99], anglicycline antibiotics [102, 103] and others.

In most studies, the researchers used the minimum inhibitory concentration as a criterion for antimicrobial activity of metabolites, but in some works they applied

Table 4. **Synthesis of exopolysaccharides by marine microorganisms**

Producer	Source of selection	Cultivation temperature, °C	Source of carbon, g/l	Concentration of EPS, g/l	Physico-chemical properties of EPS		Physiological role, functional properties and prospects of use EPS	Literature
					chemical composition	molecular weight, kDa		
Zunongwangia profunda SM-A87	Seawater	9.8	Serum (60.9%, volume fraction)	17.2	Glucose, mannose, galactose, xylose, fucose, glucuronic acid, non-identifiable, carbohydrate (1:0.84:0.29:0.29: 0.05:0.06:0.21)	3 760	Moisture retaining agent, antioxidant	[72]
Colwellia psychrerythraea 34H	Glacier	4	Peptone, 5	–	N-acetylquinozoamine unit and two galacturonic acid residues are combined with alanine	–	Cryoprotectant	[76]
Pseudomonas sp. ID1	Marine sediments of Antarctica	11	Glucose 20	–	Glucose, galactose, fucose (1:0.5:0.48). Available uronic acids	2 000	Cryoprotectant, emulsifier	[82]
Flavobacterium sp. ASB 3-3	Glacier	20–25	Glycerol, 30	7.25	Glucose, galactose (1:0.43)	–	Emulsifier, flocculant, cryoprotectant	[77]
Aerococcus uriaeequi HZ	Seawater	25	Sucrose, 30	2.34	D-mannose (10.71%) D-Glucose (66.99%)	2.84×10^5	Flocculant, moisture-retaining agent, antioxidant	[78]
Pseudomonas stutzeri 273	Marine bottom sediments	28	Peptone, 10	–	Glucosamine (35.4%), rhamnose (28.6%), Glucose (27.2%), mannose	190	Inhibits the formation of biofilms, antioxidant	[83]
Bacillus velezensis MHM3	Sediments of the coastal zone	25	Sucrose, 50	5.8	Uronic acids and sulfate, glucuronic acid, Glucose, fructose and rhamnose with a molar ratio of 4.00:2.00:1.00:0.13	1 145	Antitumor, anti-inflammatory.	[74]
Bacillus amyloliquefaciens 3MS	Sediments of the coastal zone	28	Glucose, 20	–	Uronic acids (12.3%) and sulfate (22.8%), Glucose, galactose and glucuronic acid in molar ratio 1.6:1.0:0.9	–	Antioxidant, anti-inflammatory and antitumor activity	[75]
Bacillus licheniformis T14	Hydrothermal vent	50	Sucrose, 50	0.366	Fructose, fucose, Glucose (1:0.75:0.28) and traces of galactosamine, mannose	1 000	Antiviral, immunomodulatory and anticytotoxic activity. Inhibits the formation of biofilms	[81, 84, 85]
Halolactibacillus miurensis T7	Place of extraction of sea salt, the coast of India	32	Glucose, 20	2.5	Galactose (61.87%), Glucose (25.17%), xylose, fructose, mannose, rhamnose	–	Antioxidant activity	[80]
Labrenzia sp. PRIM-30	Seawater	32	Dextrose, 10	0.84	Glucose, arabinose, galacturonic acid, mannose (14.4:1.2:1:0.6) Sulfated groups are available 4.76%	269	Antioxidant, emulsifier	[86]
Alteromonas sp. JL2810	Seawater	28	Glucose, 10	0.77	Glucose, mannose, rhamnose	–	Biosorption of metals	[79]

Note: «–» — no data.

the IC_{50} (concentration of the substance that caused the death of 50% of test culture cells) [93, 94].

The authors [89] found that the compound with the trivial name Flavipezine A (aromatic butyrolactone synthesized by *Aspergillus flavipes* AIL8) showed in addition to antibacterial, both antiadhesive activity and the ability to destroy the biofilm of *S. aureus*.

In [102] it was shown that the synthesis of a new anglicycline antibiotic Stremycin A with *Streptomyces pratensis* NA-ZhouS1 strain was observed in response to the so-called "metallic" stress (the presence of heavy metal salts $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $CrCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 6H_2O$). The authors found that the action of $NiCl_2 \cdot 6H_2O$ at a concentration of 100 μm activated latent genes clusters responsible for the synthesis of this antibiotic.

The summarized data on the synthesis of secondary metabolites with antimicrobial activity by marine microorganisms is shown in Table 5. As a rule, microorganisms synthesize a complex of such compounds. However, the component of the complex that exhibits the highest antimicrobial activity is shown in Table 5. These data indicates that the vast majority of metabolites are characterized by high antimicrobial activity against a wide range of test cultures, namely minimum inhibitory concentrations range from 0.25 to 16 μg/ml. Slightly lower antimicrobial activity was found for compounds synthesized by *Streptomyces* sp. G278 [103, 104] and *Engyodontium album* DFFSCS021 [97].

To obtain most of the antimicrobial metabolites, the cultivation of marine microorganisms was carried out deeply, and in [89, 96, 97, 99] solid-phase cultivation was used. The use of carbohydrates as a source of carbon nutrition for the production of antimicrobial compounds at present may be a deterrent to the organization of their industrial production.

Metabolites with antitumor activity

The number of identified anti-cancer compounds synthesized by marine microorganisms is increasing every year. Their cytotoxicity has been proven using different tumor cell lines. Thus, in [90, 93, 96, 104–118] it was found that secondary metabolites synthesized by marine microorganisms showed antitumor activity. Stachybocin B, Engidontiumon H and (2R, 4bR, 6aS, 12bS, 12cS, 14a)-4b-deoxy-β-aflatrem were characterized by antimicrobial activity (see Table 5) as well.

Most producers of antitumor compounds are mycelial fungi of the genera *Aspergillus* [90,104–106], *Engyodontium* [96], *Stachybotrys* [93], *Sarcopodium* [108], *Penicillium* [91, 109, 110], *Lasiodiplodia* [111], *Campylocarpon* [112], *Eutypell* [113], *Acaromyces* [116] and others. By the way, some of them are endophytic fungi.

Identified compounds of marine microorganisms have different mechanisms of action on tumor cells *in vitro*. Thus, indole diterpenoid synthesized by *Aspergillus flavus* OUCMDZ-2205 exhibits cytotoxic activity in a model of pulmonary epithelial carcinoma (A549), stopping the cell cycle in phase S [90].

The tetranorditerpenoids wentilactone A and B produced by *Aspergillus wentii* EN-48 were found to induce mitochondrial apoptosis of cancer cells in a model of lung cancer and hepatoma *in vitro*. These compounds activated the Ras/Raf/ERK pathway, which initiated apoptosis and G2/M phase delay during tumor cell proliferation [106, 107]. Xu et al. [105] found that *Aspergillus dimorphicus* SD317 can produce wentilactone A and B. The authors optimized the cultivation conditions of strain SD317. As a result, the concentration of wentilactone A and B in the culture fluid increased by 11 times and was 13.4 and 6.5 mg/l, respectively. The optimal conditions for the synthesis of these compounds were as follows: pH 7.3, salinity 24.5‰, duration of cultivation 27 days at a temperature of 23 °C. Moreover, it was found that introduction of 3% methanol in the nutrient medium could also stimulate the synthesis of wentilactone [105].

Stemphylium globuliferum cells (strain number was not shown) produce altersolanol Q and 10-methyl altersolanol Q [114], *Phomopsis* sp. PM0409092 — altersolanol A [115], which exhibited antitumor activity *in vitro* in a mouse lymphoma model. Their cytotoxic activity was established using 34 human cancer cell lines. The mean IC_{50} and IC_{70} values were 0.005 and 0.024 μg/ml, respectively. Altersolanol A is a kinase inhibitor that induces cell death by apoptosis via a caspase-dependent pathway. The antitumor activity of these compounds is associated with pro-apoptotic and anti-invasive activity, which is manifested in the inhibition of the transcriptional activity of NF-kB [114, 115].

In [109] it was shown that the fungi *Penicillium brocae* MA-231 synthesize brocazines A, B, E and F, which show cytotoxic activity to various cancer cell lines. Brocazines

Table 5. **Synthesis of antimicrobial metabolites by marine microorganisms**

Producer	Source of selection	Source of carbon. g/l	Compound. Type	Test culture	MIC. μg/ml	IC_{50}, μM	Literature
Engyodontium album DFFSCS021	Marine bottom sediments	Rice*. Glucose	Engidontium H Polyketide xanthone	*Escherichia coli* *Bacillus subtilis*	64.0 32.0	–	[96]
Aspergillus versicolor MF359	Sea sponge *Hymeniacidon perleve*	Rice *	5-Methoxides-hydrosterigmatocystine Polyketide xanthone	*Staphylococcus aureus* *Bacillus subtilis*	12.5 3.125	–	[97]
Aspergillus flavipes AIL8	Mangrove plant *Acanthus ilicifolius*	Rice *	Flavi pezine A butyrolactone	*Staphylococcus aureus* *Bacillus subtilis*	8.0 0.25	–	[99]
Aspergillus flavus OUC-MDZ-2205	Shrimp *Penaeus vannamei*	Glucose 10 maltose 20 mannitol 20	(2R.4bR.6aS.12bS. 12cS.14a)-4b-deoxy-β-aflatrem indole diterpenoid	*Staphylococcus aureus*	20.5 мкМ	–	[90]
Stachybotrys sp. MF347	Seawater	Glucose 10	Stachybocin B Bicyclic sesquiterpene	*Staphylococcus epidermidis* *Bacillus subtilis* *Staphylococcus aureus* MRSA	–	1.02 1.42 1.75	[93]
Trichoderma sp. MF106	Seawater	Glucose 10	Trichodin A Pyridines	*Staphylococcus epidermidis* *Bacillus subtilis* *Candida albicans*	–	24.28 27.05 25.38	[94]
Beauveria felina EN-135	Moss	Rice *	Desmethylizalidine C1 cyclohexadepsi peptide	*Escherichia coli*	8	–	[89]
Penicillium brocae MA-231	Mangrove plant *Avicennia marina*	Glucose 20	Penicibrocazin C Diketopi perazine sulfide derivatives	*Staphylococcus aureus* *Micrococcus luteus*	0.25 0.25	–	[91]
Diaporthaceae sp. PSU-SP2/4	Sea sponge	Dextrose-potato broth	Diaportalalazine five-cyclic cytochalasin	*Staphylococcus aureus* MRSA	2	–	[92]
Streptomyces pratensis NA-ZhouS1	Seawater	Starch 20	Stremycin A Angicycline polyketide antibiotic	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Klebsiella pneumonia* *Escherichia coli* *Bacillus subtilis*	16 16 16 16 8–16	–	[102]
Streptomyces sp. A6H	Seawater	–	Vinomycin A1 Angicillin antibiotic	*Staphylococcus aureus*	4	–	[103]
Streptomyces sp. G278	Echino-derms *Holothuria edulis*	Starch 10	**2.5**-Bis (5-tert-butyl-2-benzoxazolyl) thiophene	*Escherichia coli* *Salmonella enterica* *Staphylococcus aureus* *Enterococcus faecalis* *Candida albicans*	64 256 256 256 64	–	[101]

Note: «*» – solid phase cultivation; «–» — no data.

Table 6. **Antitumor activity of secondary metabolites synthesized by marine microorganisms**

Producer	Source of selection	Source of carbon, g/l	Compound. Type	Grafting cell culture	IC_{50}, μM	Literature
Acaromyces ingoldii FS121	Seawater	Dextrose-potato broth	Acaromycin A Anthraquinone derivative	MCF-7 NCI-H460 SF-268 HepG2	6.7 10 7.8 7.3	[116]
*Stemphylium globuliferum**	Mangrove plant *Avicennia marina*	Dextrose-potato agar **	Altersolanol A Anthraquinone derivative	L5178Y BXF T24 RXF 944L PRXF LN-CAP OVXF899L LXF529L GXF251L MEXF462NL	3.4 0.001 0.001 0.001 0.006 0.004 0.052 0.034	[114, 115, 119]
Sarcopodium sp. FKJ-0025	Marine bottom sediments	–	Sarcopodinol B Derivative of hydroquinones	HL-60 Jurkat Panc 1	37 47 66	[108]
Engyodontium album DFFSCS021	Marine bottom sediments	Rice*, Glucose	Engyodontiumone N Xanthone polyketide	U937	4.9	[96]
Aspergillus wentii SD-310	Marine bottom sediments	Potato juiceGlucose	Asperolide E Tetranorlabdan diterpenoid	HeLa, MCF-7 NCI-H446	10.0 11.0 16.0	[104]
Aspergillus dimorphicus SD317	Marine bottom sediments	Dextrose-potato broth	Wentilactone A Tetranorditerpenoid	NCI-H446 NCI-H460	1.9 5.56	[105–107]
			Wentilactone B Tetranorditerpenoid	SMMC-7721	18.96	
Aspergillus flavus OUC-MDZ-2205	Shrimp *Penaeus vannamei*	Glucose,10 Maltose,20 Mannitol 20	(2R,4bR,6aS,12bS, 12cS,14a)-4b-deoxy-β-aflatrem Indole diterpenoid	A549	10	[90]
Eutypella sp. FS46	Marine bottom sediments	Dextrose-potato broth	Scopararane I Pimarane-type diterpene	MCF-7 NCI-H460 SF-268	83.9 13.5 25.3	[113]
Stachybotrys sp. MF347	Seawater	Glucose,10	Stachybocin B Bicyclic sesquiterpene	NIH-3T3 HepG2	16.45 17.87	[93]
Penicillium sp. PR19N-1	Deep waters of Antarctica	–	Eremophilane-type sesquiterpene	HL-60 A549	28.3 5.2	[110]
Penicillium brocae MA-231	Mangrove plant *Avicennia marina*	Dextrose-potato broth	Brocazine A,B,E,F Diketopiperazine derivatives	Du145 HeLa HepG2 MCF-7 NCI-H460 SGC-7901 SW1990 SW480 U251	1.7–11.2 4.3–6.9 2.9–6.4 3.0–9.0 0.89–12.4 2.4–8.0 2.1–6.4 1.2–2.0 3.5–6.1	[109]
Lasiodiplodia sp. 318	Mangrove plant *Exoecaria agallocha*	Rice*	Lasiodiplodine (2,4-dihydroxy-6-nonylbenzoate) Resorcinic acid lactone	MMQ GH3	5.2 13.0	[111]

Table 6 (End)

Producer	Source of selection	Source of carbon, g/l	Compound. Type	Grafting cell culture	IC_{50}, μM	Literature
Campylocarpon sp. HDN13-307	Mangrove plant *Sonneratia caseolaris*	Glucose, 10 Maltose, 20 Mannitol, 20	Campyridone D Pyridone alkaloid	HeLa	8.8	[112]
			Ilicicolin H Pyridone alkaloid		4.7	
Arthrinium arundinis ZSDS1-F3	Sea sponge *Phakellia fusca*	Sorbitol, 20 Maltose, 20	10-phenyl-[12]-cytochalazine Z16 Aminopolyketide	K562 A549 H1975 MCF-7 U937 BGC823 HL60 HeLa MOLT-4	6.2 1.1 14.2 18.5 3 18.8 6.2 3.2 4.1	[117]
Simplicillium obclavatum EIODSF020e	Deep waters	Glucose, 10 Maltose, 20 Mannitol, 20 Starch 0,5	Simplicilliumtide A Linear peptide	HL60	64.7	[118]
			Simplicilliumtide E Linear peptide	K562	39.4	

Note: «*» — strain number is not given; «**» — solid phase cultivation; «–» — no data; IC_{50} — cytotoxicity index; the concentration of the compound that causes 50% lysis of the monolayer of cancer cell.

A and B showed high antitumor activity to the SW480 cell line (IC_{50} 2.0 and 1.2 μM, respectively), while brocazine F — to the DU145 and NCI-H460 cell lines (IC_{50} 1.7 and 0.89 μM in accordance).

Over the past eight years, a number of antitumor compounds synthesized by marine microorganisms have been identified (Table 6). By chemical nature, it is a diverse group of compounds, including tetranorditerpenoids [105–107], scopararane I [113], engidontium H [96], stachybocin B [93], campyridone D [115], anthraquinone derivatives [114–116, 119] and diketopiperazine [109], peptides [118], and others. Their anti-cancer effect has been shown in various models of *in vitro* tumors — breast adenocarcinoma [104, 109, 113, 116, 117], hepatocellular carcinoma [93, 109, 116], leukemia [108, 110, 117, 118], cervical cancer. [104, 109, 112, 117], lungs [90, 110, 117] and others.

Despite a large number of newly identified microbial compounds, only some of them showed better antitumor activity compared to standard anticancer drugs [119]. However, taking into account the chemical diversity of metabolites of marine microorganisms, it could be assumed that rational derivatization could lead to compounds with a wide range of antitumor activity.

Thus, the analysis of literature data showed that marine microorganisms synthesize a wide range of practically valuable enzymes, surfactants, exopolysaccharides, as well as secondary metabolites with diverse biological activity (antimicrobial, antitumor, cytotoxic). However, at present, they can hardly be considered as potential biological agents for use in biotechnological processes. There are several reasons for this.

Firstly, in most studies, researchers do not provide indicators of the synthesis of a target product, and the ability to synthesize it is often established based on qualitative reactions. If in some works the concentrations of certain metabolites (in g/l) are indicated, they are significantly lower than those synthesized by existing industrial strains.

Secondly, marine organisms synthesize practically valuable metabolites growing on expensive carbohydrate substrates (Table 1, 2, 4–6). There are only isolated reports of the use of industrial wastes for example for the synthesis of surfactant lipopeptides (Table 2). Also, high-value complex nutrient media are often used to cultivate marine microorganisms.

Thirdly, in many studies, researchers, have established the ability of marine microorganisms to form a specific target product, do not try to optimize at least the composition of the nutrient medium to increase its synthesis or scale the process of biosynthesis to fermentation equipment.

To predict the possible organization of industrial production with the use of marine microorganisms as producers in the nearest future, it might be biotechnology for

the production of hydrolytic enzymes that decompose plant and algal polymers. The need for such enzymes is due to the use of plant biomass in biofuel production. Also, the genetic potential of marine microorganisms can be used in biotechnology as a source of genes encoding the synthesis of new biologically active substances with unique properties, including antimicrobial and antitumor.

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ПРАКТИЧНО ЦІННІ МЕТАБОЛІТИ МОРСЬКИХ МІКРООРГАНІЗМІВ

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В огляді наведено дані сучасної літератури щодо синтезу грибами, актинобактеріями та бактеріями, виділеними з морських екосистем (морська вода, донні відкладення, поверхня флори та фауни, мангрові біоми, льодовики), практично цінних метаболітів. Морські мікроорганізми синтезують широкий спектр практично цінних ензимів (холодоактивна галактозидаза, агараза, альгінатліаза, фукоїдаза, хітиназа та ін.), поверхнево-активних гліко- та ліпопептидів з емульгувальною, антимікробною та антиадгезивною активністю, екзополісахаридів, а також вторинних метаболітів з різноманітною біологічною активністю (антимікробна, протипухлинна, цитотоксична). Разом з тим використання морських продуцентів у біотехнологічних процесах стримується їхньою невисокою синтезувальною здатністю і великими витратами на біосинтез (складні живильні середовища і дорогі вуглеводні субстрати). У біотехнології морські мікроорганізми можуть бути використані як джерела генів, що кодують синтез нових біологічно активних речовин з унікальними властивостями, зокрема антимікробними та протипухлинними.

Ключові слова: морські гриби, бактерії, біологічно активні речовини.

ПРАКТИЧЕСКИ ЦЕННЫЕ МЕТАБОЛИТЫ МОРСКИХ МИКРООРГАНИЗМОВ

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В обзоре приведены данные современной литературы по синтезу грибами, актинобактериями и бактериями, выделенными из морских экосистем (морская вода, донные отложения, поверхность флоры и фауны, мангровые биомы, ледники), практически ценных метаболитов. Морские микроорганизмы синтезируют широкий спектр практически ценных энзимов (холодоактивная галактозидаза, агараза, альгинатлиаза, фукоидаза, хитиназа и др.), поверхностно-активных глико- и липопептидов с эмульгирующей, антимикробной и антиадгезивной активностью, экзополисахаридов, а также вторичных метаболитов с различной биологической активностью (антимикробная, противоопухолевая, цитотоксическая). Вместе с тем использование морских продуцентов в биотехнологических процессах сдерживается их невысокой синтезирующей способностью и значительными затратами на биосинтез (сложные питательные среды и дорогие углеводные субстраты). В биотехнологии морские микроорганизмы могут быть использованы в качестве источников генов, кодирующих синтез новых биологически активных веществ с уникальными свойствами, в частности антимикробными и противоопухолевыми.

Ключевые слова: морские грибы, бактерии, биологически активные вещества.

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INTEGRATED MATHEMATICAL MODEL FOR IMITATION OF THE COURSE OF VIRAL DISEASE AND CORRECTION OF THE INDUCED HYPOXIC STATE

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The aim of the work was to create a complex mathematical model simulating the course of the disease caused by the SARS-CoV-2 virus on the level of interaction between functional systems of organism and pharmacological correction of organism hypoxic states arising in the complicated course of the disease. In the present work the methods of mathematical modeling and theory of optimal control of moving objects were used. The proposed integrated mathematical model consisted on the mathematical models of functional systems of respiration and blood circulation, thermoregulation, immune response, erythropoesis, and pharmacological correction. Individual patient data were taken for this model, and the disturbing effect in the form of viral disease was simulated. The reactions of functional respiratory and blood circulatory systems were predicted. Partial pressures of respiratory gases in alveolar spaces and their tensions in lung capillaries blood, arterial and mixed venous blood, and tissue fluid were calculated. Further the intravenous injection of antihypoxant was simulated and the values of the same parameters were calculated. In such a way it was possible to choose the most optimal way of hypoxic state correction for any individual. This model is theoretical only for today because the models of respiratory and blood circulation systems were designed for the average person and it does not suppose peculiarities of individual persons infected with SARS-CoV-2. In particular, this concerns the pequliarities of gas exchange in the alveolar space and characteristics of respiratory gases diffusion through the alveolar-capillary and capillary-tissue membranes. However, it is one of possible directions for solving the complex tasks related to treatment of the disease caused by SARS-CoV-2 virus. In the result of the work the complex of information support for the imitation of viral disease course was developed at the level of interaction of organism functional systems, as well as pharmacological correction of caused by it hypoxic states.

Key words: SARS-Cov-2 virus, immune response model, mathematical model of the respiratory system, hypoxic state, infection lesion

New coronavirus infection burst had happened in Republic of China with epicenter in Wuhan (Hubei Province) in late 2019. The World Health Organization officially named it COVID-19 (“Corona virus disease 2019”) on February 11, 2020. The International Committee on Viruses Taxonomy had assigned the official name to the agent of this infection — SARS-CoV-2 on February 11, 2020.

The information on epidemiology, clinical features, prevention, and treatment of this disease is limited until now. The most common clinical manifestation of the new variant of coronavirus strain infection was bilateral pneumonia: the development of acute respiratory distress syndrome was registered in 3–4% of patients [1]. This potentially severe acute respiratory infection causes dangerous disease [2]. It can occur both in the form of

acute respiratory viral infection with mild course [3, 4] and in severe form with such specific complications as viral pneumonia caused acute respiratory distress syndrome or respiratory failure with a risk of the death [5]. However, full clinical picture is not yet clear [6]. There are no specific antiviral agents for the treatment or prophylaxis of this disease [7]. In most cases (approximately 80%) it turns out that no specific treatment is required, and recovery takes place on its own [2, 8]. In severe cases, specific means and methods are used to maintain functions of vital organs [9]. Respiratory insufficiency development is also possible against the background of this infection [3]. Less than a third of patients demonstrated the development of acute respiratory distress-syndrome [2]. In case of acute respiratory distress-syndrome, tachycardia, tachypnoea or cyanosis may also be appeared to accompany hypoxia [6].

Inflammatory processes can influence on cardiovascular system resulting in arrhythmias and myocarditis. Acute heart insufficiency is mostly found in severely or critically ill patients. Infection can occure long-term influences on the health of cardiovascular system. In case of patients with cardiovascular diseases in anamnesis, strict monitoring of their conditions may be required [2].

There is no specific antiviral therapy against SARS-CoV-2 virus [9] and there is no evidence of effective immunomodulating therapy [10]. Patients receive mainly symptomatic and supportive therapy. In severe cases, treatment aims to maintain vital functions of organs [9].

Although unlicensed drugs and experimental therapies are used today in practice of coronaviral disease treatment, for example, with the use of antiviral agents, such treatment should be carried out within the framework of ethically based clinical trials [2]. Critically important is the use of tools that are justified both ethically and scientific researches [11, 12].

