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RESEARCH ARTICLE

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PHARMACY & PHARMACOLOGY

Cerebroprotective effect of sitagliptin and aminoguanidine combination in disorders of cerebral circulation

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The aim of the study was to evaluate a cerebroprotective activity of the sitagliptin and aminoguanidine combination in rats with an acute and chronic cerebral circulation insufficiency, as well as with a traumatic brain injury.

Materials and methods. The study was carried out on male Wistar rats in 3 stages using, respectively, a model of a chronic pathology: a chronic cerebral circulation insufficiency (CCCI), as well as 2 models of the acute brain injury (BI): an acute cerebral circulation insufficiency (ACCI), and a traumatic brain injury (TBI). A CCCI was modeled by a bilateral stenosis of the common carotid arteries (by 50%), a model of a hemorrhagic stroke caused by an intracerebral injection of the autologous blood was used as a stroke, a TBI was modeled by a mechanical damage to the brain tissue. To assess the pathology course severity, the following tests were used: Adhesion test, Open field, Morris water maze test, as well as Garcia and Combs&D'Alecy scales. In the animals with an acute damage to the brain at the end of the experiment, the severity of edema of the affected hemisphere was also determined. The treatment was with sitagliptin (10 mg/kg), aminoguanidine (25 mg/kg), or a combination thereof. The obtained data were subjected to the statistical processing.

Results. In the course of the study, it was found out that the administration of a sitagliptin and aminoguanidine combination, unlike each of the components, had a cerebroprotective effect in the animals with a chronic or acute damage to the brain, reducing the severity of psychoneurological (cognitive and sensory-motor) disorders, as well as the brain edema.

Conclusion. Aminoguanidine, as an iNOS blocker, enhances the action of sitagliptin, preventing the brain