Bases for used methodology. Therapy prescriptions should not be based on hypotheses, but on clinical studies that confirm the effectiveness of such therapy. Hypotheses, however, may be the basis for a planned clinical trial [13]. Therefore, it seems reasonable to apply simulation modeling of coronaviral disease course and exposure to pharmacological drugs.

The methods of information technologies and mathematical modeling complement those of experimental biology and medicine. Modern diagnostic methods, whatever perfect they may be, give only a "slice" of current organism state. Therefore, the mathematical modeling of organism functional systems and an organism as a whole became widespread in the last third of the last century, allowing to simulate various processes taking place in the organism and to study these processes at the level inaccessible to the modern methodical diagnosis level, for example, to simulate extreme organism disturbances and forecast the functional state of organs and systems with this disturbance.

Mathematic model of functional respiratory system, developed by the united efforts of the scientists from Glushkov Institute of Cybernetics and Bogomoletz Institute of Physiology both of the National Academy of Sciences of Ukraine was based exactly on these principles.

The purpose of the work was to create integrated mathematical model to simulate the course of the disease caused by SARS-CoV-2 virus and pharmacological correction of complications — organism hypoxic states.

Mathematical models of respiration and blood circulation systems

Many mathematical models of various functional systems and organism as a whole exist nowadays. Let's observe the models related to the respiratory and blood circulatory systems because of several reasons. First, according to the current information, exactly these systems are the most affected by the SARS-CoV-2 virus [14–27]. Secondly, in the theory of adaptation developed by Meyerson, exactly these systems responded most noticeably to changes of living conditions [28, 29]. Thirdly, in a number of publications there were shown that if we consider the human organism from the point of view of reliability theory, and assume it as a "chain with a weak link", then such "weak links" are exactly the respiratory and blood circulatory systems [30–37].

First of all, Gray model should be highlighted, in which the respiratory system was presented as a feedback system and thus the background for studying the relationships between alveolar ventilation V and oxygen pressures pO_2, carbon dioxide pCO_2 and the arterial blood acidity pH was laid [38].

The next qualitatively important step was the model of Grodins, who suggested that the respiratory system should be considered as a dynamic system, which made it possible to use the appropriate mathematical apparatus [39, 40]. The ventilation dynamics was studied

when the concentration of carbon dioxide in respiratory system changed. Therewith elements of system analysis were used. The control and controlled systems responsible for process of gas exchange were given up, tissue reservoirs of an organism in which oxygen was consumed and carbon dioxide was released were subdivided. Two reservoirs were identified as "brain" and "non-brain". The first reservoir included vitally important organs, the second one — peripheral organs and tissues. Grodins derived the differential equations describing the dynamics of partial pressures and tensions of respiratory gases in the lungs, blood and tissues, basing on the principles of material balance and continuity of the flow [39, 40]. A significant disadvantage of the model was the assumption that during inspiration, a constant pCO_2 was maintained in the respiratory mixture, alveoli and blood.

Mathematical models of respiratory and blood circulatory systems: their use for the solution of practical and theoretical problems in medicine and physiology

Further development of Grodins model was a model of mass transfer and mass exchange of respiratory gases in human body and dolphin, proposed by Kolchinskaya and Misyura [41]. The model considers the process of mass transfer and mass exchange of respiratory gases through the alveolar-capillary and capillar-tissue membranes, taking into account their structural and functional pequliarities. This approach enabled to study gases transportation in human body during respiratory cycle: inspiration, expiration and pause, taking into account the biophysical and biochemical characteristics of the processes. Besides, tissue reservoirs were differentiated in the model, tissues of brain, heart, liver, kidneys, skeletal muscles, and etc. were defined. This made it possible to elaborate the models of gases saturation and to study the process of hypoxia development in them [41]. The proposed model contained equations for determining of alveolar ventilation and systemic blood flow obtained on the basis of experimental data. However in order to calculate oxygen and carbon dioxide regimes of human organism under changes in living conditions, it was required the data that were impossible to obtain at the current methodological level of bioexperiment. Therefore, it is quite problematic to use such type of models for the cases upon changing the levels of energy consumption, environmental conditions without solving the problem concerning control of respiratory system function.

In addition, blood circulatory system, contrary to respiratory one, is multifunctional, and this causes certain difficulties linked with determination of optimality criterion. Consequently, the concept of organism's oxygen regimes regulation formulated by Kolchinskaya and Lauer was an actual one [42]. According to this concept, the regulation in organism is carried out by one complex system that coordinates joint functioning of various mechanisms and subordinates this system to its main task — to maintain optimal oxygen parameters along the oxygen pathes in organism. Herewith, the delivery speed should match the oxygen demand in tissues. In accordance with this concept, mathematical models should consider the united action of the systems of external respiration, blood circulation, and tissue respiration, aimed on the providing of tissues demand in oxygen.

There are numerous other mathematical models [43–52]. Let's observe exactly the models developed by Onopchuk and representatives of his scientific school [37, 53–59]. Basing on above-described approach, few mathematical models of heat transfer and heat exchange [60–62], immune system [63–65], system of energy supply [66] and erythropoesis [67, 68] were developed.

These models were used to solve a number of practical and theoretical problems in medicine and physiology. Namely, the theoretical problems linked with investigations of cerebral blood circulatory tensions in operators of continuous interaction system were solved [69–74], compromise resolution of conflict situations in the problem of optimal control in decisions making in difficult situations was studied [37, 75–77], the role of hypoxia, hypercapnia and hypometabolism during adaptation of the respiratory system to intensive muscular activity and stay in conditions of hypoxic hypoxia were investigated [78–82], mathematical models of short-term, medium-term and long-term adaptation of the respiratory system to extreme environmental influences were developed [35, 37, 83, 84], parameters of self-organization of the rescue command members breathing system during short-term and medium-term adaptation to hypoxic hypoxia were studied [35, 82], the tasks of modeling of the hypoxic and hypercapnic stages of training athletes were considered [85, 86], dependence of parameters of functional self-organization for high qualification

women-athletes on the hormonal status of their organisms were studied [87–89], algorithm for predicting of fatigue development in highly skilled athletes with refined muscular activity was constructed [90, 91], mathematic models for the development of hypoxia at coronary heart disease were developed [92–97], algorithm for the selection of data models and algorithms for their processing to build an integrated estimation of the reliability and performance of athletes was proposed [98–101]. Separately, it is necessary to highlight the use of these models in sports of the highest achievements, for the sportsmen specializing in cyclic sports [102], martial arts [103–107], alpinism [108], their practical application in research at the Elbrus Medical and Biological Station of Bogomoletz Institute of Physiology of the National Academy of Sciences of Ukraine [109–121], for solution of a broad range of problems connected with the examination of operators of continuously interacting systems and flying personnel.

Separately, it is necessary to write about the works [122–124] associated with the development of software for the improving of the tools and methods for operational data mining, processing and analysis of functional diagnostic data, and the person's stay in hyperbaric environment [125, 126].

There is also a number of works devoted to the research and identification of organism reserves under the extreme disturbances [127–132] and optimization of the recovery and rehabilitation processes after the extreme loads on an organism [133,134], thermoregulation processes under the extreme influences [116].

Therefore, the idea to apply such models for new class of problems related to studying and treatment of infectious organism lesions infected with SARS-CoV-2 seems quite reasonable and appropriate.

Integrated model of the functional system of respiration, blood circulation, heat transfer, and immune response

To simulate the hypoxic state caused by SARS-CoV-2 virus we proposed to use integrated mathematical model of the functional respiratory and blood circulatory system, thermoregulation, and immune response to predict the course of viral disease [37, 54, 55, 57, 60–65].

When studying the organism adaptation to one or another disturbances, including infectious disease, it is advisable to take into consideration the possibility of participation of intersystem mechanisms in process of organism state stabilizing, taking into account both intra-systemic and intersystemic conflict situations. In response to the environment disturbing influence (external or internal), all organism functional systems react against it to some extent, trying to stabilize the organism state, despite the contradictions between goals and interests. The structural scheme of complex mathematical model for investigation of the main functional systems (respiration, blood circulation, heat transfer, immune), their pharmacological correction as well as mechanisms of their interaction and interconnection during the life activities in extreme conditions of the external and internal environment was shown on Fig. 1.

Let's give a description of the models of individual functional systems. Briefly,

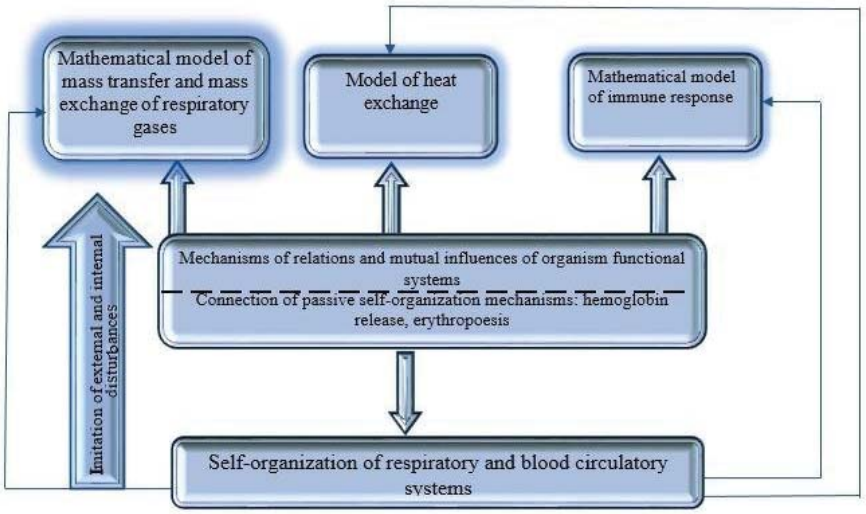

Fig. 1. **Integrated model of the functional system of respiration, blood circulation, heat transfer, and immune response**

the mathematical model of the functional respiratory system could be represented as follows. Mathematical model of respiratory and blood circulatory system is a controlled dynamic system, the phase state of which is characterized by partial pressures and tensions of respiratory gases in each element of the system.

The controlled part of the model is based on differential equations describing changes in average partial pressures of respiratory gases in each part of respiratory cycle — during inspiration, expiration and pause. Briefly, the model can be submitted as follows:

$$\frac{dp_iO_2}{d\tau} = \varphi(p_iO_2, p_iCO_2, \eta_i, \dot{V}, Q, Q_{t_i}, G_{t_i}O_2, q_{t_i}O_2) \quad , (1)$$

$$\frac{dp_iCO_2}{d\tau} = \psi(p_iO_2, p_iCO_2, \eta_i, \dot{V}, Q, Q_{t_i}, G_{t_i}CO_2, q_{t_i}CO_2) \, , (2)$$

where the functions φ and ψ are described in detail in [54, 55], V is ventilation, η is a degree of hemoglobin saturation with oxygen, Q is volumetric velocity of systemic and Q_{t_i} — local blood flows, $q_{t_i}O_2$ is oxygen consumption rate by i-th tissue reservoir, $q_{t_i}O_2$ is the rate of carbon dioxide release in i-th tissue reservoir. The velocities $G_{t_i}O_2$ of oxygen flows from the blood into the tissue and $G_{t_i}O_2$ of carbon dioxide from the tissue into the blood are determined by the ratio:

$$G_{t_i} = D_{t_i}S_{t_i}(p_{ct_i} - p_{t_i}), \qquad (3)$$

where D_{t_i} are gas permeability coefficients through the airhematic barrier, S_{t_i} is gas exchange surface area.

In this model, respiratory, cardiac and vascular smooth muscles are the active mechanisms of self-regulation. Accordinly V, Q, Q_{t_i}, $i = 1,m$ are the control parameters in the dynamic system, which are determined as a result of solving the task of optimal output of the disturbed dynamic system into a stable equilibrium state characterized by the following retios:

$$G_{t_i}O_2 - q_{t_i}O_2 = 0, \quad i = \overline{1,m} \quad , \qquad (4)$$

$$G_{t_i}CO_2 + q_{t_i}CO_2 = 0, \quad i = \overline{1,m} \, . \qquad (5)$$

The optimal values are those that provide a minimum of the functional:

$$I = \int_{t_0}^{T} \left(\rho_1 \sum_{t_i} \lambda_{t_i} \left(G_{t_i}O_2 - q_{t_i}O_2 \right)^2 + \rho_2 \sum_{t_i} \lambda_{t_i} \left(G_{t_i}CO_2 + q_{t_i}CO_2 \right)^2 \right) dt, \quad (6)$$

under the restrictions:

$$\dot{V}^{\min} \le \dot{V} \le \dot{V}^{\max}, \quad Q^{\min} \le Q \le Q^{\max}, \quad Q_{t_i}^{\min} \le Q_{t_i} \le Q_{t_i}^{\max}, \quad \sum_{t_i} Q_{t_i} = Q. \quad (7)$$

In (7) ρ_1, ρ_2 are organism sensitivity coefficients to the oxygen deficiency and carbon dioxide excess, λ_{t_i} characterize functionally the morphological features of tissue region.

The dynamics of infectious lesion of organism was given by Marchuk as a system of ordinary nonlinear differential equations with delay [135]. Let's consider one of the equations of this system:

$$\frac{dm}{d\tau} = \sigma\nu(1-m) - \mu_m, \qquad (8)$$

where $m(\tau)$ is relative characteristics of an affected organ. If M is characteristics of healthy organ (mass or area), and M' is corresponding characteristic of the healthy part of affected organ, then

$$m = 1 - \frac{M'}{M}, \qquad (9)$$

is a relative characteristic of lesion of an organ-target. The factor $(1 - m)$ in (8) determines the effect of antigens on unaffected part of an organ-target.

Decrease in this characteristic occur due to the regenerative activity of an organism with μ_m coefficient characterizing the rate of mass recovery of the affected organ.

The pathological state of an organism that developed due to the infectious lesion can be considered as disturbance during modeling of blood circulatory system. Then σ and μ_m in (8) are the functions depended on Q_{t_i}. When considering joint modeling of respiratory, circulatory and immune systems and their regulation, it is necessary to add the term

$$\rho_{\eta_i} f_i^2(m(\tau), V(\tau)), \qquad (10)$$

to the quality criterion of regulation (6) into the integration element, where ρ_{η_i} is a coefficient characterizing the influence degree of the simulated disease type on the level of gas homeostasis. The function $f_i(m, V)$determines the damage degree of target-organ at current moment. At control points, this function was taken as:

$$f_i(m,V) = a_i m + b_i v \qquad (11)$$

It could be assumed that the flow of energy processes in the tissues of an organ-target is supplied only due to its unaffected part. Then the mass of metabolizing part of the organ will

be determined:

$$v_{t_i}(\tau) = v_{t_i}^0(1 - m(\tau)), \quad (12)$$

where $v_{t_i}^0$ is a total mass (volume) of tissues of healthy organ.

In case of infectious disease, it is natural to assume a reaction of thermoregulatory system. Let's complete our model of the dynamics of the course of infectious disease by introducing the variable T (the temperature of internal sphere of organism [136, 137]) in the equation below:

$$\frac{dT_{t_k}}{d\tau} = K_T(F\nu - (F\nu)^*)\chi(F\nu - (F\nu)^*) - \mu_T(T_{t_k} - T_{t_k}^*), \quad (13)$$

where K_T, μ_T are coefficients, $F\nu$ is concentration of $F\nu$ complexes, $(F\nu)^*$ is maximal permissible concentration of complexes, $T_{t_k}^*$ is normal temperature of core of organism, χ is Heaviside function. In this case, it was natural to put the coefficients in model (8)–(12) in the form of functions depending on T_{t_k}:

$$\overline{\beta}(T_{t_k}) = \frac{\overline{\beta}(T_{t_k}^*)}{1 + \alpha_{T_k}(T_{t_k} - T_{t_k}^*)}, \quad (14)$$

$$\overline{\alpha}_{T_{t_k}} = \overline{\alpha}(T_{t_k}^*)[1 + b_{T_{t_k}}(T_{t_k} - T_{t_k}^*)], \quad (15)$$

where $\overline{\beta}(T_{t_k}^*) = \overline{\beta}, \overline{\alpha}(T_{t_k}^*) = \overline{\alpha}$, $\alpha_{T_{t_k}}, b_{T_{t_k}}$ are coefficients.

It is natural to assume that at the initial stage of disease, the passive mechanisms of self-regulation such as erythropoesis, release of hemoglobin and mioglobin into blood were involved. An increase of the content of red blood cells and the content of hemoglobin in them is powerful regulatory mechanism for maintaining of organism stable state in conditions that lead to oxygen deficiency under the various disturbances. In [67] the linear dependences of erytropoetin (EPO), *Ht* and *Hb* were obtained and than they were introduced into the mathematical model of functional respiratory and blood circulatory system to enhance the regulation of respiratory system main function in hypoxia.

Further, due to the fact that severe hypoxia develops in organism as a result of lung damage, the injection of antihypoxants into the organism is advisable in order to study the possible ways of organism state relief in case of hypoxia. The integrated model described above for this case has to be supplemented by the equations of transport of pharmacological preparations in organism in forms, suggested previously [107, 118, 138]. The algorithm for the application of this approach is given in Fig. 2.

Our developed mathematical model of pharmacological correction of hypoxic states clarifies the role of pharmacological preparation use for prevention of hypoxic states development in organism (for organism state perfection). It was assumed that the withdrawal of antihypoxant *f* from the organism is carried out through the kidneys. It was assumed as well that we use pharmacological preparations that improve oxygen permeability through the capillary tissue membranes of blood vessels. According to this scheme it was assumed that the most effective was intravenous administration of antihypoxant, although the model enabled to simulate as well as respiratory, oral and intramuscular way of antihypoxants administration.

Procedure for the work with the model

1. Patient examination is carried out.

2. The data obtained from the survey are the source for calculation of organism oxygen regimes [121, 122].

3. The data obtained during patient examination and some data obtained as a result of calculation of organism oxygen regimes were taken as input source data in the models of functional respiratory system, blood circulatory system and thermoregulation. In such a way the models individualization was fulfilled.

4. Further, using the model of immune response, the effect of virus is simulated; with the interaction and interinfluence of the models, the partial pressures and tensions of respiratory gases in all parts of respiratory system, alveolar ventilation and systemic blood flows are calculated.

5. The next step is to simulate the effects of pharmacological preparations and, consequently, the values of the same indicators have to be calculated again.

6. The obtained data are analyzed and further, in case of unsatisfactory result, another effect of antihypoxant is simulated, or if the obtained indicators are acceptable, then this scheme of pharmacological preparation use is chosen.

Thus in this publication, the results of development of comprehensive integrated mathematical model for simulation of the course of disease caused by SARS-CoV-2 were suggested. It could be used

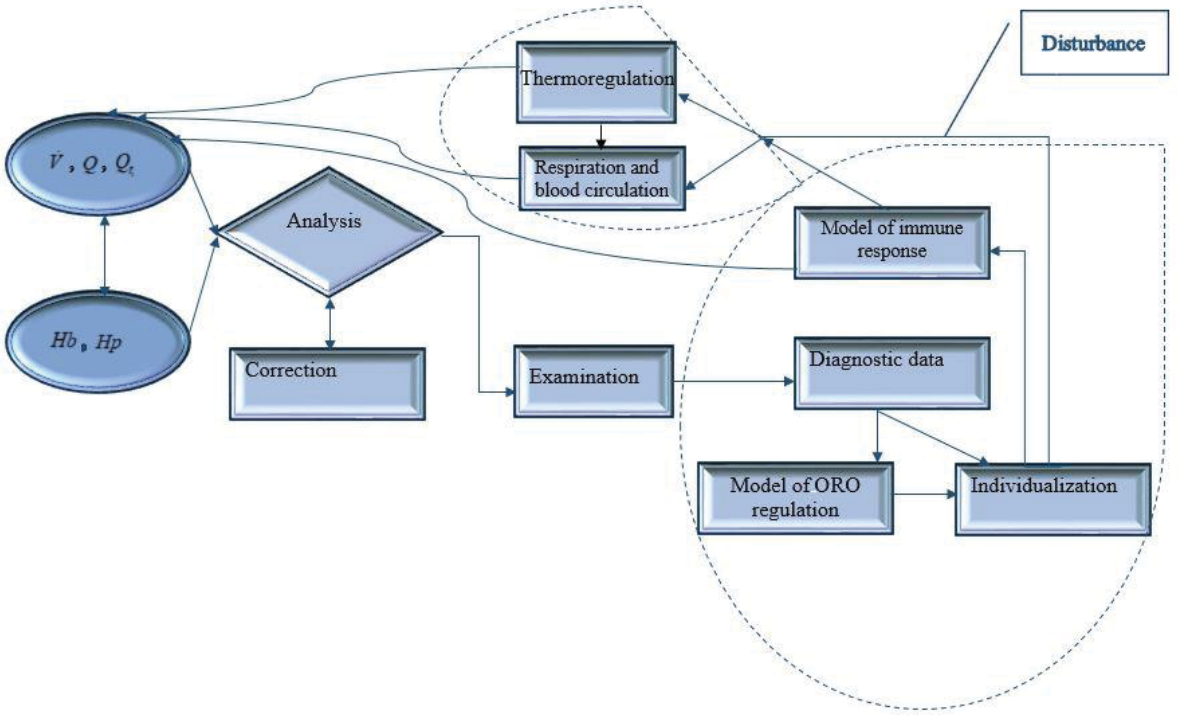

Fig. 2. **Scheme of mathematical model for simulating the course of viral disease and its pharmacological correction:** *Hb*, *BH* — concentrations of hemoglobin and buffer bases in blood; Q — volurimetric velocity of systemic blood flow; Q_{t_j} — volurimetric velocity of local blood flows; *ORO* — oxygen regimes of organism; ν — alveolar ventilation (air volume that pass through alveolar space during 1 min)

for pharmacological correction of hypoxic states that occur with the complication of disease course as well. The bases for the used methodology were observed as well as mathematical models of respiration and blood circulation systems. The information about the developed models of respiratory and blood circulatory systems and their use for the solution of practical and theoretical problems in medicine and physiology were suggested. For simulation of hypoxic state caused by SARS-CoV-2, we proposed to use the integrated mathematical model of functional respiratory and blood circulatory systems, thermoregulation, and immune response one to forecast the course of viral disease. The structural scheme of complex mathematical model for the investigations of main functional systems (respiration, blood circulation, heat transfer, and immune response), their pharmacological correction as well as mechanisms of their interaction and interconnection during the life activities in extreme conditions of the external and internal environment was demonstrated. In the result, the complex of information support for imitation of viral disease course as well as for it pharmacological correction caused by the organism hypoxic states were developed.

For today, this mathematical integrated model has theoretical significance only. It is based on the information about the clinically registered manifestations of coronaviral (SARS-CoV-2) disease available in the public domain. Therefore, this model requires further perfection. In particular, it seems necessary to clarify some characteristics of respiratory gases transport through the alveolar-capillary membrane, peculiarities of gas exchange in the alveolar space, which cause the decrease of blood oxygenation. These are the problems that need to be solved in close collaboration with the professionals in medicine. At the same time, the imitation on this model the development of infectious disease and associated hypoxic state is one of the possible and quite effective tool for solving the tasks associated with the support of patients in acute hypoxic respiratory and heart failure caused by the complications of viral (SARS-CoV-2) disease.

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ІНТЕГРОВАНА МАТЕМАТИЧНА МОДЕЛЬ ДЛЯ ІМІТАЦІЇ ПЕРЕБІГУ ВІРУСНОГО ЗАХВОРЮВАННЯ ТА КОРЕКЦІЇ СПРИЧИНЕНОГО НИМ ГІПОКСИЧНОГО СТАНУ

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Метою роботи було створення комплексної математичної моделі, що імітує перебіг захворювання, спричиненого вірусом SARS-CoV-2, та фармакологічної корекції гіпоксичних станів організму в разі ускладнення цього захворювання. В цій роботі було використано методи математичного моделювання та теорії оптимального керування рухомими об'єктами. Запропонована математична модель складалася з математичних моделей функціональних систем дихання та кровообігу, терморегуляції, імунної відповіді, еритропоезу та фармакологічної корекції. Для цієї моделі було взято індивідуальні дані пацієнта і здійснено імітацію вірусного захворювання. Прогнозували реакції органів дихання та кровообігу: розраховано парціальний тиск дихальних газів у альвеолярних просторах та їхню напругу в крові легеневих капілярів, артеріальної та змішаної венозної крові та тканинної рідини. Далі імітували ін'єкцію антигіпоксанту та розраховували значення тих самих параметрів. Таким чином можна було вибрати найбільш оптимальний спосіб корекції гіпоксичного стану для будь-якої людини. На сьогодні ця модель є суто теоретичною, оскільки моделі системи дихання та кровообігу було розроблено на усереднені дані, і вони не враховують особливостей окремих осіб, інфікованих SARS-CoV-2. Зокрема, це стосується газообміну в альвеолярному просторі можливих особливостей проникності дихальних газів через альвеолярно-капілярну мембрану. Однак це один із можливих напрямів вирішення складних завдань, пов'язаних з лікуванням захворювання, спричиненого вірусом SARS-CoV-2. У результаті було розроблено комплекс інформаційної підтримки для імітації перебігу вірусних захворювань, а також фармакологічної корекції спричинених ними гіпоксичних станів.

Ключові слова: вірус SARS-CoV-2, модель імунного відгуку, математична модель дихальної системи, гіпоксичний стан, інфекційне ураження.

ИНТЕГРИРОВАННАЯ МАТЕМАТИЧЕСКАЯ МОДЕЛЬ ДЛЯ МОДЕЛИРОВАНИЯ ТЕЧЕНИЯ ВИРУСНОГО ЗАБОЛЕВАНИЯ И КОРРЕКЦИИ ВЫЗВАННОГО ИМ ГИПОКСИЧЕСКОГО СОСТОЯНИЯ

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Целью работы было создание комплексной математической модели, имитирующей течение заболевания, вызванного вирусом SARS-CoV-2, и фармакологической коррекции гипоксических состояний организма, возникающих в случае осложнения этого заболевания. В этой работе использовались методы математического моделирования и теории оптимального управления движущимися объектами. Предлагаемая математическая модель состояла из математических моделей функциональных систем дыхания и кровообращения, терморегуляции, иммунного ответа, эритропоэза и фармакологической коррекции. Для этой модели были взяты индивидуальные данные пациента и смоделирован эффект в виде вирусного заболевания. Спрогнозированы реакции дыхательной и кровеносной систем: рассчитаны парциальное давление дыхательных газов в альвеолярных пространствах и их напряжение в крови капилляров легких, артериальной и смешанной венозной крови и тканевой жидкости. Далее имитировали инъекцию антигипоксанта и рассчитывали значения тех же параметров. Таким образом можно было выбрать наиболее оптимальный способ коррекции гипоксического состояния для среднестатистического человека. На сегодняшний день эта модель является чисто теоретической, поскольку модели системы дыхания и кровообращения были разработаны усредненные данные, и не учитывающие особенности отдельных лиц, инфицированных SARS-CoV-2. В частности, это касается газообмена в альвеолярном пространстве и возможных особенностей проницаемости дыхательных газов через альвеолярно-капиллярную мембрану. Однако это одно из возможных направлений решения сложных задач, связанных с лечением заболевания, вызванного вирусом SARS-CoV-2. В результате был разработан комплекс информационной поддержки для имитации течения вирусных заболеваний, а также фармакологической коррекции вызванных ими гипоксических состояний.

Ключевые слова: вирус SARS-CoV-2, модель иммунного отклика, математическая модель дыхательной системы, гипоксическое состояние, инфекционное поражение.

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PREVENTION OF CISPLATIN TOXICITY AGAINST NORMAL CELLS BY COMPLEXATION WITH C_{60} FULLERENE

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The aim of this study was to evaluate the toxicity of noncovalent nanocomplex of C_{60} fullerene with cisplatin (C_{60}-Cis-Pt) against normal cells. The toxicity of the C_{60}-Cis-Pt nanocomplex compared to the free Cis-Pt was studied by estimating kidney human embryonic (HEK293) cells viability using MTT assay and rat erythrocytes resistance to acid haemolysis. It was shown that free 40 μM Cis-Pt changed the morphology and reduced the viability of HEK293 cells, as well as increased the number of haemolyzed erythrocytes compared to the control. According to the investigated parameters analysis no cytotoxic effects of C_{60}-Cis-Pt nanocomplex was observed at Cis-Pt equivalent concentration. The prevention of Cis-Pt toxic action against normal cells by its complexation with C_{60} fullerene opens the prospect of nanostructure usage as an effective cytoprotector and a target carrier in tumor cells.

Key words: C_{60} fullerene, cisplatin, nanocomplex, HEK293 cells, cytotoxicity, erythrocytes, haemolysis.

The use of biologically active nanomaterials for targeted drug delivery, enhancement of the traditional anticancer drugs therapeutic efficacy and prevention of its side effects is a significant and complex problem of modern biotechnology. The representative of carbon nanostructures C_{60} fullerene is promising in this direction. It is a chemically stable, nanosized (0.72 nm), almost spherical and hydrophobic molecule that penetrates through biological membranes, localizes within cells [1–3]. As is known, chemical modification affects the physical, chemical and biological properties of C_{60} fullerene. Pristine C_{60} fullerene and its water-soluble derivatives do not cause toxic effects [1, 4, 5]. The accumulation of C_{60} fullerene in tumors of the liver, stomach, intestine, lungs, bones and its selective damaging effect on malignantly transformed cells was detected [6–8]. The surface structure of C_{60} molecule with a system of double π-conjugated electron-deficient bonds is unique and determines the properties of this nanostructure as an antioxidant (free radical scavenger) [9, 10] as well as its ability to generate reactive oxygen species (ROS) after UV-Vis light irradiation [11–14] that can be used in photodynamic therapy of tumors. Besides, C_{60} fullerene can form stable complexes with chemotherapeutic drugs [15–17], that can be used to optimize their action.

The traditional broad-acting anticancer drug is cisplatin (cis-diaminodichloroplatinum, cis-[Pt(II)$(NH_3)_2Cl_2$], Cis-Pt), the cytotoxic effect of which is caused by DNA damage and oxidative stress induction [18–22]. Despite the negative side effects of Cis-Pt, it is widely used in antitumor therapy. Clinical usage of

Cis-Pt might be increased with improving its selectivity, overcoming drug resistance and reducing toxicity.

To increase the effectiveness of Cis-Pt antitumor effect and minimize its side effects, a noncovalent nanocomplex of C_{60} fullerene with Cis-Pt (C_{60}-Cis-Pt) was created [23]. Estimation of C_{60}-Cis-Pt nanocomplex toxicity is an important prerequisite of its usage for biomedical purposes. Thus, the aim of this work was to estimate the toxic effect of free Cis-Pt against normal cells in comparison with C_{60}-Cis-Pt nanocomplex.

Materials and Methods

Creation of C_{60}-Cis-Pt nanocomplex. C_{60} fullerene aqueous colloid solution (C_{60}FAS) (150 μg/ml, $2–10^{-4}$ M, purity 99.95%) was prepared at the Technical University of Ilmenau (Germany) as described in [24, 25]. C_{60} FAS is characterized by a high C_{60} fullerene concentration and stable up to 12 months at +4 °C.

To generate C_{60}-Cis-Pt nanocomplexes, the C_{60} solution (150 μg/ml) and Cis-Pt (Sigma, USA) solution in 0.9% NaCl saline (150 μg/ml) were mixed in 1:1 volume ratio. The mixture was sonicated with ultrasound (22 kHz, 20 min) and stirred (400 rpm, 18 h). Final concentrations of C_{60} fullerene and Cis-Pt were 75 μg/ml (104 μM) and 75 μg/ml (250 μM), respectively. The stability of C_{60}-Cis-Pt nanocomplexes in the aqueous medium was confirmed by the results of the dynamic light scattering technique [26]. The calculated dissociation constant for the obtained noncovalent C_{60}-Cis-Pt nanocomplex is ~ 2 mM [27].

Cell culture. Non-tumor HEK293 (human embryonic kidney 293) cells were kindly supplied by the Bank of Cell Cultures and Transplantable Experimental Tumors of Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the National Academy of Sciences of Ukraine (Kyiv, Ukraine). Cells were maintained in DMEM (Sigma-Aldrich Co, Ltd, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co, Ltd, USA), 50 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were incubated for 24 h with or without free Cis-Pt or C_{60}-Cis-Pt nanocomplex in Cis-Pt equivalent concentration.

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich Co, Ltd, USA) reduction assay [28]. At indicated time points of incubation 100 μl aliquots ($10×10^3$ cells) were placed into the 96-well microplates Greiner (Sigma-Aldrich Co, Ltd, USA), 10 μl of MTT solution (4 mg/ml in phosphate-buffered saline (PBS)) was added to each well and the plates were incubated for another 2 h at 37 °C. Precipitates were dissolved with 100 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co, Ltd, USA). Diformazan formation was determined by measuring absorption at 570 nm with a microplate reader μQuant (BioTEK, USA).

Curve fitting and calculation of the half-maximal inhibitory concentration (IC_{50} value) were done using GraphPad Prism 7 (GraphPad Software Inc., USA). Briefly, individual concentration-effect curves were generated by fitting the logarithm of the tested compound concentration versus corresponding normalized percent of cell viability values using nonlinear regression.

Cells morphology was investigated using phase-contrast microscopy (Olympus CKX41SF, Japan). For light microscopy images an Olympus SP-500UZ (Indonesia) camera was used.

Erythrocytes haemolysis. Erythrocytes isolated from the heparinized rat blood, were incubated at 37 °C with or without C_{60}-Cis-Pt nanocomplex. Erythrocytes haemolysis was induced by addition of hydrochloric acid to the final concentration of 0.001 N [29]. Measurements of the haemolysis dynamics were carried out for 2 min with a 10 s interval on the spectrophotometer (Scinco, Germany) at $\lambda = 630$ nm.

All experiments with animals in this study were performed according to the Bio-Ethics Committee of the abovementioned institution.

Statistical analysis was performed using two-way ANOVA followed by post Bonferroni tests. The IC_{50} value was represented as M ± SD of more than four independent experiments. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Viability and morphology of HEK293 cells. Cytotoxic activity of C_{60}-Cis-Pt nanocomplex against HEK293 cells in Cis-Pt equivalent concentrations of 5–40 μM in comparison with the free drug was studied by MTT test at 24 h of incubation. The viability of cells incubated without additions of C_{60} fullerene, Cis-Pt, or C_{60}-Cis-Pt nanocomplex was taken as 100% (control).

No effect of C_{60} fullerene used alone in the range of 2.8–16.6 μM concentrations,

equivalent to those in C_{60}-Cis-Pt nanocomplex, on HEK293 cells viability during the incubation period was detected (data are not presented). The calculated IC_{50} values for C_{60} fullerene (IC_{50} = 530 μM) (Table 1) action on HEK293 cells showed that it is a low toxic compound. These results are in a good agreement with the literature data. Thus, the toxic effect of pristine C_{60} fullerene against normal baby hamster kidney BHK-21 cells were observed only at high 440 μM concentration [30].

We have detected the cytotoxic effect of Cis-Pt against HEK293 cells at 40 μM concentration. Under the action of the drug at this concentration cell viability at 24 h was reduced by 28% compared to the control (Fig. 1). The decrease of HEK293 cells viability by 43% compared to the control under the action of 50 μM Cis-Pt was also demonstrated in [31]. The calculated IC_{50} value for Cis-Pt was shown to be 75 μM (Table 1).

Table 1. **IC_{50} values for C_{60} fullerene, Cis-Pt and C_{60}-Cis-Pt nanocomplex in HEK293 cells ($M \pm m$, $n = 6$)**

Compounds	IC_{50} (μM), 24 h
C_{60} fullerene	530 ± 43
Cis-Pt	75 ± 5.6
C_{60}-Cis-Pt nanocomplex	90 ± 6.8*

Note: * — $P < 0.05$ in comparison with Cis-Pt.

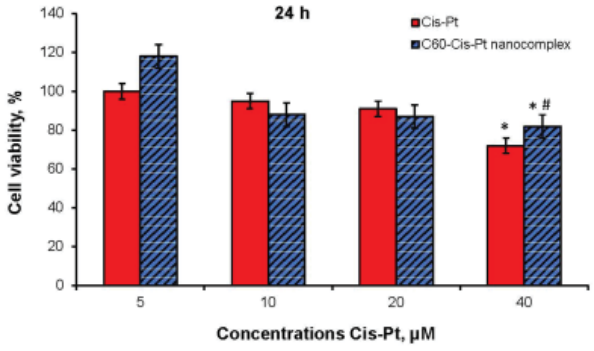

Fig. 1. **Viability of HEK294 cells treated with free Cis-Pt or C_{60}-Cis-Pt nanocomplex in Cis-Pt equivalent concentrations at 24 h of incubation ($M \pm m$, $n = 6$):**
* $P < 0.05$ in comparison with control (untreated cells); # $P < 0.05$ in comparison with Cis-Pt

With the action of 5 μM C_{60}-Cis-Pt nanocomplex the viability of HEK293 cells was increased by 20% compared to the control (Fig. 1), probably due to the initial adaptive response to the compound. C_{60}-Cis-Pt nanocomplex at 40 μM Cis-Pt equivalent concentration inhibited HEK293 viability, but the toxic effect appeared to be only 18% as compared to control (Fig. 1). The calculated value of IC_{50} for C_{60}-Cis-Pt nanocomplex was higher (90 μM) than that for free Cis-Pt (Table 1) confirming the decreased cytotoxicity of Cis-Pt against non-tumor cells at complexation with C_{60} fullerene.

Morphological studies showed that untreated (control) HEK293 cells formed elongated epithelioid structures and dense monolayer in some areas, a large number of intercellular contacts were observed. The cytotoxic effect of 40 μM Cis-Pt on HEK293 cells was evidenced by morphological changes of cells (Fig. 2, Table 2).

Most of HEK293 cells treated with 40 μM Cis-Pt were characterized by atypical morphology and smaller size (Table 2). No evident effect of C_{60}-Cis-Pt nanocomplex in equivalent 40 μM Cis-Pt concentration on cells morphology was detected.

Therefore, the toxic effect of Cis-Pt on normal cells at complexation with C_{60} fullerene was reduced. The protective effects of C_{60} fullerene against the toxic effects of Cis-Pt may be due to the antioxidant properties of the carbon nanostructure [32, 33, 34]. We have previously shown that C_{60} fullerene prevented ROS production in thymocytes and prevented the decrease of thymocytes viability induced by Cis-Pt [35, 36].

Erythrocytes haemolysis. The use of platinum-based drugs in chemotherapy is limited due to its high haematotoxicity, that is the cause of haemolytic anemia and bone marrow disease [37]. So the search for erythrocyte protection pathways against drug damage is of current interest.

The effect of Cis-Pt and C_{60}-Cis-Pt nanocomplex at the level of cells plasma membrane was estimated by the dynamics of erythrocytes haemolysis, which reflects the dynamics of erythrocyte plasma membrane destruction and the release of haemoglobin into the environment. Erythrocytes haemolysis of the control (untreated red blood cells) was accelerated at 40 s after the treatment with haemolytic and reached the maximum at 60 s, the number of haemolyzed cells was 30 ± 2% (Fig. 3).

Haemolysis of erythrocytes treated with 40 μM Cis-Pt slightly slowed, however, the

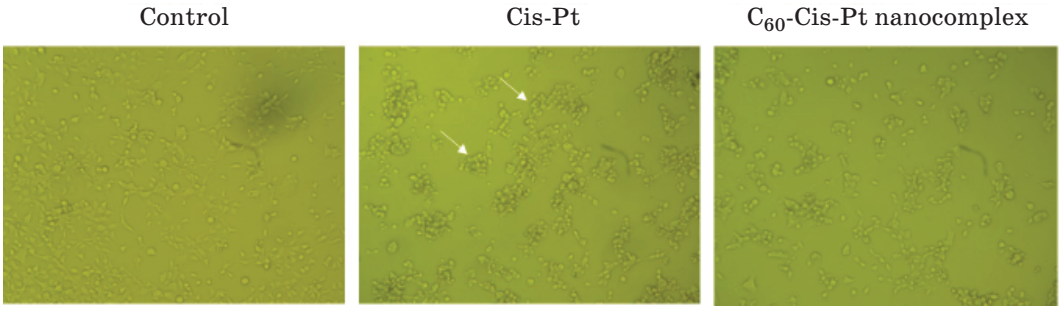

Fig. 2. **Microphotographs of HEK293 cells incubated for 24 h in the presence of free 40 μM Cis-Pt or C_{60}-Cis-Pt nanocomplex** (phase-contrast microscopy, × 400)

Table 2. **Morphological features in HEK293 cells at 24 h after action 40 μM Cis-Pt or C_{60}-Cis-Pt nanocomplex** ($M \pm m$, $n = 6$)

Compounds	Changes		
	Atypical cell morphology	Smaller cell size	Roud shape and nonadherent pattern
Cis-Pt	++++	++	++++
C_{60}-Cis-Pt nanocomplex	+	–	+

Note: + few, ++ moderate, +++ severe, ++++ many.

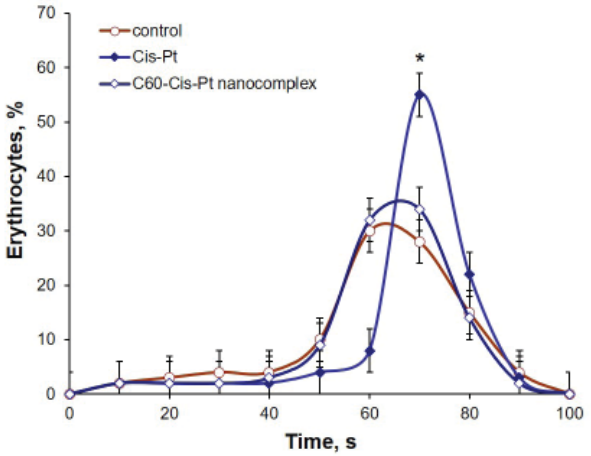

Fig. 3. **Erythrocytes haemolysis after action 40 μM Cis-Pt or C_{60}-Cis-Pt nanocomplex** ($M \pm m$, $n = 6$): * $P < 0.05$ in comparison with control (untreated cells)

number of haemolyzed cells reached 55 ± 2% (Fig. 3), indicating on Cis-Pt-induced decrease of cells resistance to haemolysis. As it was shown in [38], Cis-Pt at 35 μM concentration caused morphological changes of human erythrocyte membrane by embedding into the inner layer of cells. The extranuclear mechanism of Cis-Pt cytotoxic action is associated with its high affinity to phosphatidylserine the inner monolayer of the erythrocyte plasma membrane [39].

C_{60} fullerene at 16.6 μM concentration equivalent to that in C_{60}-Cis-Pt nanocomplex did not change parameters of erythrogram, indicating on the absence of haemolytic effect of carbon nanostructure (data are not presented). As it was shown by us previously [40, 41], the slight haemolytic activity of C_{60} fullerene was detected at concentrations > 50 μM.

No haemolytic effect was detected when erythrocytes were treated with C_{60}-Cis-Pt nanocomplex at 40 μM Cis-Pt equivalent concentration. The parameters of erythrogramm were the same as in control (Fig. 3) indicating that C_{60} fullerene weakened Cis-Pt interaction with erythrocyte membrane and was able to increase the resistance of erythrocyte membrane to Cis-Pt induced haemolysis.

The data obtained showed that the cytotoxic effect of Cis-Pt against normal cells was prevented at complexation with C_{60} fullerene and that C_{60}-Cis-Pt nanocomplex has a potential to be used for minimization of anticancer drug side effects.

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The authors declare that they have no conflicts of interest.

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ЗАПОБІГАННЯ ТОКСИЧНІЙ ДІЇ ЦИСПЛАТИНУ ЩОДО НОРМАЛЬНИХ КЛІТИН ЗА КОМПЛЕКСОУТВОРЕННЯ ІЗ C_{60} ФУЛЕРЕНОМ

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Метою роботи було оцінити токсичність нековалентного нанокомплексу C_{60} фулерену з цисплатином (C_{60}-Cis-Pt) щодо нормальних клітин. Токсичність C_{60}-Cis-Pt нанокомплексу, порівняно з вільним Cis-Pt, вивчали на клітинах ембріональної нирки людини (HEK293), оцінюючи їх життєздатність за допомогою МТТ-тесту та на еритроцитах щура за їхньою стійкістю до кислотного гемолізу. Було показано, що вільний 40 мкМ Cis-Pt змінював морфологію та знижував життєздатність клітин HEK293 на 28%, а також збільшував кількість гемолізованих еритроцитів на 25% порівняно з контролем. C_{60}-Cis-Pt нанокомплекс за еквівалентної концентрації Cis-Pt не впливав на досліджувані показники і не спричиняв цитотоксичних ефектів. Запобігання токсичній дії Cis-Pt на нормальні клітини за його комплексоутворення із C_{60} фулереном відкриває перспективу використання наноструктури як ефективного цитопротектора і таргетного носія у пухлинні клітини.

Ключові слова: C_{60} фулерен, цисплатин, нанокомплекс, HEK293 клітини, цитотоксичність, еритроцити, гемоліз.

ПРЕДОТВРАЩЕНИЕ ТОКСИЧЕСКОГО ДЕЙСТВИЯ ЦИСПЛАТИНА НА НОРМАЛЬНЫЕ КЛЕТКИ ПУТЕМ КОМПЛЕКСООБРАЗОВАНИЯ С C_{60} ФУЛЛЕРЕНОМ

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Целью работы было оценить токсичность нековалентного нанокомплекса C_{60} фуллерена с цисплатином (C_{60}-Cis-Pt) относительно нормальных клеток. Токсичность C_{60}-Cis-Pt нанокомплекса, в сравнении со свободным Cis-Pt, изучали путем оценки жизнеспособности клеток эмбриональной почки человека (HEK293) с помощью МТТ-теста и на эритроцитах крысы по их устойчивости к кислотному гемолизу. Было показано, что свободный 40 мкМ Cis-Pt вызывал морфологические изменения и снижение жизнеспособности клеток HEK293 на 28%, а также увеличение количества гемолизированных эритроцитов на 25% по сравнению с контролем. C_{60}-Cis-Pt нанокомплекс в эквивалентной концентрации Cis-Pt не влиял на исследуемые показатели и не вызывал цитотоксических эффектов. Предотвращение токсического действия Cis-Pt на нормальные клетки при комплексообразовании с C_{60} фуллереном открывает перспективу применения наноструктуры как эффективного цитопротектора и таргетного носителя в опухолевые клетки.

Ключевые слова: C_{60} фуллерен, цисплатин, нанокомплекс, HEK293 клетки, цитотоксичность, эритроциты, гемолиз.

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STUDIES OF EFFICIENCY OF THE COMPOSITE SYSTEM "LYMPHOSILICA" IN MODELING EXPERIMENTAL OBESITY IN RATS

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The features of the influence of the nanocomposite system (Lymphosilica supplement), which was created on the basis of hydroconsolidated finely divided silica and plant materials with a high content of polyphenols, flavonoids, terpenes, polysaccharides on liver cytophysiological characteristics under the conditions of the development of metabolic syndrome and hepatotoxicity under simulated alimentary, were studied.Biochemical studies of blood serum and histological analysis of liver tissue were performed. It was shown that a high-calorie diet of rats over 100 days leads to the development of individual manifestations of obesity and metabolic syndrome, which are expressed in an increase in body weight and visceral fat, the development of the initial and middle stages of parenchymal fatty liver dystrophy with a decrease in morphological manifestations of the synthetic activity of the nucleus, with slight deviations at the biochemical level.The introduction of a phytocomposite does not lead to pathological changes in the organism of rats, but in some cases leads to manifestations of "adaptive stress" of liver hepatocytes, both with a standard diet and with a high-calorie diet. The positive effect of the composite system on reducing the proportion of visceral fat (by 38%) in rats with a standard diet was shown, and against the background of simulated alimentary obesity, normalization of the level of bilirubin (25 % decrease compared with alimentary obesity) and its fractions, lower levels cholesterol and alkaline phosphatase (in both cases by 19% compared with initial obesity), increased globulin fractions of blood serum, protective effect against dystrophic changes in liver tissue.

Key words: nanocomposite, Lymphosilica, nanosilica, rats, alimentary obesity, hepatocytes of the-liver, visceralfat.

The efficacy (biological activity) of pharmacological preparations can be enhanced by inclusion in the dosage form the substances that provide a dosed, prolonged release of active substances, their delivery in an intact state to the absorption site and activity sensitizers, which create accelerated mass transfer zones in the absorption sites ensuring increased drugs bioavailability. It was assumed that creation of such systems would reduce the pharmacological burden on a body by increasing the bioactivity and bioavailability of pharmaceuticals and eliminate the possibility of temporary hyperconcentration of toxic substances in various parts of the body. In particular, the inclusion of drugs in a matrix of biocompatible polymers, such as polyacrylamide, polyurethane, polyvinyl alcohol, etc. would significantly slow down (from several minutes to several days) the process of active substances releasing [1, 2]. However, notwithstanding the polymers used are easily combined with body tissues and do not cause adverse reactions, their elimination is a long multi-stage process. An alternative direction is the use of biodegradable polymers and biopolymers in composites with highly dispersed fumed silica (HDS). At the same time, HDS can perform several functions. It creates a spatially ordered system that

provides optimal conditions for bioactive drugs releasing; serves as a rate controller of substances releasing from the cellulose matrix of medicinal plants; acts as a means of drug delivery to the intestinal mucosa and influences actively on mass transfer in the absorption zone due to the formation of nanostructured layers of aqueous solutions with increased solubility of weakly polar organic compounds (which are related the majority of drugs) [3].

Silica is widely used for biomedical purposes not only as a concomitant substances giving the dosage forms the necessary physico-chemical properties, but also as an independent drug with a pronounced detoxifying effect, well established in the treatment of food poisoning, bacterial and wound infections [4–6]. In recent years, technologies have also been developed for the use of porous silicas as means of targeted drug delivery, involving the creation of nanoscale channels in silica film samples into which a drug can be introduced [7–9]. One of the most important applications of such nanotechnologies is the development of new systems for the controlled release of drugs, with their protection against physiological degradation or elimination [10, 11].

As a medicine for creating a nanocomposite system based on hydrocondensed HDS, the complex highly dispersed plant composite system "Lymphosilica" was used, developed at Chuiko Institute of Surface Chemistry of the National Academy of Sciences of Ukraine [12], containing the components of raw Echinacea purpurea, Sudanese rose, dandelion root, enriched with polyphenols, in particular flavonoids (anthocyanins, quercetin), terpenes, polysaccharides.

The effectiveness of the composite system is due to the fact that plant polyphenolic compounds are able to mobilize their own mechanisms of homeostasis in a living organism, stimulate the function of the adrenal cortex, and the secretion of glucocorticoid hormones. Besides, due to HDS presence, it is detoxicant and has a high antioxidant, wound healing, antimicrobial, antifungal, and protistocidal action [13, 14]. Polyphenolic compounds are aplied in medical practice for acute and chronic colitis, enteritis, gastritis, inflammatory processes of the oral cavity, ulcers, for detoxification and recovery of the body in chronic diseases. Particularly important is the positive effect of polyphenols on pathogenesis of such conditions as metabolic syndrome, obesity, diabetes, fatty liver. Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver damage worldwide [15]. Over the past decades, the use of herbal preparations for NAFLD has received increasing attention due to their wide availability, low side effects and proven therapeutic mechanisms [16].

The aim of this work was to study the effects of "Lymphosilica" composite system created on the basis of hydrocondensed silica and plant materials on the manifestations of metabolic syndrome and hepatotoxicity in simulated alimentary obesity in rats.

Materials and Methods

Research drugs. We used finely dispersed silica of the A-300 grade obtained by the Kalush experimental plant of the Chuiko Institute of Surface Chemistry of the National Academy of Sciences of Ukraine. The composition of the phytocomposite "Lymphosilica" includes hydroconsolidated silica (Hidrosil, TU U 20.1-3291669-015:2016) with a bulk density of ρ_d = 300 mg/cm^3 and components of micronized plant material — *Hibiscus sabdariffa, Taraxacum officinale, Calluna vulgaris, Calendula officinalis, Trifolium pratense, Echinacea purpurea, Elytrigia repens*. When creating the composite system "Lymphosilica", silica and phytocomponents were mixed by four-hour processing in a ball mill. The content of the plant component was 30 wt.%.

Experimental groups of animals. The rat parameters of seven experimental groups were analyzed in the work (n = 8 in each group) (Table 1). The initial body weight of the animals was 120 ± 7 g. An initial experiment was preliminarily carried out. During 95 days it was modeling alimentary obesity in animals. Herewith the use of highly dispersed silica (HDS) both in pure form and in the said of a nanocomposite system was applied. At the same time, some of the animals were kept on a standard diet, while the rest simulated the development of the metabolic syndrome through the use of high-calorie diets enriched in fats and carbohydrates [17]. The animals received pure HDS, pure phytopreparation (a mixture based on raw materials of Echinacea, Sudanese rose, dandelion rhizome, enriched with polyphenolic compounds), or a composite system based on HDS and phytocomposite ("Lymphosilica") as well. The drugs were administered orally with feed water in the form of an aqueous suspension in a volume of 2 ml per animal (in the morning, 1 hour before a meal).

In all groups of animals, at the end of the period of modeling obesity after instant decapitation, the general morphometric parameters of the body (body weight, growth-weight coefficient (body mass index — Ketle index), and the mass of visceral and subcutaneous fat) were determined. For biochemical and histological analysis, blood serum and organs of the digestive, excretory and endocrine systems, as well as heart and adipose tissue of various types and localizations (white and brown fat, subcutaneous and visceral fat) were selected.

All animal experiments were performed in compliance with the international principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), Article 26 of the Law of Ukraine "On the Protection of Animals from Cruel Treatment" (No. 3447-IV, 02.21.2006), as well as all the norms of bioethics, biological safety and general ethical principles of animal experiments adopted by the First National Congress of Ukraine on Bioethics (September 2001).

Biochemical analysis of blood serum. In the blood serum of all the studied rats, the levels of total bilirubin and its individual fractions were determined [18] as well as total plasma protein, its albumin and globulin fractions [19, 20], glucose [21], cholestrol [22], and the activity of the enzymes alanine and aspartate aminotransferase (ALT and AST, respectively) [23, 24], alkaline phosphatase (ALP) [25]. The level of bilirubin and its fractions was determined using the Biosystems S.A. diagnostic kit. (Spain). The levels of all other indicated serum components and its enzymatic activity were determined using standard LiquickCor diagnostic kits (Cormay, Poland). Spectrophotometric measurements were performed on a Sinnowa BS-3000M biochemical analyzer.

Histological analysis. In order to study the effect of nanocomposite systems on the development of histopathological processes in the liver, a macroscopic analysis was done (description of the liver in appearance, anatomical structure, color of the organ, state of the parenchyma and capsules), and histological analysis of this organ. For histological examination, a standard preparation technique was used. To do this, the liver was fixed in 10% formalin, pieces of the organ 1.5×1.5 cm in size were dehydrated, enlightened and soaked in paraffin. The sections of 5–6 μm thick were stained with hematoxylin and eosin to obtain an examination histological specimen.

Subsequently, the obtained histological preparations were analyzed using a light microscope in order to reveal morphological changes in the structure of the histostructure of the lobules of the liver and hepatocytes (the area of hepatocyte nuclei, the state of blood vessels, pathomorphological changes). To do this, micrographs were taken using the System Microscope installation with an Olympus BX 41 system video camera equipped with a Camedia C-5050 zoom digital camera and Olympus DP 80 FT 3.2 software based on a Pentium 4 computer with Windows XP operating system. To evaluate morphometric changes in digital micrographs using the ImageJ program, we measured the area of hepatocyte nuclei (150–250 values per each

Table 1. **Experimental groups of animals (n = 8 in each group)**

Group designations	Experiment Conditions
1 K	Control, standard food and water regimen
2 A	Standard food regimen + aqueous suspension of HDS (0.3 g/kg body weight, in the morning, 1 hour before meal)
3 F	Standard food regimen + phytopreparation suspension (0.3 g dry matter/kg body weight, in the morning, 1 hour before meal)
4 AF	Standard food regimen + composite system (HDS suspension with phytopreparation, 0.3 g/kg, in the morning, 1 hour before meal)
5 O	High-calorie diet (modeling of obesity, without additional administration of other substances)
6 OF	High-calorie diet + suspension of herbal remedies (0.3 g/kg, in the morning, 1 hour before meal)
7 OAF	High-calorie diet + composite system (0.3 g/kg, in the morning, 1 hour before meal)

series) and calculated the percentage of 1- and 2-nuclear cells and performed statistical analysis using the Student's *t*-test, while the difference was considered probable at $P<0.05$.

Results and Discussion

Morphometric parameters of rats in groups with different diets and the use of additives based on silica.

It was found that differences in the weight of animals in groups with different diets were observed, starting from 75 days of modeling obesity. During this period, a decrease in body weight gain was revealed in animals that received pure HDS, phytopreparation, or a composite system of HDS + phytopreparation (Fig. 1). This primarily concerns animals that were on a standard diet (groups 2A, 3F, 4AF). In animals on a high-calorie diet, a decrease in weight gain with the introduction of a sorbent, herbal preparation or composite system (groups 6 OF, 7 OAF) did not cause significant changes compared with animals that were on the same diet, but without additives.

During the experimental period, no significant difference was found in the growth-weight coefficient (the Ketle body mass index varied in different groups in the range of 6.51–7.34) and in the weight of subcutaneous fat in animals of all groups, except for the group (6 OF), receiving herbal medicine against the background of a high-calorie diet (Fig. 2). At the same time, the weight of visceral fat, which accumulation is one of the manifestations of the metabolic syndrome, was significantly lower in animals treated with both free herbal medicine (by 34%) and a composite system (by 38%) (Fig. 2). However, this decrease applies only to animals that were on a standard diet, while against a background of high-calorie nutrition, the mass of visceral fat not only keep up decrease, but even increases with additional introduction of phytopreparation (Fig. 2), which, obviously, promotes absorption excess carbohydrate and lipid components.

Biochemical parameters of rat blood serum in groups with different diets and when using additives based on silica.

Determination of total protein and protein fractions in blood serum gives an idea of the level of protein nutrition and helps to diagnose manifestations of hepatopathy and nephropathy. The concentration of total protein in serum depends mainly on the synthesis and decomposition of two main protein fractions: albumin and globulins.

Our studies showed a significant (11%) increase in total blood protein in groups 2 A (standard diet with HDS) and 7 OAF (composite nanosystem against a high-calorie diet) (Table 2). These changes are due to an increase in the absolute and relative content of globulins, with a constant absolute content of albumin. Such growth is small (6–9%), but significant, both when pure HDS is introduced into the diet of animals in comparison with the control group, and in the groups receiving the studied additives (phytopreparation or composite system) against the background of obesity. An increase in the level of globulins may be due to activation of the synthetic activity of the liver in response to xenogenic substances and enhancement of the immune properties of the blood [22].

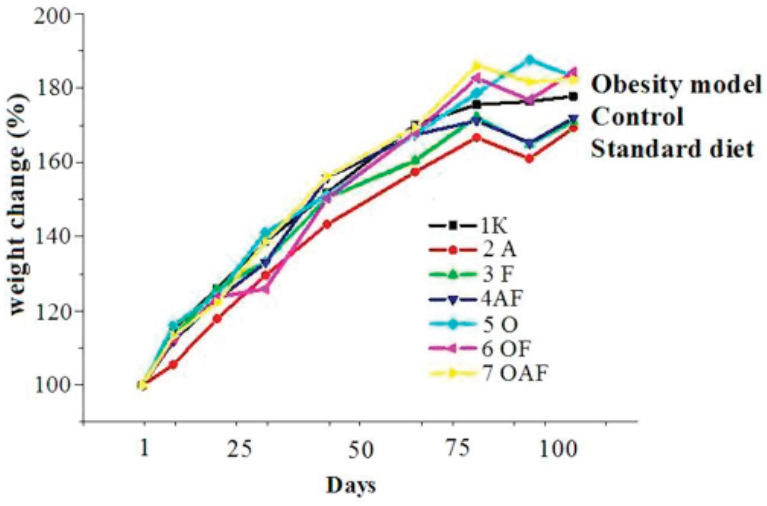


Fig. 1. **Change in weight of rats using a standard and high-calorie (O) diet, as well as additional substances -HDS (A), herbal medicine (F), composite system (AF)**

Table 2. **Indicators of body mass parameters (growth-weight Quetelet index, subcutaneous and visceral fat mass) of rats using standard and high-calorie diets and additional substances**

Group	Ketle Index			Subcutaneous fat. g			Visceral fat. g		
	M	σ	m	M	σ	m	M	σ	m
1 K	6.95	0.30	0.12	2.31	0.39	0.16	5.2	0.81	0.33
2 A	6.79	0.56	0.21	2.25	0.55	0.21	4.78	1.95	0.74
3 F	6.68	0.61	0.23	2.23	0.25	0.09	3.43*	0.51	0.19
4AF	6.72	0.91	0.32	2.14	0.23	0.08	3.24*	0.93	0.33
5 O	7.00	0.70	0.26	2.68	0.45	0.17	6.81	1.89	0.71
6 OF	7.34	0.64	0.24	3.17*	0.56	0.21	8.15*	2.12	0.80
7 OAF	6.51	0.66	0.27	2.90	0.91	0.37	7.09	4.44	1.81

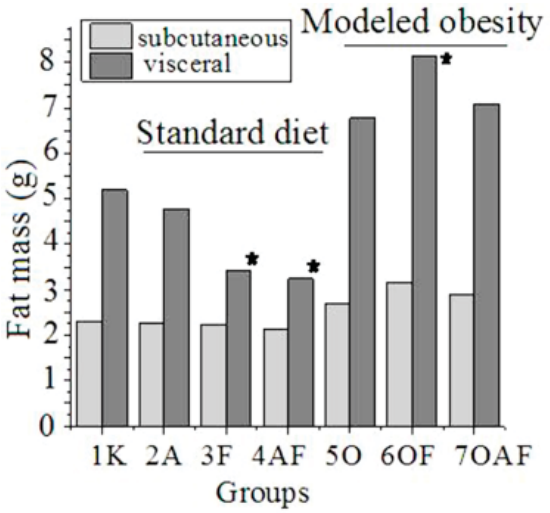


Fig. 2. **Weight of visceralf when using a standard and high-caloriediet and additional substances — silica (2A), polyphenolic herbal medicine (3F), composite system (4AF)**
* — $P < 0.05$ 05 compared with the control group

When studying the level of bilirubin fractions in blood plasma, an insignificant but reliable increase in its level (from 37% to 70%) was found as with the use of HDS, herbal medicine and composite system in a midth of a standard diet, as with alimentary obesity (Fig. 3, *a*), although it is well within the physiological norm. At the same time, in case of high-calorie diet using a phytopreparation and nanocomposite system, the level of total and direct bilirubin significantly decreases compared to obese animals that did not receive these additives. The described changes are determined mainly by the indirect bilirubin fraction (Fig. 3, *b*), the first stage of bilirubin formation from hemoglobin, which is carried out by the cells of the reticuloendothelial system, most of which are localized in liver (80%, Kupffer cells), as well as in spleen and bone marrow. Preliminarily, an increase in the level of indirect bilirubin can be explained by the "adaptive stress" of liver cells in response to the intake of foreign substances and excess nutrition.

Direct bilirubin (conjugated and soluble form) is formed in hepatocytes to remove bilirubin from the body. Its going down to the control level when it is used affected by obesity, a phytopreparation, and especially a nanocomposite system based on HDS (Fig. 3, *c*), demonstrates normalization of bile formation by hepatocytes and its outflow through both the intrahepatic and extrahepatic ducts under such conditions.

The hepatoprotective effect of the studied systems is also in evidence when they affect the level of aminotransferases — enzymes that indicate the state of hepatocytes. Under the influence of both a free phytocomplex and the use of a composite system (but not pure HDS), an insignificant (by 15–20%), but reliable decrease in the activity of alanine aminotransferase (ALT) was found in animal groups on both standard and high-calorie diets (Table 3). The level of aspartate aminotransferase (AST) remained unchanged in all groups.

Alkaline phosphatase (ALP) is an enzyme that is extremely common in human and animal tissues, especially in the intestinal mucosa, osteoblasts, and walls of the bile ducts of the liver. It catalyzes the removal of phosphoric acid from its organic compounds with an optimum pH in an alkaline environment (pH 8.6–10.1). The enzyme is contained in the cell membrane and is involved in the transport of phosphorus. The serum alkaline phosphatase activity represents the total activity of its

Table 2. **Levels of total protein and protein fractions in rat blood serum using standard and high-calorie diets and additional substances —silica (A), polyphenol herbal medicine (F) and composite system (AF)**

Group	Total protein, g/l			Albumen,%			Globulin,%		
	M	σ	m	M	σ	m	M	σ	m
1 K	81.0	7.13	2.52	50.75	1.58	0.56	49.25	1.58	0.56
2 A	90.0*	3.38	1.19	46.25*	2.053	0.73	53.75*	2.05	0.73
3 F	84.75	2.76	0.98	51.75	3.57	1.26	48.25	3.58	1.26
4 AF	82.75	6.86	2.43	51.75	1.39	0.49	48.25	1.39	0.49
5 O	82.0	2.51	0.89	50.25	3.499	1.23	49.75	3.49	1.23
6 OF	80.0	3.62	1.28	47.5*	1.60	0.57	52.5*^	1.60	0.57
7 OAF	89.0*^	2.00	0.71	46.5*^	0.93	0.33	53.5*^	0.93	0.33

Note: * — $P < 0.05$ compared with the control group (1K);
^ — $P < 0.05$ compared with the group with simulated obesity (5O).

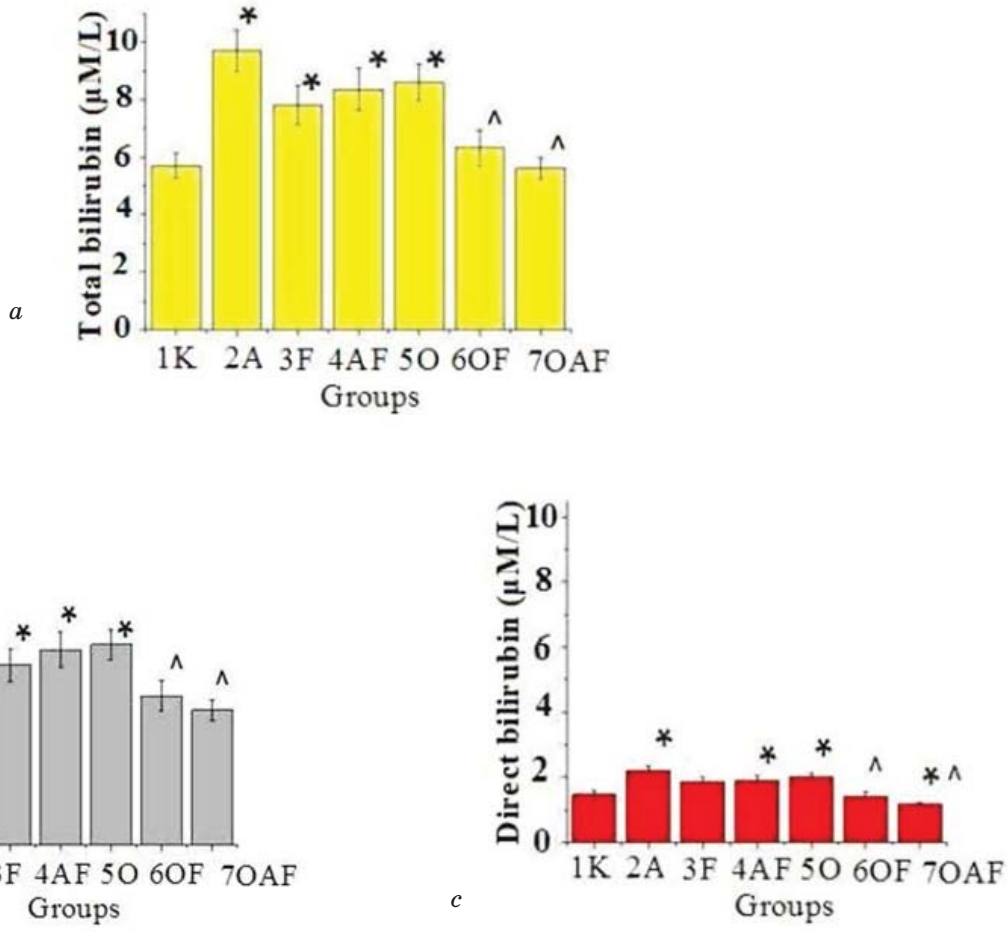


Fig. 3. **The levels of bilirubin (*a* — total; *b* — indirect; *c* — direct) in the blood serum of rats using a standard and high-calorie diet, as well as additional substances — HDS (A), herbal medicine (F), composite AF system:**
* — $P < 0.05$ compared with the control group (1K);
^ — $P < 0.05$ compared with the group with simulated obesity (5O)

Table 3. **The activity levels of alanine and aspartate aminotransferase in rat blood serum using standard and high-calorie diets and additional substances — HDS (A), herbal medicine (F), composite system (AF)**

Group	АЛТ			АСТ		
	M	σ	m	M	σ	m
1 К	118.75	13.61	4.81	306.50	46.83	16.56
2A	122.00	11.81	4.17	291.25	24.58	8.69
3F	98.25*	5.47	1.93	290.75	31.77	11.23
4AF	96.25*	5.68	2.01	315.50	24.73	8.74
5O	92.00*	10.90	3.85	306.75	61.50	21.74
6OF	64.98*^	5.60	1.98	271.25	47.03	16.63
7OAF	101.57	11.93	4.22	351.00	76.52	27.05

Note: * — $P < 0.05$ compared with the control group (1K);
^ — $P < 0.05$ compared with the group with simulated obesity (5O).

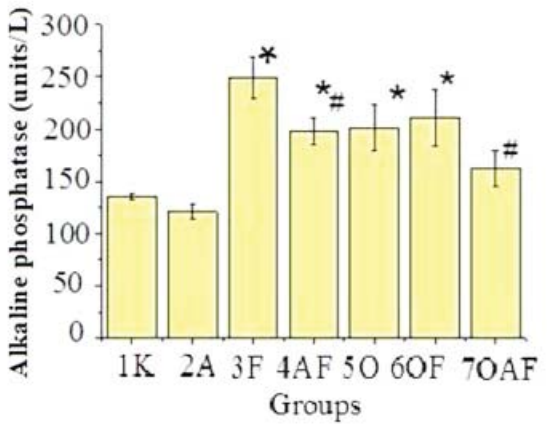


Fig. 4. **Levels of activity of alkaline phosphatase in rat blood serum using a standard and high-calorie diet and additional substances — HDS (A), herbal medicine (F), composite system (AF)**
* — $P < 0.05$ compared with the control group (1 K);
# — $P < 0.05$ compared with the group of the corresponding diet with the addition of a free herbal remedy.

isoenzymes. Hepatic and bone fractions of alkaline phosphatase are always present in the blood serum of adults, and the activity of the hepatic fraction is especially high. Its increase is noted in liver diseases, in particular, bile duct obstruction and inflammation [22, 25].

In our studies, the activity of alkaline phosphatase did not change with daily use of HDS for a month. However, it grew when modeling obesity and especially when using a herbal preparation against the background of such obesity. This can be explained by the known stimulating effect of the use of various medicinal substances on the functional state of the liver and, in particular, on the activity of alkaline phosphatase [22]. At the same time, the use of phytocomponents as part of a composite system with HDS (both on the standard and high-calorie diets — groups 4 AF and 7 OAF) led to a significant decrease in such activity (Table 5, Fig. 4). Obviously, this is explained by a slower release of phytocomponents from the composite system and, accordingly, a decrease in their stimulating effect (as xenobiotics) on the enzymatic activity of hepatocytes.

Cholesterol is a secondary monoatomic cyclic alcohol that is constantly formed in a body and is used to build cell membranes, synthesize sex and steroid hormones, and vitamin D. Cholesterol enters the body with food, but most of it is synthesized in a liver. The content of cholesterol and triglycerides in blood are the most important indicators of lipid metabolism. There is a direct correlation between an increase in plasma cholesterol concentration and the risk of atherosclerotic coronary artery disease.

Over the period of studies, the level of cholesterol in the blood serum of laboratory animals with a high-calorie diet does not differ from that in animals that were on a standard diet, which is obviously explained by a very short period of modeling the state of obesity, when changes in metabolic processes are at the initial stages of changes. However, an increase in cholesterol level (by 24%, which does not go beyond the physiological norm) was observed in animals receiving pure HDS and a significant

decrease (by 18% in comparison with both the control and the modeled obesity group) in animals receiving a nanocomposite system with a high-calorie diet) (Fig. 5). The highest increase in cholesterol in the group treated with pure silica sorbent, according to our assumptions, could be associated with the adsorption capacity of silica and a corresponding decrease in protein fractions of blood plasma, which were also involved in the transportation of cholestrol in the bloodstream. However, such an assumption requires separate experimental evidence.

Morphological studies of the liver under conditions of application of various diets and additives based on silica and phyto-raw materials.

Today, one can consider the association of fatty degeneration of liver with metabolic disorders in a body such as obesity, diabetes mellitus, dyslipidemia, hypertension and a high risk of cardiovascular disorders against the background of this pathology. Moreover, it was shown that not only insulin resistance could lead to fatty hepatosis, but fatty liver disease itself could be the cause of hepatic insulin resistance, especially for type II diabetes, which is registered in more than 370 million people in the world, and the annual increase in patients with this pathology accounts for 5–7% [25, 26]. According to various authors, the prevalence of fatty hepatosis in human's ranges from 6.3% to 33% [28]. Moreover, in patients with various metabolic disorders, such as obesity, diabetes

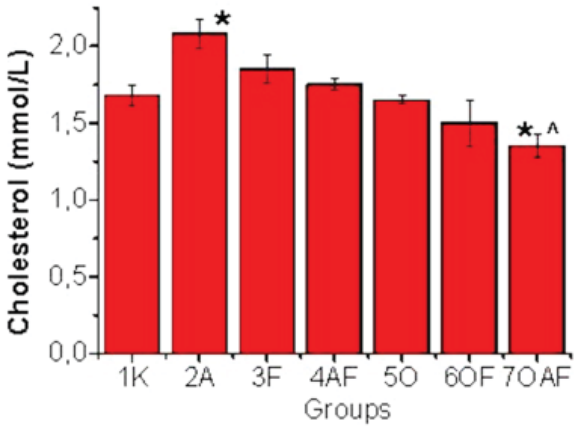


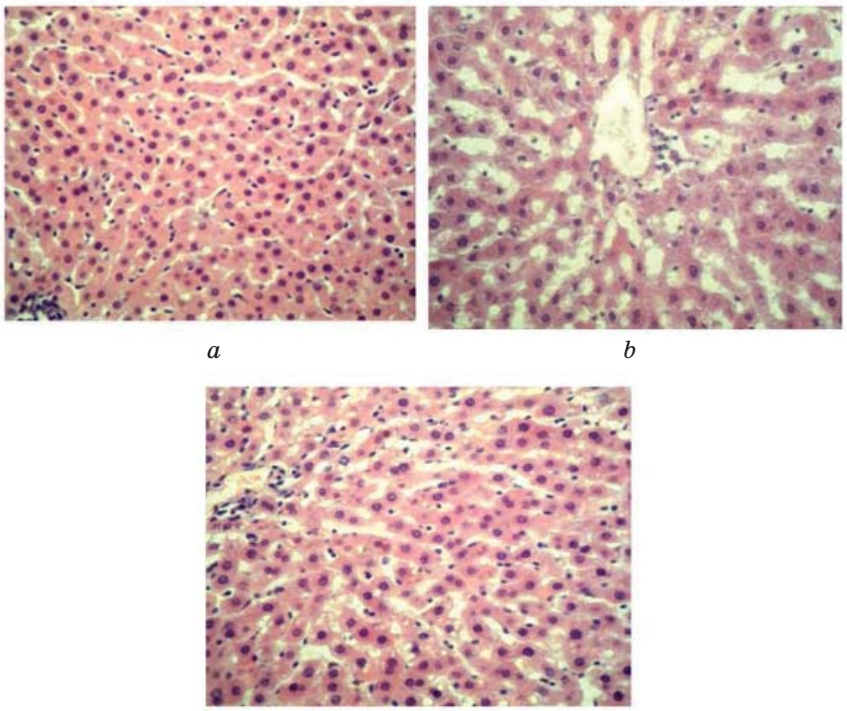
Fig. 5. **The level of cholesterol in the blood plasma of rats using a standard and high-calorie diet and additional substances — HDS (A), herbal medicine (F), composite system (AF):**
* — $P < 0.05$ compared with the control group (1K);
^ — $P < 0.05$ compared with the group with simulated obesity (5O)

mellitus, dyslipidemia, the presence of fatty liver disease can reach 75–100%.

Fatty change of liver is microscopically displayed by sharp increase in the content and change in fat composition in hepatocytes. In the liver cells, dusty granules of lipids first appear (the so-called pulverized obesity), then their small drops (so called small-drop obesity), then they merge into large drops (large-drop obesity) or one fatty vacuole that fills the entire cytoplasm and moves the nucleus to the periphery. Modified in this way the liver cells resemble fat. It is proved that more often fat deposition in liver begins on periphery, less often in the center of the lobules. Lipid deposition is diffuse in expressive fatty degeneration of liver cells [27–29].

It is found that in the rats' liver of all groups (both control and experimental), all its macroscopic parts are well defined, the capsule is not strained, the organ surface is smooth, the parenchyma in the section has a uniform red-brown color. Thus, with rat autopsy, no visual deviations from the norm were detected in liver of all the studied groups in comparison with control animals. Based on the analysis of histological preparations, it was found that the liver histostructure in intact animals (control group 1K) has the usual structure, in particular, the structure of the lobule parenchyma and the radial orientation of the hepatic trabeculae are preserved, the interlobular connective tissue layers are moderately expressed. Hepatocytes have a polygonal shape, contain clearly visible nuclei (mainly 1) with 2–3 nucleoli, the cytoplasm is slightly pink (Fig. 6). The severity of anisonucleosis is negligible, the number of binuclear cells is moderate. No disturbances were detected in the microvasculature (Fig. 6).

So, we can conclude about the moderate functional state of the liver of rats in the control group. When intact rats of three series were administrated orally a composite system based on silica, pure silica, plant material (herbal medicine) respectively, the liver histostructure was practically unchanged compared to the control. The trabecular pattern of hepatocytes inside the lobules was preserved, the severity of anisonucleosis was negligible and could be compared with the intact control. On most micropreparations, hepatocytes retained their polygonal shape, homogeneous color of the cytoplasm, and a clear structure of the nuclei. Microcirculatory disorders were not detected. Sinusoidal capillaries did not contain blood, were moderately dilated (Fig. 6). The measurements of the area of hepatocyte nuclei



a *b*

c

Fig. 6. **Micrographs of sections of the liver of rats of the control group (*a*), with alimentary obesity (group 5 O, *b*) and with the introduction of a composite system against obesity (group 7 OAF, *c*)**
Hematoxylin and eosin staining. ×600

in these experimental series did not change (Fig. 7). So, according to the cytoarchitectonics of lobules, trabeculae and the state of hepatocytes, the liver of these animals with the introduction of a composite system, HDS or phytopreparation corresponded to the physiological norm as in the control series of the experiment.

In the animals' liver that were on a carbohydrate and fat-fortified diet (alimentary obesity), significant dystrophic changes in the organ parenchyma were found that corresponded to small and large droplet parenchymal fatty degeneration of hepatocytes. In particular it was the presence of clusters of small drops, sometimes a very large "vacuole" in the cytoplasm of hepatocytes. Hepatocytes in the form of fat cells were appeared. In some places, the normal trabecular structure of particles was significantly disturbed and sinusoidal cavities containing blood cells, mainly red blood cells, expanded dramatically. Anisonucleosis is intensified, and elongated and irregular in shape pyknotic nuclei appeared. At the same time, the synthetic activity of the nuclei decreased compared with the control, since their area was significantly reduced (Fig. 6, 7).

Under conditions of alimentary obesity and introduction of a composite system based on silica or the initial phytopreparation to rats, an improvement in the morphological and functional state of liver was revealed. It manifested itself in normalization of liver trabecular structure, pronounced narrowing of sinusoidal cavities, which practically did not contain blood. Herewith, the sections of hepatocytes, mainly with dust- and small-droplet obesity, are sometimes found on histological preparations. An analysis of morphometric measurements of the area of hepatocyte nuclei enable to suggest that in the run-up to a composite system or plant material, the functional activity of liver cells increases, since the size of the nuclei increases significantly (Fig. 7). These nuclei are light, rounded, which is consistent with an increase in their transcriptional activity.

Thus, the obtained data indicate an improvement in the histostructure of the organ when using both a composite system and pure silica or free herbal medicine against the background of alimentary obesity, which is probably the result of an increase in compensatory-adaptive reactions of liver tissue.

It has been shown that a high-calorie diet of rats over 100 days leads to the development of individual manifestations of obesity and

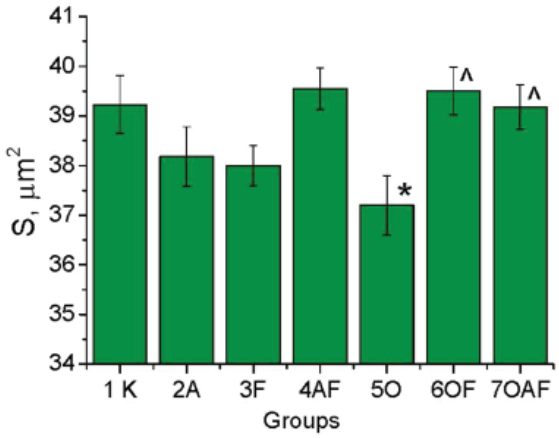


Fig. 7. **The size of the area of the nuclei of hepatocytes (S, μm^2) in different experimental groups:**
* — $P < 0.05$ compared with the control group (1K);
ˆ — $P < 0.05$ compared with the group of nutritional obesity (5O) without the use of additional drugs

metabolic syndrome, which are expressed in an increase in body weight and a significant increase in visceral fat mass (control — 5.2 g, obesity — 6.81 g).

From the histophysiological characteristics of the liver, the development of the initial and middle stages of parenchymal fatty degeneration (small- and large-droplet obesity of hepatocytes) with morphological manifestations of a decrease in the synthetic activity of the nucleus, pronounced anisonucleosis, and expansion of sinusoidal ducts filled with blood cells were revealed.

With the development of simulated alimentary obesity on liver histological preparations the signs of small- and large-droplet obesity of liver hepatocytes (parenchymal fatty degeneration), the decrease in synthetic activity of hepatocytes, pronounced anisonucleosis, and expansion of sinusoidal cavities with impregnation of blood cells were revealed.

At the biochemical level, deviations from the norm at this stage of the development of obesity are weak and are manifested mainly in a slight increase in the level of total bilirubin (8.2 μM/l for obesity compared to 5.8 μM/l in the control group) and alkaline phosphatase (K — 135.325 u/l and obesity 200.95 u/l).

The additives used are HDS, the composite system "Lymphosilica" or the original phytopreparation do not cause pathological changes in the liver histostructure even with their long-term use.

The phytopreparationinjection with a high content of polyphenols, as well as flavonoids, polysaccharide terpenes, leads in some cases to manifestations of "adaptive stress", in which liver enzymatic activity is activated.

It is shown the positive effect of the composite "Lymphosilica" system based on HDS and herbal remedies on:

– proportion lowering of visceral fat in rats when kept on a standard diet (in the absence of such an effect against the simulated obesity);

– a decrease in the proportion of visceral fat in rats when kept on a standard diet for F (herbal medicine) — 3.43 ± 0.19 g and for AF (Lymphosilica) — 3.24 ± 0.33 g (at a control level of 5.2 ± 0,33 g), against the background of simulated obesity, this effect was not detected;

– normalization of bilirubin level and its fractions against the simulated obesity (decline from 8.2 μM/l for obesity to 6.02 μM/l (OF) and — 5.3 μM/l (OAF);

– lowering cholesterol (from 1.65 ± 0.03 mM/l for obesity to 1.35 ± 0.08 mM/l) and alkaline phosphatase (from 200.95 ± 22.19 u/l for obesity, up to 162.25 u/l) under the action of the combined drug Lymphosilica against the background of simulated obesity;

– increased globulin fractions of blood serum (by 8.6% in relation to control).

With the introduction of the composite system "Lymphosilica" or the initial phytopreparation against the background of alimentary obesity, the morphological signs of activation of synthetic processes in the nuclei of hepatocytes are strengthened, which indicates an increase in the compensatory capabilities of hepatocytes and a decrease in the manifestation of parenchymal fatty degeneration, but minor foci of hepatocytes with mainly dust remain.

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БІОМЕТРИЧНІ ДОСЛІДЖЕННЯ ЕФЕКТИВНОСТІ КОМПОЗИТНОЇ СИСТЕМИ «ЛІМФОСИЛІКА» НА ОСНОВІ ЗБОРУ ЛІКАРСЬКИХ РОСЛИН ТА НАНОКРЕМНЕЗЕМУ

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Досліджено вплив нанокомпозитної системи на основі високодисперсного кремнезему та рослинної сировини з високим вмістом поліфенолів (добавка «Лімфосиліка») на цитофізіологічні особливості печінки в умовах розвитку метаболічного синдрому при моделюванні аліментарного ожиріння у щурів. Проводилися біохімічні дослідження сироватки крові та гістологічний аналіз тканини печінки. Показано, що висококалорійний раціон щурів протягом 100 діб призводить до розвитку окремих проявів ожиріння і метаболічного синдрому, які виражаються в збільшенні маси тіла і вісцерального жиру, розвитку початкових і середніх стадій паренхіматозної жирової дистрофії печінки зі зниженням морфологічних проявів синтетичної активності ядра, з незначними відхиленнями на біохімічному рівні. Введення фітокомпозиту з високим вмістом поліфенолів, а також флавоноїдів, терпенів полісахаридів не зумовлює патологічні зміни в організмі щурів, але в деяких випадках призводить до проявів «адаптаційного напруги» гепатоцитів печінки, як при стандартному раціоні, так і на тлі висококалорійного раціону. Показано позитивний вплив композитної системи на зниження частки вісцерального жиру (на 38%) в організмі щурів при стандартному раціоні харчування, а на тлі модельованого аліментарного ожиріння — нормалізація рівня білірубіну (зниження на 25% порівняно з аліментарним ожирінням) і його фракцій, зниження рівня холестролу та лужної фосфатази (в обох випадках на 19% порівняно з початковим ожирінням), підвищення глобулінових фракцій сироватки крові, протективну дію стосовно дистрофічних змін в тканині печінки.

Ключові слова: нанокомпозит, Лімфосиліка, нанокремнезем, щурі, аліментарне ожиріння, гепатоцити печінки, вісцеральний жир.

БИОМЕТРИЧЕСКИЕ ИССЛЕДОВАНИЯ ЭФФЕКТИВНОСТИ КОМПОЗИТНОЙ СИСТЕМЫ «ЛИМФОСИЛИКА» НА ОСНОВЕ СБОРА ЛЕКАРСТВЕННЫХ РАСТЕНИЙ И НАНОКРЕМНЕЗЕМА

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Исследовано влияние нанокомпозитной системы на основе высокодисперсного кремнезема и растительного сырья с высоким содержанием полифенолов (добавка «Лимфосилика») на цитофизиологические особенности печени в условиях развития метаболического синдрома при моделируемом алиментарном ожирении у крыс. Проводились биохимические исследования сыворотки крови и гистологический анализ ткани печени. Показано, что высококалорийный рацион крыс в течение 100 суток ведет к развитию отдельных проявлений ожирения и метаболического синдрома, которые выражаются в увеличении массы тела и висцерального жира, развития начальных и средних стадий паренхиматозной жировой дистрофии печени со снижением морфологических проявлений синтетической активности ядра, с незначительными отклонениями на биохимическом уровне. Введение фитокомпозита с высоким содержанием полифенолов, а также флавоноидов, терпенов полисахаридов не приводит к патологическим изменениям в организме крыс, но в ряде случаев ведет к проявлениям «адаптационного напряжения» гепатоцитов печени, как при стандартном рационе, так и на фоне высококалорийного рациона. Показано положительное влияние композитной системы на снижение доли висцерального жира (на 38%) в организме крыс при стандартном рационе питания, а на фоне моделированного алиментарного ожирения — нормализация уровня билирубина (снижение на 25% по сравнению с алиментарным ожирением) и его фракций, снижение уровня холестрола и щелочной фосфатазы (в обоих случаях на 19% по сравнению с начальным ожирением), повышение глобулиновых фракций сыворотки крови, протективное действие по отношению к дистрофическим изменениям в ткани печени.

Ключевые слова: нанокомпозит, Лимфосилика, нанокремнезем, крысы, алиментарное ожирение, гепатоциты печени, висцеральный жир.

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CHEMICAL COMPOSITION AND ANTIMICROBIAL PROPERTIES OF ESSENTIAL OIL FROM *Origanum vulgare* L. IN DIFFERENT HABITATS

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Essential oils are widely used in beauty therapy, medicine and food industry, and they are considered to be a valuable consumer product. At the same time, the biochemical composition and properties of essential oils, including their antimicrobial activity, varies depending on the habitat, climatic conditions and plant chemotype. The purpose of our work is to study the qualitative and quantitative composition of essential oils and their antimicrobial properties, from *Origanum vulgare* plants harvested in eastern Slovakia and Lviv region, Ukraine.

In the wild, *O. vulgare* L. were gathered in close vicinity of the village of Trostianets, Lviv region , Ukraine, in 2019. In Slovakia, the plants were grown by Agrokarpaty Company, Plavnica. Essential oils were extracted by hydrodistillation (2 hours) in a Clevenger apparatus, according to the European pharmacopoeia.

The analysis of the biochemical properties of essential oils extracted from plant populations from Lviv region , showed that the contents of essential oils were within 0.35 ± 0.05%. The composition of essential oils shows that *O. vulgare* L. plants from the natural population grown in Lviv region , belongs to the monoterpene chemotype. Monoterpene hydrocarbons α-terpinene and α-terpineol together accounted for 29–33%, acyclic monoterpenes — β-myrcene — 7%, linalool — 4%, while the polyphenol compound p-cymene accounted for only 15%.

The *O. vulgare* plants from Slovakia were characterised by the essential oil content of 0.15 to 0.50%, and the composition which allowed us to refer them to the carvacrol chemotype, with phenols as its main ingredients — carvacrol and thymol (together 71%), and isopropyltoluene (4.0%). Monoterpene hydrocarbons terpinene (5.0%) and terpineol alcohol (6.0%) jointly accounted for 11%; acyclic monoterpene mycrene — 3%; and sesquiterpene β-caryophyllene — 4,5%.

Essntial oil from *O. vulgare* harvested in Slovakia demonstrated high antimicrobial activity against reference and clinical isolates of opportunistic microorganisms. Essential oil from the samples gathered in Lviv region, showed low antimicrobial activity.

Thus, it has been shown that the reviewed plants referred to different chemotypes, which calls forth the prospect of the use of essential oils extracted from different plant chemotypes for different purposes, depending upon their biochemical composition and properties.

Key words: oregano essential oil, antimicrobial activity, biochemical properties.

Common oregano, or wild marjoram (*Origanum vulgare* L.), genus *Origanum* (*Lamiaceae* Martinov) has somewhat been 'forgotten' in Ukraine as a medicinal plant, though it remains widely spread in the Mediterranean where it is used as an essential-oil-bearing and aromatic plant. This herb demonstrates antibacterial and antioxidant activity, and antifungal property. It owes its high biological activity to the presence of essential oils, flavonoids, and glycosides.

O. vulgare is a polymorphic species, and *Flora Europaea* (1972) distinguishes several subspecies of this species: *O. vulgare* ssp.

vulgare, *O. vulgare* ssp. *hirtum*, *O. vulgare* ssp. *viride*. The subspecies differ by the structure of their reproductive organs, location of the essential-oil trichomes, and composition of the respective essential oils [1].

Academic literature distinguishes chemotypes of populations that form "biochemical varieties" or "physiological forms" in botanic species. Say, Italian scholars have singled out four reasons affecting the population chemotype of *O. vulgare* and its subspecies. First of all, it is the environmental conditions, especially climate, that affect the biosynthetic way of phenol compounds; secondly, the sexual polymorphism and the genetic mechanism; thirdly, the state of the plants (fresh or dried); fourth, the phenophase of the plants' development.

The phytochemical analysis of plants from 51 populations in 17 European countries has established that active cymyl and/or acyclic linalool/linalyl acetate synthesis of essential oils is peculiar for plants from the Mediterranean, whereas active sabinyl was peculiar for the plants growing in the continental [2]. In these plants, the contents of essential oils amounted to 0.03–4.6%, and their composition included sabinene, myrcene, p-cymene, 1.8-cyneol, β-ocimene, γ-terpinene, sabinene hydrate, linalool, α-terpineol, calvacrol methyl ether, linalyl acetate, thymol and carvacrol, β-caryophyllene, germacrene D, germacrene D-4-ol, spathulenol, caryophyllene oxide, and oplopanone.

The chemotype with phenolic prevalence was typical for southern Italy. Four variants of thymol- and carvacrol-chemotype were distinguished in the *O. vulgare* ssp. *hirtum* populations from different parts of Sicily. The author connects such differences with the mixture of genetic and environmental factors affecting the biosynthesis of essential oils [3]. In the *O. vulgare* ssp. *hirtum* populations from Campagna [4], carvacrol/thymol chemotype prevailed along the sunny coast; another, tymol/α-terpineol chemotype was peculiar for the populations located in the mainland part of the country characterised by lower air temperatures. The third chemotype, with prevalence of linalyl acetate and linalool (the lowest content of phenols) was spread along the mountainous coastline.

In Bulgaria, carvacrol, whose content reached as much as 73.4%, dominated in all samples [5]. The samples of *Origanum vulgare* ssp. from southern Croatia picked in different seasons belonged to the thymol/carvacrol type [6]. The plants of *O. vulgare* ssp. *hirtum* collected from northern Greece were rich in thymol, and those collected from southern Greece — in carvacrol [7]. Studies of the contents of essential oils from *O. vulgare* grown in the south-eastern part of Spain showed the following results: (E)-β-caryophyllene (0.5–4.9%), thymol (0.2–5.8%), p-cymene (3.8–8.2%), γ-terpinene (2.1–10.7%) and carvacrol (58.7–77.4%) [8].

Another chemotype characterised by prevalence of sesqui- or monoterpenes, was typical for the populations from the continental climate with significantly lower solar radiation and temperatures [9]. In the *O. vulgare* plants from China and Pakistan, from 11 to 46 components of essential oil were isolated, in which oxygenic monoterpenes prevailed, and, besides, two populations showed high contents of sesquiterpene hydrocarbons — 33.7% and 43.7%, respectively [10].

In the essential oil from *O. vulgare* grown in the Ukrainian Polissia region, 24 components were identified, of which the following ones prevailed: α-cadinol (14.24%), germacrene D (13.76%), β-caryophyllene (12.23%), 1,6-germacrediene-5-ol (11.12%), epi-α-cadinol (8.56%), α-farnesene (5.75%), terpinene-4-ol, thymol, cis-sabinenhydrate, linalool, γ-terpinene-trans-ocimene, geraniol, and neral [11].

Quite often, different chemotypes of a population are linked with different subspecies of *O. vulgare*. As seen from the literature analysis, thymol-chemotype is typical for *O. vulgare* ssp. *hirtum*, whereas linalool is the main volatile component of *O. vulgare* ssp. *virens*, where the content of thymol is insignificant [12].

Thereby, both the composition and the properties of *O. vulgare* depend upon the natural climatic conditions as well as the genetic conditionality of the plants' biochemical properties.

The purpose of our work is to study the qualitative and quantitative composition and antimicrobial properties of essential oils from *O. vulgare* plants collected in eastern Slovakia and Lviv oblast, Ukraine.

Materials and Methods

Oregano herb growing and harvesting conditions

The demand of oregano raw material is satisfied by large-scale cultivation by the Agrokarpaty Company, Plavnica, Eastern Slovakia (N 49° 16′ 28″, E 20° 46′ 50″, altitude above sea level: 530 m). The production of

oregano monocultures needs a light, rich, well-drained soil with pH of 6.0–8.0. Growth occurs between 4 and 32 °C, optimally at 23 °C. Oregano plants are propagated vegetatively to obtain plants with the desired flavour and aroma characteristics of their parents (the Krajova variety). The first harvest of leaves and tender tops occurs just as flowering commences. The plants are cut 60–100 mm from the ground. In the region 3–4 cuts can be made over a single year. Where commercially grown, oregano plantings are productive for 4–5 years. The leaves are thoroughly dried, cleaned, and stored as soon as possible. The area of cultivated parcels varied from 2 to 5 ha in the last years. Typically, yields of 1,500–3,000 kg per ha of dried herb are obtained.

Plant samples from the natural habitat of *Origanum vulgare* L. were taken in 2019 in the stow which is a geological nature sanctuary of local significance. It is located by the road in the western part of the village of Trostianets, Mykolaiv rayon, Lviv oblast (geographical coordinates: 49°33′03″ north latitude and 24°00′28″ east longitude; average altitude above sea level — 283 m). The site represents Tortonian sandstone — tightly pressed sand, white-coloured with a yellowish tint; with patches of hard rock with small grottoes, where leftovers of the Tortonian layer flora had been found.

January is the coldest month in Lviv oblast; its mean temperature is by 2–3 °C lower than in December. All winter months in the Opillia region are characterised by a big variability of air temperature (2.5–4.7 °C). The temperatures in July have been observed to drop insignificantly (to 17.0–17.5 °C) in the elevations of Roztochia and Opillia, whereas Little Polissia and Precarpathia have shown the highest monthly mean temperatures (18.0–18.5 °C). Lviv oblast is peculiar for rather significant yearly precipitation totals, varying between 579 and 1,070 mm. The largest amount of precipitation falls on June — July (90–140 mm a month); the lowest — on January — February (24–40 mm a month). Thus, the amount of precipitation in summer is by 2 to 3 times higher than in winter.

Origano oil isolation

The essential oil from this raw-material was prepared by hydro-distillation (2 hours) in Clevenger-type apparatus according to the European Pharmacopoeia and a mixture of hexane was used as a collecting solvent. The essential oils stored under N_2 at + 4 °C in the dark space before their composition identification.

GC-FID analyse

The analysis of oregano essential oils was carried out using a Vega Series Carloerba Gas Chromatograph, connected to a Spectrophysics SP 4270 integrator. The following operating conditions were used: column: DB5, 30 m×0.32 mm inner diameter (i. d.), film thickness: 0.25 mm, carrier gas: nitrogen, adjusted to a flux of 1 mL per min, injection and FID-detector temperatures: 220 °C, respectively 250 °C. Components were identified by their GC retention times, and the resulting values were comparable to those of literature. Oil component standards for comparison were supplied by Extrasynthese Ltd. (France).

GC/MS analyse

GC/MS analyses were carried out on a Varian 450-GC connected with a Varian 220-MS. The separation was achieved using a Factor Four TM: Capillary Column VF 5 ms (30 m × 0.25 mm i. d., 0.25 µm film thickness). Injector type 1177 was heated on temperature 220 °C. Injection mode split less (1 µl of a 1:1,000 n-hexane/diethyl ether solution). Helium was used as a carrier gas at a constant column flow rate of 1.2 mL per min. Column temperature was programmed: initial temperature 50 °C for 10 min, then to 100 °C at 3 °C per min; isothermal for 5 min and then continued to 150 °C at 10 °C per min. Total time for analysis of one sample took 54.97 min. Identification of components was done by comparison of their mass spectra with those stored in NIST 02 (software library) or with mass spectra from the literature [13] (Adams, 2007) and a home-made library, as well as on comparison of their retention indices with the standards.

Antimicrobial assay

Antimicrobial activity of Origano EO was determined using agar diffusion test [14]. The bacterium inocula 100 µL in the physiological solution were adjusted to the equivalent of 0.5 McFarland standard, and evenly spread on the surface of Muller-Hinton agar (incubated at 37 ± 2 °C for 24 hours); yeasts — on SDA agar (incubated at 35 ± 2 °C for 48 hours). The extracts 20 µL were introduced into wells 6 mm in diameter. The diameters of the inhibition zones were measured in millimetres including the diameter of the well. Each antimicrobial assay was performed at least three times.

As test cultures, the following bacteria and yeasts from the American Type Culture Collection were used: *Candida albicans* ATCC 885-653; *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; *Enterococcus*

faecalis ATCC 29212; *Streptococcus pyogenes* ATCC 19615; reference *S. aureus* CCM 4223 biofilm-forming strain. We also used clinical strains of bacteria and yeasts (*S. aureus, E. coli, S. pyogenes, E. faecalis, C. albicans*) isolated from the oral cavities of patients suffering from inflammatory periodontium and pharynx. We chose the clinical strains with multiple resistance at least to two classes of antibiotics. As a positive control were used: gentamicin (10 mg/disk) for Gram-negative bacteria, ampicillin (10 mg/disk) for Gram-positive bacteria, and nystatin (100 UI) for *Candida*. As negative control, DMSO were used.

Statistical analysis

Values mentioned are the mean with standard deviations, obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student's *t*-test. A value of $P < 0.05$ was considered as statistically significant.

Results and Discussion

The State Pharmacopoeia of Ukraine [15] lists *O. onites* L. or *O. vulgare* L. subsp. *hirtus*, or mixture of the two species (Origani herba, OREGANO). The contents of essential oil are no less than 25 ml/kg on anhydrous basis; the aggregate total of carvacrol and thymol in essential oil is no less than 60%. Origanum^N — national description; dried *O. vulgare* grass harvested during the blossoming period. Contents: the total of flavonoids — at least 1.5% calculated with reference to luteolin 7-glucoside and on anhydrous basis; essential oil — at least 1 ml/kg on anhydrous basis.

As a result of the study, it was established that dried *O. vulgare* grass grown in Slovakia predominantly contained essential oils (0.15–0.50%), in which such phenols as carvacrol and thymol (together — 71.25%) and isopropyltoluene (4.0%) were the main ingredients. Such monoterpene hydrocarbons as terpinene and terpineol alcohol jointly made up 11%: acyclic monoterpene myrcene — 3%; and sesquiterpene β-caryophyllene — 4.5% (Table 1).

According to our study, *O. vulgare* grass harvested from a sunny hill close to the village of Trostianets, Lviv oblast, Ukraine contained in total 1.14 ± 0.04% of flavonoids. According to the Origanum^N national description, *Origanum vulgare* L. should contain the flavonoid total of at least 1.5% calculated with reference to luteolin 7-glucoside ($C_{23}H_{24}O_{10}$; M. м. 460) and on anhydrous basis. The population from Lviv oblast demonstrated high aggregate contents of essential oils — 0.35 ± 0.05% hm (3,5 g/kg of dry weight), with a 10.5% humidity loss. In the reviewed *O. vulgare* isolated during blossoming phase, 16 essential oils were identified (Table 1).

Monoterpene hydrocarbons α-terpinene and α-terpineol together made up 29–33%, i. e. a third part of all essential oils, with α-terpineol alcohol making two thirds of them. The survey also showed high contents of acyclic monoterpenes — β-myrcene (7%) and linalool — 4%, and only 15% fell on such polyphenol compound as p-cymene. Besides, the reviewed species was observed to synthesize quite a big amount of sesquiterpene β-caryophyllene — 7%. Linalool and α -terpineol are odorous volatile alcohol monoterpenes. All other essential oils: geraniol, terpinolene, cineole, limonene, thujon, borneol, bornylacetate, fenchol, carvacrol, and thymol were observed to be contained from 2% down to trace amounts.

Accumulation of essential oils depends upon the growth environment — solar radiation, climate, and topographic conditions. The [16] ascertained that in the presence of phenol compounds plants synthesize essential oils by transforming γ-terpinene into *p*-cymene with subsequent hydroxylation of *p*-cymene to thymol or carvacrol, subject to solar radiation.

In the environmental conditions of Lviv oblast, Ukraine, due to the considerably lower summer air temperatures, higher amounts of precipitation, especially in summer, and on poor sandy soils, *O. vulgare* tended to form the monoterpene chemotype, where monoterpenes made up 41%, sesquiterpenes — 7%, and the phenol compound of *p*-cymene — only 15%; carvacrol and thymole together accounted for approx. 2%. In Slovakia, the reviewed plants belonged to the carvacrol chemotype, typical for the Mediterranean region with warm climate.

Plant phenophase was also observed to be affecting the contents and the character of essential oils. In spring, p-cymene prevailed over carvacrol in *O. vulgare* subsp. *hirtum*; but by the end of the growth season the ratio would reverse. Such regularity was observed within one plant, where the young leaves contained more cymene than the older ones [17]. Kokkini et al. [7] showed that the contents of essential oils (γ-terpinene, p-cymene, thymol and carvacrol) would change during the season: in autumn, the

Table 1. **Composition of essential oils from medicinal herbal material *Origanum vulgare* ($n = 3$, $x \pm SD$)**

Essential oil components	Essential oil content, %	
	Trostianets, Ukraine	Plavnica, Slovakia
Acyclic monoterpenes		
Geraniol	0.7 ± 0.1	n/a
β-Myrcene	7.0 ± 0.5	3.0 ± 0.5
Linalol	4.0 ± 0.5	n/a
Monocyclic monoterpenes		
α-Terpinene	11 ± 1	5.0 ± 1.0
α-Terpineol	18 ± 1	6.0 ± 2.5
Terpinolene	1.8 ± 0.2	n/a
Cyneol	1.8 ± 0.2	n/a
Limolene	traces	n/a
Bicyclic monoterpenes		
Thujone	1.2 ± 0.2	n/a
Borneol	1.0 ± 0.1	n/a
Bornyl acetate	0.6 ± 0.1	n/a
Fenchol	traces	n/a
Sesquiterpenes		
β-Caryophyllene	7.5 ± 0.5	4.5 ± 0.5
Aromatic (phenol) compounds		
p-Cymene	15 ± 1	n/a
Carvacrol	1.6 ± 0.2	55.21 ± 3.0
Thymol	0.3 ± 0.1	16.04 ± 1.5
Isopropyltoluene	n/a	4.0 ± 1.5

plants had more phenols compared with those harvested in midsummer. The number of oils in the populations of *O. vulgare* ssp. *virens, (O. vulgare* ssp. *viridulum)* in southern Italy achieved maximum values during full blossoming of the plants [18]. The contents of phenols is as a rule high during blossoming of plants of phenolic type [5].

The volatile constituents of *O. vulgare* L. ssp. *hirtum* grown in Croatia were established to be affected by both the time of harvesting and desiccation of the plants. The content of p-cymene reached its maximum in August. Upon drying of the plant material, all samples demonstrated insignificant decrease in the yield of essential oils compared with the fresh plants. Drying at room temperature did not affect the qualitative composition of oregano oil [6].

Among the phenolic essential oils extracted from the reviewed plants from Lviv oblast, Ukraine, p-cymen dominated (15%); the rest (1.9%) fell on carvacrol and thymol. Whereas the plants were in the blossoming phenophase, it would be reasonable to check their chemical composition by the end of this phenophase — at the beginning of fructification.

The antimicrobial properties of essential oils of *O. vulgare* collected in Lviv oblast, with domination of monoterpenes (nearly 50%) — monoterpene essential oils (α-terpinene and α-terpineol — 29–33%), acyclic monoterpenes (β-myrcene and linalool — 11%); and only 17% fell on polyphenol compounds — were very weak (Table 2).

The studies showed that essential oil from *O. vulgare* L. grown in Slovakia demonstrated antimicrobial activity upon all isolates taken into the experiment — both typical and clinical ones (Figure). High antimycotic activity of essential oil was established. It was also revealed that oil is active against methicillin-resistant and biofilm-forming isolates of *S. aureus*. At the same time, the antimicrobial activity of the oil received from the plants of the local population was low. Such pattern may have been caused by the low contents of carvacrol and thymol, which play a decisive role in the antimicrobial activity of plants of the given species.

The composition of essential oils affects the pharmacological properties of the raw materials. Say, the essential oil with maximum content of carvacrol was received from the plants *O. vulgare* ssp. *virens* from southern Italy [18] demonstrated the highest antibacterial activity. Somewhat lower antibacterial activity was shown by the oil received from the populations of *O. vulgare* ssp. *viridulum* from "Ricigliano", which were characterised by a big amount of thymol, whereas the populations from "Acerno" had the lowest contents of phenols and demonstrated the least antimicrobial activity.

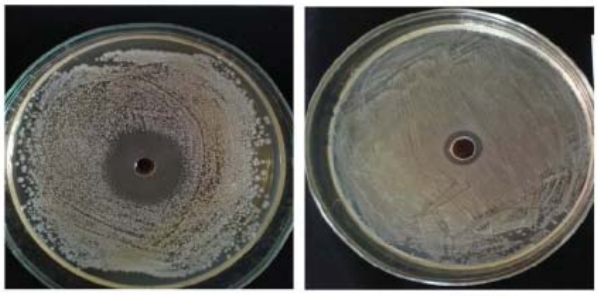
Extract of essential oils from *O. vulgare* with carvacrol, thymol and cymol being the main components, showed an expressed inhibiting activity against enteropathogenic bacteria of *E. coli* and *S. enteritica* var. *enteritidis*.

Owing to the presence of cinnamaldehyde, carvacrol, thymol and eugenol, essential oils of cinnamon, oregano, thyme and cloves showed strong antimicrobial activity against *L. monocytogenes*, *S. typhimurium*, *E. coli* O157: H7 and bacteria causing food spoilage (*B. thermosphacta* та *P. fluorescens*)

Table 2. **Antimicrobial activity of essential oil from *Origanum vulgare*** ($n = 3$, $x \pm SD$)

Test culture	Plavnica, Slovakia	Trostianets, Ukraine
S. aureus ATCC 25923	26.7 ± 0.58	10.5 ± 0.58
S. aureus clinic biofilm creation	25.7 ± 0.58	9.50 ± 0.33
S. aureus MRSA clinic	25.5 ± 0.5	–
E. coli ATCC 25922	21.3 ± 0.58	9.0 ± 0.50
E. coli clinic	25.8 ± 0.29	8.50 ± 0.25
E. faecalis ATCC 29212	20.3 ± 0.58	10.5 ± 0.25
E. faecalis clinic	19.3 ± 0.58	9.8 ± 0.25
S. pyogenes ATCC 19615	31.3 ± 0.58	–
S. pyogenes clinic	29.7 ± 0.58	–
C. albicans ATCC 885-653	36.3 ± 0.58	12.0 ± 0.58
C. albicans clinic	35.3 ± 0.58	11.0 ± 0.30

Note: * the data were statistically significant as compared with the control ($P < 0.05$) as a control were used: ampicilin — for gram-positive bacteria; gentamicine — for gram-negative bacteria; nystatin — for microscopic fungi; control of antibiotic — no inhibition; control of methanol — no inhibition.



Antimicrobial activity of essential oil from *O. vulgare* against clinical isolates of *E. coli*

[19]. The mixture of essential oils from *O. vulgare* (carvacrol (66.9%)) and *Rosmarinus officinalis* (1.8-cineolom (32.2%)) used as spices, provided for the inhibition of the growth of bacteria located on food products (*L. monocytogenes*, *Y. enterocolitica* and *A. hydrophilla*, *P. fluorescens*) [20]. It was proved that *O. vulgare* can be used as condiment to inhibit the growth of *S. aureus*, and to inhibit the synthesis of staphylococcal enterotoxins [21].

In our previous studies, we showed that by their biochemical properties and antimicrobial activity, essential oils can be used to inhibit opportunistic and pathogenic microorganisms, and as natural preserving agents [22–24].

Aqueous extract of *O. vulgare* was revealed to exert a high antimicrobial activity upon ten bacteria: *E. coli* ATCC 25922, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa* ATCC 27835 et al. Their activity was stronger against gram-positive pathogens *S. epidermidis* (ATCC 12228) than against gram-negative *E. coli* [4].

Thus, owing to the presence of aromatic essential oils (carvacrol and thymol), flavonoids (rosmarinic acid) and derivatives of pyrocatechin acid, the essential oil received from *O. vulgare* grown in Slovakia has a high antimicrobial activity, compared with the monoterpene essential oil of the plants grown in Lviv oblast, Ukraine. The obtained data have proved the possibility of the use of essential oil from Slovakia as an antimicrobial medication. At the same time, it appears relevant to proceed with the studies of the properties of essential oils introduced in other climatic and paedological conditions, and use of plants from Slovakia as planting material.

Conclusions

Accumulation of essential oils has been shown to depend upon the growth conditions — solar radiation, climate, and topographic conditions. In sunlit places and warm climate, phenol compounds were accumulated, whereas under continental conditions monoterpenes prevailed, which was typical for populations of *O. vulgare* L.

The populations growing in Lviv oblast, Ukraine, were characterised by the monoterpene chemotype, with the contents of essential oil amounting to 0.35 ± 0.05%. The essential oil contained monoterpene hydrocarbons α-terpinene and α- terpineol (29–33%), acyclic monoterpenes — β-myrcene

(7%), linalool (4%); whereas the polyphenol compound p-cymene accounted for 15% only.

In the plants *O. vulgare* from Slovakia, essential oil accounted for 0.15–0.50% of their composition, with domination of the carvacrol chemotype where phenols are the main ingredients — carvacrol and thymol (71% together), and isopropyltoluene (4.0%). Monoterpene hydrocarbons terpinene (5.0%) and terpineol alcohol (6.0%) jointly accounted for 11%; acyclic monoterpene myrcene — 3%, and sesquiterpene β-caryophyllene — 4.5%.

The essential oil from *O. vulgare* collected in Slovakia demonstrated high antimicrobial properties against reference and clinical isolates of opportunistic microorganisms. The essential oil from the samples taken in Lviv oblast, Ukraine showed low antimicrobial activity.

The study was conducted in the framework of bilateral cooperation between Ukraine and Slovakia.

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ХІМІЧНИЙ СКЛАД ТА АНТИМІКРОБНІ ВЛАСТИВОСТІ ЕФІРНОЇ ОЛІЇ *Origanum vulgare* L. РІЗНИХ МІСЦЕЗРОСТАНЬ

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Ефірні олії широко використовують у різних галузях косметології, медицини, харчової промисловості і вони є високовживаним продуктом. Водночас біохімічний склад, властивості, зокрема антимікробна активність, олій варіюють залежно від місця зростання, кліматичних умов та хемотипу рослин. Метою нашої роботи є дослідження якісного та кількісного складу

ХИМИЧЕСКИЙ СОСТАВ И АНТИМИКРОБНЫЕ СВОЙСТВА *Origanum vulgare* L. РАЗНЫХ МЕСТООБИТАНИЙ

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Эфирные масла широко используются в различных отраслях косметологии, медицины, пищевой промышленности и являются высокопотребляемым продуктом. В то же время биохимический состав, свойства, в том числе антимикробная активность, масел варьируют в зависимости от места произрастания, климатических условий и хемотипа растений.

ефірних олій *O. vulgare* та антимікробних властивостей, рослин, що зібрані у Східній Словаччині та у Львівській області України.

У природних умовах *Origanum vulgare* L. було зібрано в околицях села Тростянець, Львівська область, у 2019 р. У Словаччині рослини були вирощені компанією Агрокарпати, Плавниця. Ефірну олію із сировини відганяли гідродистиляцією (2 години) в апараті Клівенгера згідно з Європейською фармакопеєю.

Дослідження біохімічних властивостей ефірних олій популяцій рослин, які ростуть в умовах Львівщини, показали, що вміст ЕО становить 0,35 ± 0,05%. Склад ефірних олій вказує на належність рослин природної популяції *Origano vulgare* L., що зростає у Львівській області, до монотерпенового хемотипу. Монотерпенові вуглеводні α-терпінен і α-t-терпінеол становили в сумі 29–33%, ациклічні монотерпени — β-мірцен 7% і 4% — ліналол і лише 15% становила поліфенольна сполука р-цимен.

Рослини *O. vulgare*, вирощені у Словаччині, характеризувались вмістом ефірної олії 0,15–0,50% і компонентним складом, який дав змогу віднести їх до карвакрольного хемотипу, де основним інгредієнтом є феноли — карвакрол і тимол (разом 71%), ізопропілтолуол (4,0%). Монотерпенові вуглеводні терпінен (5,0) і спирт терпінеол (6,0) становили в сумі 11%, ациклічний монотерпен — мірцен (3%) і сесквітерпен β-каріофіллен (4,5%).

Ефірна олія *O. vulgare,* зібрана в Словаччині, виявляла високу антимікробну дію на референтні та клінічні ізоляти умовно патогенних мікроорганізмів. Ефірна олія із зразків, зібраних на Львівщині, виявляла низьку антимікробну активність.

Отже, досліджувані рослини належать до різних хемотипів, що обумовлює перспективу їх використання для різних потреб залежно від біохімічного складу та властивостей.

Ключові слова: ефірна олія орігано, антимікробна активність, біохімічні властивості.

Целью работы является исследование качественного и количественного состава эфирных масел *O. vulgare*, а также антимикробных свойств, растений, собранных в Восточной Словакии и во Львовской области Украины.

В естественных условиях *Origanum vulgare* L. были собраны в окрестностях села Тростянец, Львовская область, в 2019 году. В Словакии растения были выращены компанией Агрокарпаты, Плавница. Эфирное масло из сырья отгоняли гидродистилляцией (2 часа) в аппарате Кливенгера согласно Европейской фармакопее.

Исследование биохимических свойств эфирных масел популяций растений, произрастающих в условиях Львовщины, показали, что содержание єфирных масел составляет 0,35 ± 0,05%. Состав эфирных масел указывает на принадлежность растений природной популяции *Origano vulgare* L., которая произрастает во Львовской области к монотерпеновому хемотипу. Монотерпеновые углеводороды α-терпинен и α-терпинеол составляли в сумме 29–33%, ациклические монотерпены — β-мирцен (7%) и линалол (4%) и только 15% полифенольное соединение р-цимен.

Растения *O. vulgare*, выращенные в Словакии, характеризовались содержанием эфирного масла 0,15–0,50% и компонентным составом, который позволил отнести их к карвакрольному хеотипу, в котором основным ингредиентом являются фенолы — карвакрол и тимол (в сумме 71%), изопропилтолуол 4,0%. Монотерпеновые углеводороды терпинен (5,0) и спирт терпинеол (6,0) составляли в сумме 11%, ациклический монотерпен — мирцен (3%) и сесквитерпен β-кариофиллен (4,5%).

Эфирное масло *O. vulgare*, собранное в Словакии, проявляло высокое антимикробное действие на референтные и клинические изоляты условно патогенных микроорганизмов. Эфирное масло из образцов, собранных на Львовщине, прояляло низкую антимикробную активность.

Таким образом, исследуемые растения относились к различным хемотипам, что обусловливает перспективу их использования для различных нужд в зависимости от биохимического состава и свойств.

Ключевые слова: эфирное масло оригано, антимикрбная активность, биохимические свойства.

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In vitro ACTIVITY OF THE ANTIBIOTIC BATUMIN AGAINST *Candida albicans* BIOFILM

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The aim of this work was to study action of batumin on the strains of *Candida albicans* and *Candida krusei* in planktonic and biofilm form and also to obtain more detailed insights into the influence of batumin on biofilm formation by using atomic-force microscopy. The Minimum Inhibitory Concentration (MIC) of batumin was studied according to CLSI standards. Formation of a biofilm was studied by the photometric O'Toole method by means of a plate photometer ELx808 (BioTek, USA) at wavelength of 630 nanometers.

The batumin has a high selective activity against staphylococci (MIC ≥ 0,25 μg/ml), at the same time, antibiotic, being not active concerning yeast of the genus *Candida* (MIC ≥ 512 μg/ml) showed the inhibiting action on biofilm formation of these microorganisms. Batumin influence on biofilm formation was studied in type, collection strains *C. albicans*, *C. krusei* and clinical isolates. Presence 0,125 μg/ml of batumin in the broth (1/2 MIC for staphylococci) reduced the biofilm formation at 55.6% of the studied strains. Their biofilm formation values varied for *C. albicans* from 1.5–3.9 CU (conventional unit: OD_{630} in experimental samples/OD_{630} in control samples), for *C. krusei* of 2.3–3.0. Batumin was more effective against *Candida* strains with strong biofilm formation.

Atomic force microscopy revealed qualitative changes in the exopolymeric matrix due to batumin treatment, as well as a significant reduction in the number of cells adhered to the coverslip, preventing formation of *C. albicans* 127 biofilm, However, *C. albicans* ATCC 24433 a significant reduction in the number of cells adhered to the coverslip weren't observed.

The data obtained by an Atomic force microscopy confirm ability of a batumin to prevent formation of a biofilm at the studied strains that allows to consider it as the preventive agent at treatment of the infections caused by yeast-like fungi of the genus *Candida*.

Key words: batumin, *Candida*, biofilm, atomic force microscopy.

Candida spp. are serious causes of hospital-acquired blood and urinary tract infections, and the most of these infections are associated with implanted medical devices such as central venous and bladder catheters with biofilm formation within these devices [1].

A relevant characteristic of *Candida* biofilms is resistance to antifungal agents, which can be intrinsic or acquired by transfer of genetic material between biofilm cells [2].

It has already been shown that antibacterial drugs can affect *Candida* biofilm formation. Tigecycline, for instance, is highly active against growing and mature biofilms of *Candida albicans* [3], whilst rifampicin can induce biofilm formation by this *Candida* species [4].

The polyketide antibiotic batumin synthesized by the producer strain *Pseudomonas batumici* has a high selective activity against staphylococci. At the same time strains of the yeast species *C. tropicalis*, *C. utilis* and *C. albicans* are resistant to batumin (MIC ≥ 512 μg/ml) [5, 6]. However, our data indicate that the addition of antibiotic in concentration of 0.125 μg/ml reduces the formation of biofilm not only in staphylococci strains, but in *C. albicans* as well [7].

The objective of this work was to study action of batumin on the strains of *C. albicans* and *C. krusei* in planktonic and biofilm form and also to obtain more detailed insights into the influence of batumin on biofilm formation by using atomic-force microscopy.

Materials and Methods

Batumin, obtained by fermentation of *Pseudomonas batumici*, was purified by silica gel preparative chromatography to 85% of purity.

Batumin is commercially available from Santa Cruz Biotechnology (Santa Cruz, CA) or Enzo Life Sciences Antwerp, Belgium).

The object of the study were type and collection strains of *C. albicans* (n = 33) and *C. krusei* (n = 12) (Table 1), isolated from patients after examination for intestinal dysbiosis, from skin microbial dysbiosis (microbial collection of the Institute of Cellular and Intracellular Symbiosis, Ural Branch of Russian Academy of Sciences, Orenburg, Russia).

Identification of *C. albicans* and *C. krusei* was carried out on the basis of morphological, cultural and biochemical properties with the use of the commercial test system API20CAUX (bioMerieux, France).

The Minimal inhibitory concentration (MIC) of batumin was studied according to CLSI Standards in Mueller-Hinton agar [8]. The microbial load of *C. albicans* was 0.5×10^7 cfu/ml and the Petri dishes were incubated at 37 °C for 24 hours.

Concentration of batumin (0.125 μg/ml) was used to study its effect upon biofilm formation in *Candida*. Biofilm formation was studied by a photometric method determining the bacterial capacity to adhere to the 96-well polystyrole plane-table surface (Thermo Scientific, USA) with subsequent crystal violet staining [9]. An antibiotic was added into culture medium simultaneous with the culture of fungi and cultivated 24 hours. At the study of influence of batumin (0.125 μg /ml) on the different stages of biofilm formation by *C. albicans* strains the antibiotic was added into culture medium simultaneously with yeast-like fungi in 90 minutes, 24 and 48 hours from the beginning of incubation.

Optical density measurement was done using a photometer ELx808 (BioTek, USA) at a wavelength of 630 nm. Degree of biofilm formation was presented in conditional units (CU) which was the optical density of the broth after growth of the strain relative to the nutrient broth optical density.

For the study of batumin effect on biofilm formation by atomic force microscopy, we used *C. albicans* ATCC 24433 as test-culture and *C. albicans* 127 (clinical isolate). For testing the influence of batumin on biofilm production, glass coverslips were immersed into Luria-Bertani broth with 0.125 μg/ml of batumin and incubated for 48 h at 37 °C.

Visualization of the biofilms was done by atomic force microscopy using the SMM-2000 microscope (Proton-MIET Closed JOINT Stock Company, Russia), in contact mode in an air environment [10] [11].

Statistic analysis was performed by non-parametric method using Mann Whitney U-test [12].

Results and Discussion

All the studied 45 strains of *C. albicans* and *C. krusei* were highly resistant to batumin (MIC of 512 μg/ml), in correspondence with earlier data [6]. The resistance of strains to batumin was studied according to CLSI Standards in Mueller-Hinton agar [8]. At the same time, our preliminary research showed effectiveness of the antibiotic on inhibition of formation of a biofilm in the cultures of the genus *Candida* [7, 13].

In experiments on batumin effect on biofilm formation by strains of *Candida* we used concentration 0.125 μg/ml, which causes only modification of biological properties, including biofilm formation and did not influence on grows properties of cultures.

The obtained results showed that the effect of batumin on the formed biofilm in the representatives of the genus *Candida* was variable for different strains and species (Table 1).

The biofilm formation values varied for *C. albicans* from 1.5 to 3.9 CU, for *C. krusei* from 2.3 to 3.0. Presence of batumin (0.125 μg/ml) in the broth reduced the biofilm formation for 55.6% in the studied strains of fungi, whereas for staphylococci this was 85% [14].

Of special interest is the fact that the change in biofilm in the presence of batumin differs for different strains of *Candida*, and that these differences are caused at a concentration of only 0.125 μg/ml, for strains resistant to the studied antibiotic (Table 1).

Apparently, the changes we have detected in the biofilms of yeast-like fungi under the influence of batumin made them unstable, incapable of persistence and dissemination in the human body.

It should be noted that batumin is more effective against *C. albicans* strains with

Table 1. **Batumin effect against biofilm formation by *Candida albicans* and *Candida krusei***

Strains		Source	Biofilm formation before batumin		Biofilm formation after 0.125 μg/ml batumin	
			optical density (OD_{630})	CU*	optical density (OD_{630})	CU*
1	*C. albicans* 24433	Reference ATCC 24433^T	**0.12**	**2.4**	**0.13**	**2.6**
2	*C. albicans* 3	Vaginal	0.2	4.0	0.105	2.1
3	*C. albicans* 4	Vaginal	0.155	3.1	0.135	2.7
4	*C. albicans* 8	Vaginal	**0.075**	**1.5**	**0.085**	**1.7**
5	*C. albicans* 13-2	Vaginal	0.195	3.9	0.075	**1.5**
6	*C. albicans* 27	Vaginal	**0.136**	**2.7**	**0.135**	**2.7**
7	*C. albicans* 18s	Skin	0.185	3.7	0.13	2.6
8	*C. albicans* 118	Colon	0.145	2.9	0.085	1.7
9	*C. albicans* 53	Skin	**0.115**	**2.3**	**0.115**	**2.3**
10	*C. albicans* 54b	Skin	**0.105**	**2.1**	**0.11**	**2.0**
11	*C. albicans* 1-n	Nose	**0.075**	**1.5**	**0.086**	**1.7**
12	*C. albicans* 123	Colon	**0.09**	**1.8**	**0.1**	**2.0**
13	*C. albicans* 127	Colon	0.145	2.9	0.09	1.8
14	*C. albicans* 128 c	Colon	0.215	4.3	0.125	2.5
15	*C. albicans* 139	Colon	0.256	5.1	0.195	3.9
16	*C. albicans* 145-1	Colon	0.25	5.0	0.135	2.7
17	*C. albicans* 146	Colon	0.47	3.4	0.106	2.1
18	*C. albicans* 147	Colon	0.16	3.2	0.101	2.0
19	*C. albicans* 172	Colon	0.225	4.5	0.14	2.8
20	*C. albicans* 173	Colon	**0.105**	**2.1**	**0.105**	**2.1**
21	*C. albicans*174	Colon	**0.09**	**1.8**	**0.1**	**2.1**
22	*C. albicans* 175-2	Colon	**0.1**	**2.0**	**0.105**	**2.1**
23	*C. albicans*176	Colon	**0.09**	**1.8**	**0.09**	**1.8**
24	*C. albicans* 177	Colon	**0.11**	**2.2**	**0.1**	**2.0**
25	*C. albicans* 178c	Colon	**0.105**	**2.1**	**0.105**	**2.1**
26	*C. albicans* 183	Colon	**0.075**	**1.5**	**0.085**	**1.7**
27	*C. albicans* 28	Vaginal	**0.115**	**2.3**	**0.105**	**2.1**
28	*C. albicans* 29	Vaginal	**0.096**	**1.9**	**0.095**	**1.9**
29	*C. albicans* 30	Vaginal	**0.125**	**2.5**	**0.0125**	**2.5**
30	*C. albicans* 23s	Skin	0.171	3.4	0.075	1.5
31	*C. albicans* 212	Colon	0.145	2.9	0.09	1.8
32	*C. albicans* 213	Colon	0.135	2.7	0.105	2.1
33	*C. albicans* 215-1	Colon	**0.09**	**1.8**	**0.1**	**2.0**
34	*C. krusei* 9	Colon	**0.075**	**1.5**	**0.085**	**1.7**
35	*C. krusei* 10	Colon	0.16	3.2	0.115	2.3
36	*C. krusei* 21	Colon	**0.1**	**2.0**	**0.095**	**1.9**
37	*C. krusei* 22	Colon	**0.12**	**2.4**	**0.135**	**2.7**
38	*C. krusei* 23	Colon	0.24	4.8	0.13	2.6
39	*C. krusei* 2-n	Nose	0.175	3.5	0.145	2.9
40	*C. krusei* 2	Vaginal	**0.135**	**2.7**	**0.125**	**2.5**

Table 1. End

Strains		Source	Biofilm formation before batumin		Biofilm formation after 0.125 μg/ml batumin	
			optical density (OD_{630})	CU*	optical density (OD_{630})	CU*
40	*C. krusei* 2	Vaginal	**0.135**	**2.7**	**0.125**	**2.5**
41	*C. krusei* 5	Vaginal	0.19	3.8	0.13	2.6
42	*C. krusei* 6	Vaginal	0.215	4.3	0.151	3.0
43	*C. krusei* 7	Vaginal	**0.075**	**1.5**	**0.065**	**1.3**
44	*C. krusei* 25	Colon	**0.075**	**1.5**	**0.09**	**1.8**
45	*C. krusei* 26	Colon	**0.11**	**2.2**	**0.11**	**2.2**

Note: * — CU: conventional unit: OD_{630} in experimental samples / OD_{630} in control samples. The optic density of control samples is 0.05, which is the nutrient broth density; ** — the results are representative on three separate experiments. $P < 0.05$ (Mann-Whitney U-test).

strong biofilm formation (CU values between 2.6 and 3.1).

The analysis of the experimental data on batumin at the stage of biofilm formation for *C. albicans* showed dependence of batumin effectiveness at the stage of biofilm formation (Table 2).

Addition of batumin to the cultivation medium simultaneously with *C. albicans* did not influence biofilm formation of fungi in 40% of cases, and promoted reduction of biofilm formation values in 60% of cases for 63.1 ± 3.4% of the initial level ($P < 0.05$).

Addition of batumin in 90 min after incubation of *C. albicans* (an initial stage of biofilm formation) reduced values of biofilm formation in 70% of cases on average by 48.8 ± 4.4% versus control ($P < 0.05$). Addition after 24 hours of growth, the antibiotic reduced formation of biofilm in culture in 50% of cases versus 24.8% of control ($P < 0.05$), and in 50% of cases — stimulated this parameter by 32.4%.

After incubation of *C. albicans* with the antibiotic during the 48 hours in 70% of cases the lack of the preparation influence was noted and only in 30% of strains decrease in biofilm formation was noted only by 5.6%.

Thus, the biofilm of the studied strains of *C. albicans* is sensitive to batumin at early stages of its formation whereas the well-established biofilm was more resistant to studied preparation.

On the contrary, batumin in 22.2% of cases stimulated low level of biofilm formation in yeast-like fungi. Possibly, the obtained data reflect the developed relationship between species of microorganisms in a microbiocenosis, as it is known that in interaction of bacteria of the genus *Pseudomonas* and fungi of the genus *Candida* there is a mutual depression of biofilm formation of microorganisms at all stages of development [15, 16].

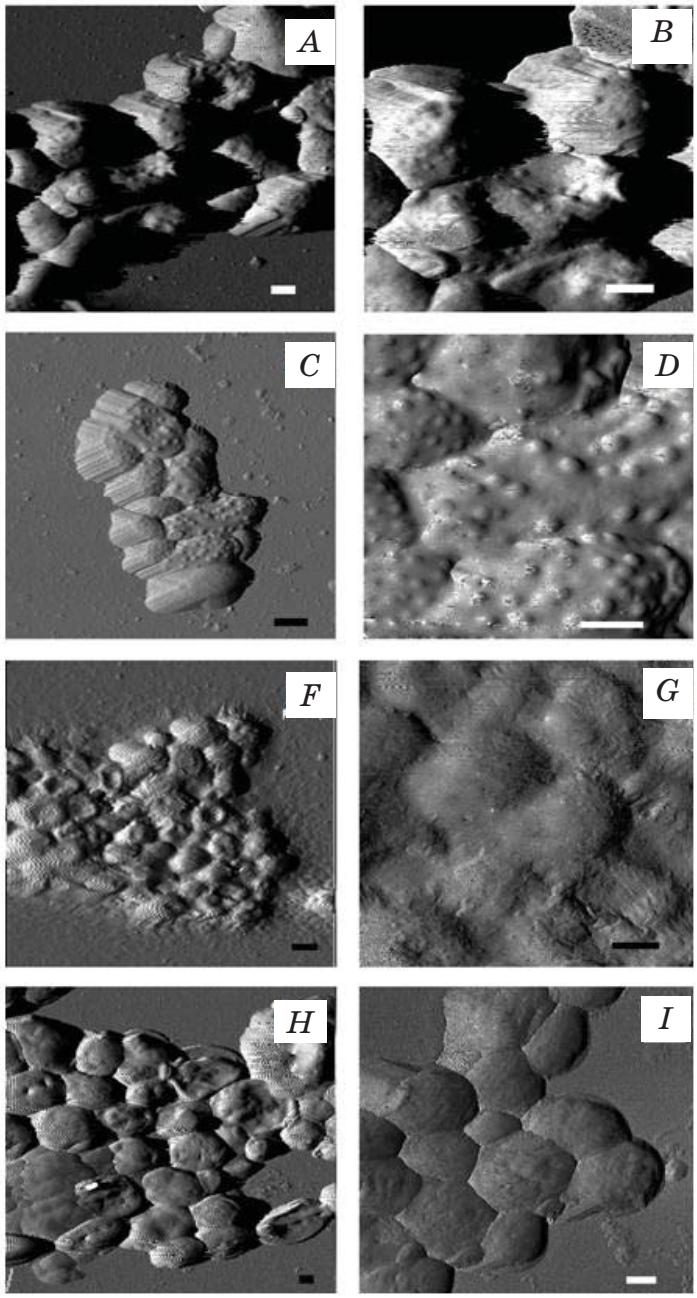
By its action on biofilms of cultures of *Candida* batumin is similar to action on biofilms of *Staphylococci* [13]. Sidrim et al. (2015) reported a similar effect when studying β-lactam antibiotics and vancomycin effects on formation of biofilms of *Candida* spp.

A more detailed study of batumin action upon *C. albicans* biofilm formation was carried out using atomic force microscopy for *C. albicans* ATCC 24433^T and *C. albicans* 127 (clinical isolate).

On the coverslip incubated with the cells *C. albicans* ATCC 24433^T without batumin the set of cells adhered to its surface (Figure, *A*). The glass coverslips were immersed into Luria-Bertani broth with 0.125 μg/ml of batumin and incubated for 48 h at 37 °C. At visualization of the surface of microorganisms, the spherical formations which are presumably gemmating daughter cells (Figure, *B*) are clearly visible. However, a significant decrease of the number of adherent cells in the presence of the antibiotic was not observed (Figure, *C*, *D*).

A detailed study of the surface of bacterial cells treated with batumin allows establishing significant reduction of their roughness values (Table 3).

The cells of *C. albicans* 127 were observed in the form of a monolayer of separate islets (Figure, *F*). This strain formed biofilm on the



Atomic force microscopy — topography images of *C. albicans* ATCC 24433^T ((*A*) (*B*)) without batumin; ((*C*) (*D*)) — in the presence of 0.125 μg/ml of batumin and *C. albicans* 127 without batumin (*F*, *G*), with antibiotic (*H*, *I*)

Scale bars: 1 μm.

surface of the glass, as can be seen. Surface biofilm was formed by an exopolymeric matrix with cells of round shape immersed in it (Figure, *G*). The addition in the medium of batumin caused, on the one hand, the lack of signs of an exopolymeric matrix (Figure, *H*, *I*), and on the other hand, the change of cell morphology. So the mean diameter of cells was equal to 1.88 ± 0.43 μm, which was significantly less than control values 2.29 ± 0.20 (Table 3).

Atomic force microscopy revealed qualitative changes in the exopolymeric matrix due to batumin treatment, as well as a significant reduction in the number of cells adhered to the coverslip, preventing formation of *C. albicans* 127 biofilm. In *C. albicans* ATCC 24433^T, a significant reduction in the number of cells adhered to the coverslip was not observed. In this case a nonspecific interaction of batumin and a surface ligand of *C. albicans* ATCC 24433^T is probable.

Table 2. **Influence of batumin (0.125 μg /ml), expressed as CU*, after addition at different stages of biofilm formation by *Candida albicans* strains**

C. albicans strains	Before	At 0 h	After 90 min	After 24 h	After 48 h
ATCC 24433^T	2.4	**2.6**	1.7	1.7	2.4
118	2.9	1.7	1.8	2.9	2.8
123	1.8	**2.0**	**1.8**	**2.3**	**2.0**
173	2.1	**2.1**	2.0	**2.8**	2.1
174	1.8	**2.1**	1.8	**2.6**	1.8
127	2.9	1.8	1.8	2.6	2.9
128c	4.3	2.5	3.0	3.5	4.0
146	3.4	2.1	2.2	2.4	3.4
147	3.2	2.1	2.5	3.0	3.2
112	2.9	1.8	1.9	**3.6**	2.9

Note: * — CU: conventional unit: OD630 in experimental samples / OD_{630} in control samples; ** — the results are representative on three separate experiments. $P < 0.05$ (Mann-Whitney U-test).

Table 3. **Morphological characteristics of *C. albicans* ATCC 24433^T and *C. albicans* 127 in the presence of batumin 0.125 μg/ml**

C. albicans strain	Batumin concentration (μg/ml)	Adherent cells,%	Length (μm)	Width (μm)	Height (μm)	Roughness values (nm)
ATCC 24433	0	100.0 ± 11.0	3.50 ± 1.17	2.09 ± 0.50	1.86 ± 0.53	49.4 ± 12.3
	0.125	47.0 ± 4.0*	3.89 ± 1.38	1.91 ± 0.61	1.09 ± 0.35*	41.0 ±16.0
127	0	100.0 ± 11.0	2.66 ± 0.45	2.29 ± 0.20	1.85 ± 0.25	45.6 ± 6.5
	0.125	15.0 ± 5.5	2.77 ± 0.42	1.88 ± 0.43*	1.62 ± 0.15	44.2 ± 14.4

Note: * — $P < 0.05$ (Mann-Whitney U-test).

The results presented in this work showed that all studied *C. albicans* and *C. krusei* strains were highly resistant against batumin (MIC ≥ 512 μg/ml). However, the antibiotic showed inhibition of biofilm formation of these microorganisms.

The biofilm of the studied strains of *C. albicans* and *C. krusei* was sensitive to batumin at early stages of its formation, whereas the well-established biofilm was more resistant to studied preparation.

The data obtained by an atomic-force microscopy confirm the ability of batumin to prevent formation of biofilm in the studied strains that allows to consider it as the preventive agent for treatment of yeast-like fungi of the genus *Candida.*

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АКТИВНІСТЬ *in vitro* АНТИБІОТИКА БАТУМІНУ ЩОДО БІОПЛІВОК *Candida albicans*

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Метою роботи було вивчити дію батуміну на штами *Candida albicans* і *Candida krusei* в планктонній та біоплівковій формі, а також одержати більш детальні відомості щодо впливу батуміну на формування біоплівки з використанням атомно-силової мікроскопії. Мінімальну інгібувальну концентрацію (МІК) батуміну досліджували відповідно до стандартів CLSI. Формування біоплівки вивчали фотометричним методом O'Toole за допомогою планшетного фотометра ELx808 (BioTek, USA) за довжини хвилі 630 нм.

Полікетидний антибіотик батумін має високу селективну активність стосовно стафілококів. Водночас антибіотик, до є неактивний стосовно дріжджів роду *Candida* (МІК 512 мкг/мл), показав інгібувальну дію на формування біоплівок у цих мікроорганізмів. Вплив батуміну на утворення біоплівки вивчали на типових і колекційних штамах *C. albicans*, *C. krusei* та клінічних ізолятах. Присутність у середовищі 0,125 мкг/мл батуміну (1/2 МІК для стафілококів) знижувало утворення біоплівки у 55,6% досліджуваних штамів. Їхні значення варіювали для *C. albicans* від 1,5 до 3,9 УО, для *C. krusei* від 2,3 до 3,0. Батумін був ефективніший щодо штамів *Candida* з високими значеннями біоплівкоутворення.

Атомно-силова мікроскопія виявила якісні зміни в екзополімерному матриксі за дії батуміну, а також значне зменшення кількості адгезованих клітин, запобігаючи утворенню біоплівки *C. albicans* 127. Однак у *C. albicans* ATCC 24433 суттєвого зменшення числа адгезованих клітин у присутності антибіотика не спостерігалося.

Отримані за допомогою атомно-силової мікроскопії дані підтверджують здатність батуміну запобігати утворенню біоплівки у досліджуваних штамів, що дає змогу розглядати його як превентивний засіб при лікуванні інфекцій, які спричинені дріжджоподібними грибами роду *Candida*.

Ключові слова: батумін, *Candida*, біоплівка, атомно-силова мікроскопія.

АКТИВНОСТЬ *in vitro* АНТИБИОТИКА БАТУМИНА В ОТНОШЕНИИ БИОПЛЕНОК *Candida albicans*

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Целью работы было изучение действия батумина на штаммы *Candida albicans* и *Candida krusei* в планктонной и биопленочной форме, а также получение более подробных сведений о влиянии батумина на формирование биопленки с использованием атомно-силовой микроскопии. Минимальную ингибирующую концентрацию (МИК) батумина исследовали в соответствии со стандартами CLSI. Формирование биопленки изучали фотометрическим методом O'Toole с помощью планшетного фотометра ELx808 (BioTek, USA) при длине волны 630 нм.

Поликетидный антибиотик батумин обладает высокой селективной активностью в отношении стафилококков, в то же время антибиотик, будучи неактивным в отношении дрожжей рода *Candida* (МИК 512 мкг/мл), показал ингибирующее действие на формирование биопленок у этих микроорганизмов. Влияние батумина на образование биопленки изучали на типовых и коллекционных штаммах *C. albicans, C. krusei*, а также клинических изолятах. Присутствие в среде 0,125 мкг/мл батумина (1/2 МИК для стафилококков) снижало образование биопленки у 55,6% исследуемых штаммов. Их значения варьировали для *C. albicans* от 1,5 до 3,9 УЕ, для *C. krusei* от 2,3 до 3,0. Батумин был более эффективен в отношении штаммов *Candida* с высокими значениями биопленкообразования.

Атомно-силовая микроскопия выявила качественные изменения в экзополимерном матриксе при действии батумина, а также значительное уменьшение числа адгезированных клеток, предотвращая образование биопленки C. albicans 127. Однако у *C. albicans* ATCC 24433 существенного уменьшения числа адгезированных клеток в присутствии антибиотика не наблюдалось.

Полученные с помощью атомно-силовой микроскопии данные подтверждают способность батумина предотвращать образование биопленки у исследуемых штаммов, что позволяет рассматривать его как превентивное средство при лечении инфекций, вызванных дрожжеподобными грибами рода *Candida*.

Ключевые слова: батумин, *Candida*, биопленка, атомно-силовая микроскопия.

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EFFECTIVE TECHNOLOGY OF PHARMACEUTICAL ENTERPRISES WASTEWATER LOCAL TREATMENT FROM ANTIBIOTICS

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The work was aimed to study the processes of industrial wastewater purification of a pharmaceutical company treatment and to recommend an effective technology for local treatment of such wastewater in accordance with the requirements of normative documents on the treated wastewater disposal into the urban sewerage system.

Model solutions of cefuroxime, a cephalosporin antibiotic, in distilled water with a concentration of 25 and 35 mg/dm^3 were used for the study. COD model solutions, which is, respectively, 90 and 120 mg/dm^3. Chemical oxygen demand was etermined by the dichromate method (according to the guiding normative document (GND) 211.1.4.021-95. "Methods for Chemical Oxygen Demand (COD) Determining in Surface and Wastewater").

The proposed technology included the following successive processes of physical and chemical treatment of wastewater: aeration — equalization of quantitative and qualitative composition of wastewater and oxidation up to 30% of organic matter; coagulation — removing of antibiotics from wastewater by their adsorption on a highly developed surface of mineral coagulants flakes; sedimentation — separating of the formed flakes; oxidation of antibiotics destruction products by hydrogen peroxide; filtration — removal of fine impurities. Effect of COD reduction rate in the coagulation and setteling of wastewater in the case of ferrous sulphate III was 79.2% and 75%, which is higher by 4,2–6,7% than when using aluminum sulphate. The COD index changing of the pharmaceutical enterprise wastewater by the stages of its purification was found as follows: "aeration — coagulation with iron sulfate III — sedimentation — oxidation — filtration". After filtering effect of reducing COD index was 95,8–100% at the initial value of 120 and 90 mg/dm^3 respectively. The technology of local wastewater treatment from antibiotics was developed, which was based on the consistent use of physico-chemical methods of treatment and enabled to remove antibiotics and related substances from wastewater in accordance with the requirements of normative documents and disposal of treated wastewater into the urban wastewater system.

Application of the developed technology of local wastewater treatment from antibiotics and related substances by the pharmaceutical companies would lead to meet the requirements of industrial wastewater disposal into the city drainage system, to a significant risk reduction of the antibiotics influence on the microorganisms of active sludge of the urban wastewater biological treatment facilities, to operating costs reduction to achieve maximum allowable discharges (MAD) of wastewater into a natural reservoir.

Key words: antibiotics, wastewater, treatment, pharmaceutical enterprises.

Environmental protection should be a priority of state policy, Ukrainians should look at all spheres of life through an ecological prism, and business should gradually move to environmentally friendly production processes. Environmental standards must be integrated into all areas of the economy and everyday life of Ukrainians. Such a system works successfully in European countries and should be tested in our country. Only the integration of environmental policy and socio-economic development will make it possible to stabilize the state of the environment in Ukraine and move to a model of sustainable development.

Ukraine has made significant progress in implementing European environmental legislation and meeting its obligations to implement environmental requirements such as those operating in EU countries. In particular, the Laws "On Environmental Impact Assessment" (which is already working successfully) and "On Strategic Environmental Assessment" were adopted, which oblige to take into account the needs of the environment

at the stage of development and decision-making.

Unfortunately, in relation to the normative documents of Ukraine on wastewater treatment from pollution, in contrast to the normative documents in the EU, USA and Canada, these documents do not take into account several important indicators of wastewater composition that directly affect the effects of wastewater treatment, active sludge properties and residual concentrations of environmentally hazardous pollutants in treated wastewater during disposal into natural reservoirs. Such indicators include the concentration of nitrogen-containing compounds in terms of total nitrogen, the concentration of phosphorus-containing compounds in terms of total phosphorus and the concentration of antibiotics and related substances.

The efficiency of municipal treatment plants (speed, treatment effects and depth of individual pollutants removal, the composition of their oxidation products, etc.) depends on many factors, among which one of the main is the physico-chemical composition of wastewater.

The most sensitive link to the negative impact of wastewater chemical components is the active sludge of biological treatment plants, namely its main technological properties such as the ability to oxidize dissolved pollutants and the ability to sediment and separate from the treated liquid during settling. An important indicator of wastewater safety for the reliability of urban treatment plants is the impact of their pollution on the sedimentation properties of active sludge and the ability to separate from the treated liquid during settling. These indicators are not always taken into account when controlling the composition of industrial wastewater supplied to the city sewer, as well as the presence of biologically active compounds like antibiotics, biocides, inhibitors, etc. in wastewater. Neglecting these characteristics causes a number of extremely negative consequences for water utilities: reducing the efficiency of urban wastewater treatment and increasing operating costs to achieve the required indicators, exceeding the MAD when discharging treated wastewater into natural reservoirs (indicators strictly controlled by environmental services), active sludge "swelling" with the prospect of its further loss, etc.

Although in Ukraine there are companies that provide local wastewater treatment after the production of liquid drugs, such as Private Joint Stock Company (PJSC) "Scientific Production Center (SPC) Borschagivsky Chemical and Pharmaceutical Plant", as well as companies that have begun work to address the issue of local wastewater treatment from antibiotics — Lekhim-Obukhiv LLC, Obukhiv city, and Chemical Plant "Chervona Zirka" PJSC, Kharkiv city, but nowadays one of the urgent problems is the presence of antibiotics in the wastewater of pharmaceutical industries.

In most cases, antibiotics and the products of their transformation do not have the ability to biological transformation, as well as they adversely affect the microorganisms of treatment plants (active sludge aeration tanks).

Physicochemical and biological methods are used to treat pharmaceutical wastewater from antibiotics.

Among the physico-chemical methods, the methods aimed at antibiotic molecules structure destroying should be noted, such as oxidation by various oxidizing agents: ozone [1, 2]; hydrogen peroxide [3, 4]; potassium permanganate [2, 5] and others.

Another group of physico-chemical methods is focused on antibiotics removal using the following methods: adsorption on coagulant or flocculant flakes [6, 7], on activated carbon [8, 9], on zeolites, etc.; membrane separation (ultrafiltration) [10, 11].

The use of biological methods of wastewater treatment from antibiotics [1, 12, 13, 14, 15] has shown good results with long-term aeration of wastewater. Thus, when aerated for 1–3 days, the purification effect by biological oxygen demand (BOD_5) reached 90–96%. To increase the treatment effect of pharmaceutical companies wastewater, in the literature the following measures are proposed:

- to dilute the concentrated wastewater of the pharmaceutical company with municipal wastewater 4–5 times;
- to use two- or three-stage biological treatment technologies with a total efficiency of 90–95%;
- to dose the compounds of inorganic phosphorus in aeration tanks in the treatment of wastewater from antibiotics, because of its very small amount in the wastewater of pharmaceutical industries;
- to increase the temperature of wastewater up to 35 °C, which allows to increase the treatment efficiency up to more than 90%, etc.

In Igor Sikorsky Kyiv Polytechnic Institute, the scientists of Ecobiotechnology and Bioenergy Department conducted the research (with practical implementation) to develop a technology for local treatment of industrial wastewater from antibiotics.

The purpose of the work is to investigate the processes of industrial wastewater treatment of a pharmaceutical enterprise and to propose

an effective technology of local wastewater treatment in accordance with the requirements of normative documents on the treated wastewater discharge into the urban sewage.

Materials and Methods

Model solutions of cefuroxime, that is cephalosporin row antibiotic, were prepared for the study in distilled water with a concentration of 25 and 35 mg/dm^3. For the preparation of solutions, a pharmaceutical form of cefuroxime was used, namely tablets (cefuroxime Sandoz). 1 tablet contains 250 mg of cefuroxime and excipients. The tablets were crushed and solutions of the above concentration were prepared.

The chemical oxygen demand indexes (COD) of model solutions were determined, which were, respectively, 90 and 120 mg/dm^3. Chemical oxygen demand (COD) was determined by the dichromate method (according to the guiding normative document (GND) 211.1.4.021-95. "Methods for Chemical Oxygen Demand (COD) Determining in Surface and Wastewater").

The following procedures of model solutions processing were investigated consistently:

1. Model solutions aeration by means of the aquarium compressor with air productivity of 20–40 dm^3/h) and the aerator installed in the vessel of 250 ml with the investigated model solution, within 16 hours;

2. Coagulation with mineral coagulants:

• ferrum III sulfate at a dose of 50 mg/dm^3 by $Fe_2(SO_4)_3$, $Fe_2(SO_4)_3 \cdot 7H_2O$ was used as a reagent;

• aluminium sulfate at a dose of 50 mg/dm^3 by $Al_2(SO_4)_3$, $Al_2(SO_4)_3 \cdot 18H_2O$ was used as a reagent.

To achieve complete coagulation of mineral salts, the pH was adjusted to 7.0–7.5 with a solution of alkali NaOH.

The model solutions were quickly mixed with the coagulant solution for 1–2 min and continued to stir slowly for 15–20 min till flakes formation and agglomeration.

3. Sedimentation the coagulated impurities for 30 minutes.

4. Clarified water selection from the vessel and formed precipitate separation.

5. Oxidation of clarified water contaminants with hydrogen peroxide at a dose of 30 mg/dm^3 using a solution of 30% concentration for 30 min with stirring using a laboratory magnetic stirrer.

6. Solution filtration through a sand filter.

Sand for filtration (fraction of 0.5–0.8 mm) was prepared as follows: thoroughly washed with running water from mechanical impurities; dried in an oven at 105 °C for 10 hours; fired in a muffle furnace at 600 °C for 2 hours to ash all residual contaminants; cooled and washed with distilled water. After that, the sand was transferred to the filter and filter layer of a 10 cm high was formed, filtration rate was 2 cm/min.

At each stage of the process, water samples were taken to determine the COD value. The analysis results and the determined purification effects at each stage are given in the Table and in the Fig. 1 and 2. The reliability of the obtained data is $P < 0.05$, i.e. statistically significant differences are found. To assess the significance of the difference between the averages of the two groups, t–test (Student's test) was used.

Results and Discussion

The results of studies using aluminium sulfate (Table) showed lower values of COD reducing effect — 80 and 75% at the initial COD 90 and 120 mg/dm^3, respectively. Therefore, a coagulant based on ferrum III sulfate has a certain advantage over aluminium sulfate.

As it is shown in Fig. 1, the greatest decrease in the COD index was observed in the coagulation processes of pollutants contained in wastewater, and in settling. For coagulation, mineral coagulants based on ferrum III sulfate and aluminium sulfate were used with pH adjustment to achieve the isoelectric region for the most complete formation of iron III and aluminium hydroxides, their coagulation and wastewater treatment from organic pollutants. The effect of COD reduction in coagulation and sedimentation of wastewater in the case of ferrum III sulfate usage was 79.2% and 75.0% in the initial COD of untreated wastewater, respectively, 90 and 120 mg/dm^3.

Increasing the initial COD index value in untreated wastewater reduces the effect of organic pollutants removal by COD when using coagulation and sedimentation by 4.0–6.5%.

The use of other purification processes according to the studied technological scheme allowed COD reduction by 25.0–33.3% during aeration, by 91.7–94.4% during oxidation with hydrogen peroxide, by up to 95.8–100% during filtration.

Based on the obtained results, the technology was developed (Fig. 3), process parameters were established (aeration duration, reagent dose, sedimentation duration, filtration rate, filter loading height, amount of precipitate formed, etc.).

The results of physico-chemical treatment technology research of a pharmaceutical company industrial wastewater according to the scheme "aeration — coagulation — sedimentation — oxidation — filtration"

No. of model solutions and cleaning effect, %	COD index value. mg/dm^3				
	before cleaning	after aeration	after settling	after oxidation	after filtration
Option with ferrum III sulfate coagulation					
1	90	60	12	5	0
Effect, %	–	33.3	86.7	94.4	100
2	120	90	25	10	5
Effect, %	–	25	79.2	91.7	95.8
Option with aluminium sulfate coagulation					
3	90	64	18	7	0
Effect, %	–	28.9	80	92.2	100
4	120	87	30	12	5
Effect, %	–	27.5	75	90	95.8

Note. The effects of COD index reduction were determined in each purification process with $P < 0.05$ relative to the initial COD values before purification.

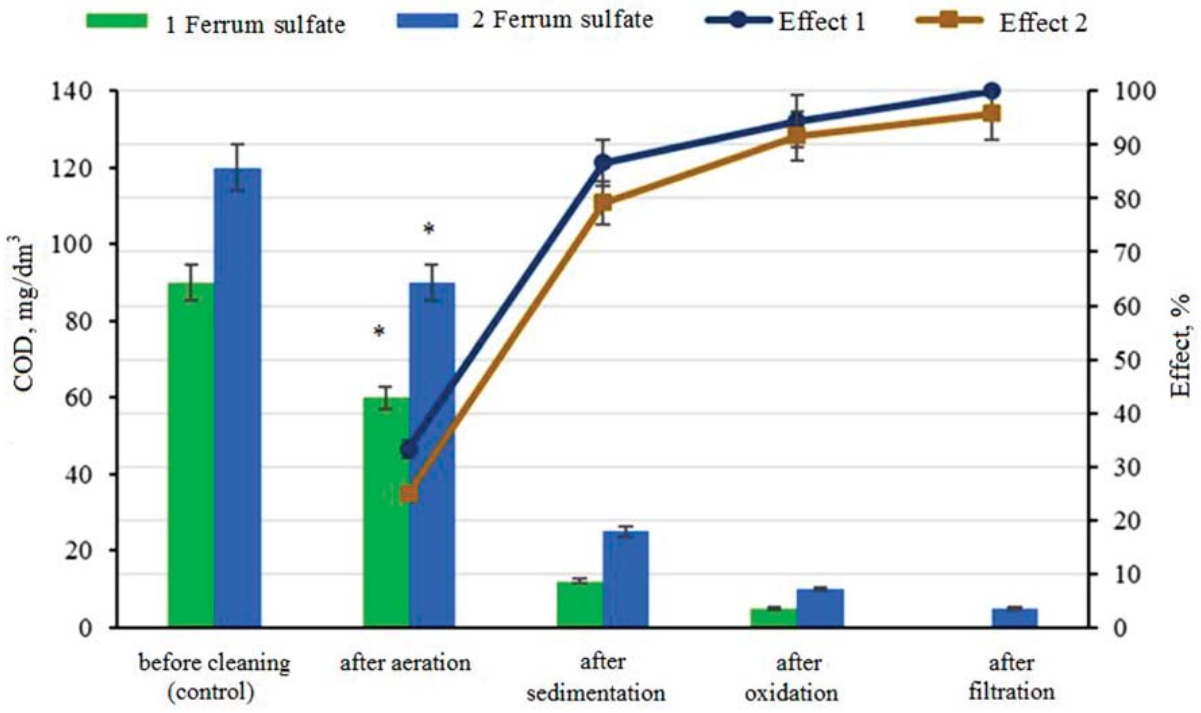

Fig. 1. **Change in the COD index of a pharmaceutical company industrial wastewater and the effect of purification by COD in purification by the technology "aeration — coagulation with ferrum III sulfate — sedimentation — oxidation — filtration":**
effect 1 was determined at a COD value in untreated wastewater of 90 mg/dm^3 in each process relative to this value; effect 2 — at a COD value in untreated wastewater — 120 mg/dm^3
* — $P < 0.05$ compared to the control without ferrum sulfate addition

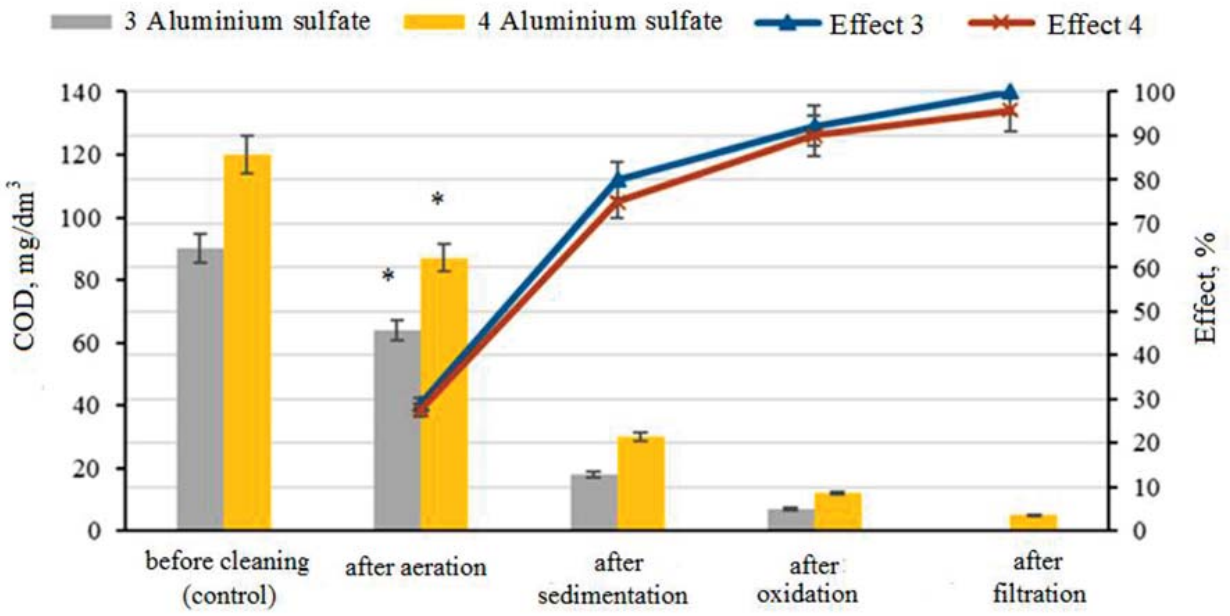

Fig. 2. **Change in the COD index of a pharmaceutical company industrial wastewater and the effect of purification by COD in purification by the technology "aeration — coagulation with aluminium sulfate — sedimentation — oxidation — filtration":**
effect 3 was determined at a COD value in untreated wastewater of 90 mg/dm^3 in each process relative to this value; effect 4 — at a COD value in untreated wastewater — 120 mg/dm^3
* — $P < 0.05$ compared to the control without aluminium sulfate addition

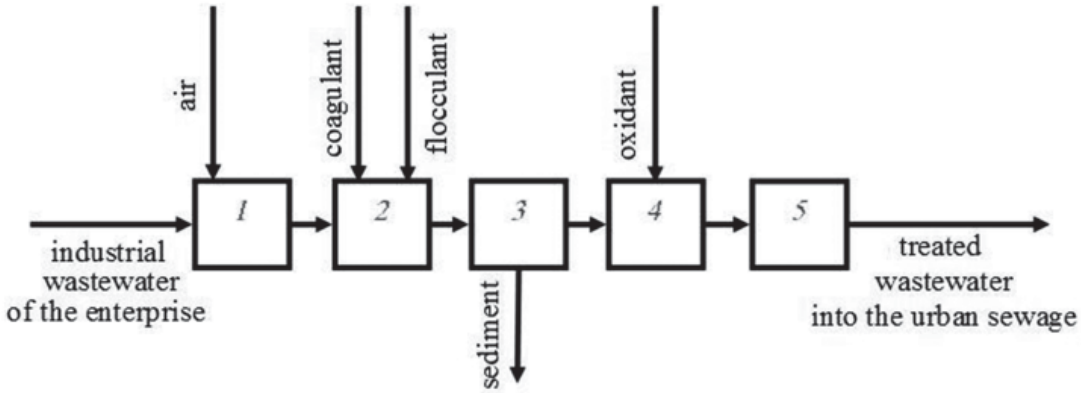

Fig. 3. **Scheme of pharmaceutical company industrial wastewater treatment technology from cefuroxime:**
1 — averaging; *2* — coagulation with mineral coagulants; *3* — settling; *4* — oxidation with hydrogen peroxide; *5* — filtration

The technology includes sequential processes of physico-chemical wastewater treatment, namely averaging, coagulation, settling, oxidation with hydrogen peroxide, and filtration.

Given the uneven drainage of industrial wastewater from pharmaceutical companies and fluctuations in pollutants concentrations during the day, to equalize the quantitative and qualitative composition of wastewater when it enters the treatment plant for local treatment, wastewater averaging is required which is carried out using an aeration system.

The use of air for wastewater aeration in the averaging unit allows in the first stage to oxidize up to 30% of organic substances contained in industrial wastewater with the help of oxygen.

The next process of wastewater treatment is coagulation of pollutants with a mineral coagulant, for example, with ferrum III sulfate — $Fe_2(SO_4)_3$ at a dose of 50–100 mg/dm^3

with alkalization of wastewater with NaOH solution up to pH 7.0–7.5. It is possible to use a flocculant, for example, Magnafloc at a dose of 1–2 mg/dm^3 to form large flocks and intensify the process of coagulant flakes sedimentation. For coagulation, aluminium sulfate $Al_2(SO_4)_3$ can also be used instead of $Fe_2(SO_4)_3$ in combination with alkali and flocculant. However, it should be noted that a more economical option is $Fe_2(SO_4)_3$.

Due to the large specific surface area of colloidal particles, they have a significant surface energy and, consequently, a high adsorption capacity, due to which the adsorption of dissolved in wastewater substances takes place on the surface of the formed colloidal particles.

The flocculation process application is based on the mechanism of flocculants action, which is based on the phenomenon of flocculant molecules adsorption on the surface of colloidal particles, formation of mesh structure of flocculant molecules, adhesion of colloidal particles due to Van der Waals forces. Under the action of flocculants, three-dimensional structures are formed between colloidal particles, capable of faster and more complete separation from the liquid phase.

Flocculation is carried out to intensify the process of iron hydroxide flakes formation in order to increase the rate of their sedimentation. Flocculants usage allows reducing the coagulants dose and coagulation process duration, and increasing the rate of the formed flakes sedimentation.

Thus, when introducing coagulant and flocculant into industrial wastewater, due to the processes of coagulation and flocculation, there is removal of contaminants from wastewater, which are impurities of varying degrees of dispersion (fine, colloidal and molecular-soluble substances) due to adsorption of these substances on the highly developed surface of mineral coagulants flakes which are formed in water at maintenance of conditions necessary for coagulation.

To separate the formed flakes with adsorbed contaminants from water, the sedimentation process is used. The taken duration of sedimentation is 1.5 hours. To increase the efficiency of sedimentation (by 25–30%) and reduce the process duration (up to 15–20 minutes), you can use a thin-layer sedimentation tank with inclined shelves of parallel working tiers of the sedimentation tank.

The sludge formed in the sedimentation tank (10–12% of the wastewater volume) must be periodically taken away for consolidations. You can use filter bags to dehydrate the sludge from the initial humidity of 95.0–99.7% to the moisture content of the compacted sludge of 70–80% for further disposal.

Further, according to the technology (Fig. 3), wastewater after sedimentation is directed to the oxidation of contaminants remaining in wastewater after coagulation, flocculation and separation of sludge, using hydrogen peroxide H_2O_2 as oxidant at a dose of 30–60 mg/dm^3 for 30 min of wastewater contact with the oxidant under stirring conditions to undergo reactions between hydrogen peroxide and contaminants. The advantages of hydrogen peroxide usage compared to other oxidizing reagents are high oxidation efficiency of organic substances, absence of residual concentrations of hydrogen peroxide in treated wastewater due to its decomposition, stability of salt content of treated wastewater, and the course of reactions without toxic intermediates formation.

The final process in the technology is the process of wastewater filtration, for example, through sand filtration loading. Due to filtration, fine impurities are removed from wastewater, which are carried away by the water flow after the sedimentation stage: small flakes of coagulant and colloidal substances that are retained on the surface of the grains of the filter loading due to the action of adhesion forces, mutual colloids coagulation, and adsorption on the loading surface.

Conclusions

Wastewater treated using the developed technology can be discharged into the urban drainage system and will not interfere with the operation of municipal treatment facilities.

1. On the basis of experimental research on wastewater treatment of pharmaceutical companies from antibiotics and related substances the local treatment technology has been developed, which is based on the consistent use of physico-chemical treatment methods, can be used for local treatment of these wastewater and for antibiotics and related substances removal from wastewater in accordance with the requirements of normative documents, and disposal of treated wastewater to the urban sewerage system.

2. The performed experimental researches allowed establishing the rational parameters

of technological processes of pharmaceutical enterprises wastewater treatment from antibiotics and related substances and recommending the offered technical solutions for introduction at the enterprises of pharmaceutical branch.

3. The developed technology of local wastewater treatment from antibiotics and related substances application by pharmaceutical companies will significantly reduce the risk of MAD exceeding on the disposal of wastewater treated in urban biological treatment plants, reduce operating costs to achieve MAD, reduce the risk of active sludge "swelling" and related operating and other economic costs.

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ЕФЕКТИВНА ТЕХНОЛОГІЯ ЛОКАЛЬНОГО ОЧИЩЕННЯ СТІЧНИХ ВОД ФАРМАЦЕВТИЧНИХ ПІДПРИЄМСТВ ВІД АНТИБІОТИКІВ

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Метою роботи було дослідити процеси очищення виробничих стічних вод фармацевтичного підприємства та запропонувати ефективну технологію локального очищення таких стічних вод відповідно до вимог нормативних документів щодо скидання очищених стічних вод у систему водовідведення міста.

Для дослідження було використано модельні розчини цефуроксиму – антибіотика цефалоспоринового ряду, у дистильованій воді з концентрацією 25 і 35 мг/дм^3. Хімічне споживання кисню модельних розчинів, становило, відповідно, 90 і 120 мг/дм^3. ХСК визначали за біхроматним методом (згідно з КНД 211.1.4.021-95. «Методика визначення хімічного споживання кисню (ХСК) в поверхневих і стічних водах»).

Ефект зниження показника ХСК при коагуляції і відстоюванні стічних вод у випадку використання сульфату заліза III становив 79,2% і 75%, що вище на 4,2–6,7% ніж при застосування сульфата алюмінію. Встановлено зміну показника ХСК стічних вод фармпідприємства за етапами їх очищення: «аерація — коагуляція сульфатом заліза III — відстоювання — окиснення — фільтрування». Після фільтрації ефект зниження показника ХСК становив 95,8–100 % при початкових значення 120 і 90 мг/дм^3 відповідно. Розроблено технологію локального очищення стічних вод від антибіотиків, яка ґрунтується на послідовному використанні фізико-хімічних методів очищення та дозволяє видалити із стічних вод антибіотики і супутні їм речовини до вимог нормативних документів та відвести очищені стічні води в міську систему водовідведення.

Застосування фармацевтичними підприємствами розробленої технології локального очищення стічних вод від антибіотиків та супутніх їм речовин призведе до забезпечення вимог скиду виробничих стічних вод у міську систему водовідведення, до суттєвого зниження небезпеки впливу антибіотиків на мікроорганізми активного мулу біологічних очисних споруд міста, до зменшення експлуатаційних витрат на досягнення гранично-допустимих скидів стічних вод у природну водойму.

Ключові слова: антибіотики, стічні води, очистка, фармацевтичні підприємства.

ЭФФЕКТИВНАЯ ТЕХНОЛОГИЯ ЛОКАЛЬНОЙ ОЧИСТКИ СТОЧНЫХ ВОД ФАРМАЦЕВТИЧЕСКИХ ПРЕДПРИЯТИЙ ОТ АНТИБИОТИКОВ

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Целью работы было исследование процессов очистки производственных сточных вод фармацевтического предприятия и предложение эффективной технологии локальной очистки таких сточных вод в соответствии с требованиями нормативных документов по сбросу очищенных сточных вод в систему водоотведения города.

Для исследования были использованы модельные растворы цефуроксима - антибиотика цефалоспоринового ряда, в дистиллированной воде с концентрацией 25 и 35 мг/дм^3. Химическое потребление кислорода модельных растворов составляло 90 и 120 мг/дм^3, соответственно. ХПК определяли по бихроматному методу (по КНД 211.1.4.021-95. «Методика определения химического потребления кислорода (ХПК) в поверхностных и сточных водах»).

Эффект снижения показателя ХПК при коагуляции и отстаивании сточных вод в случае использования сульфата железа III составил 79,2% и 75%, что выше на 4,2–6,7% чем при применении сульфата алюминия. Установлено изменение показателя ХПК сточных вод фармпредприятия по этапам их очистки: «аэрация — коагуляция сульфатом железа III — отстаивание — окисление — фильтрование». После фильтрации эффект снижения показателя ХПК составлял 95,8–100% при начальных значения 120 и 90 мг/дм^3 соответственно. Разработана технология локальной очистки сточных вод от антибиотиков, основанной на последовательном использовании физико-химических методов очистки и позволяет удалить из сточных вод антибиотики и сопутствующие им вещества с требованиями нормативных документов и отвести очищенные сточные воды в городскую систему водоотведения.

Применение фармацевтическими предприятиями разработанной технологии локальной очистки сточных вод от антибиотиков и сопутствующих им веществ приведет к обеспечению требований сброса производственных сточных вод в городскую систему водоотведения, к существенному снижению опасности воздействия антибиотиков на микроорганизмы активного ила биологических очистных сооружений города, к уменьшению эксплуатационных расходов на достижение гранично-допустимых сбросов сточных вод в естественный водоем.

Ключевые слова: антибиотики, сточные воды, очистка, фармацевтические предприятия.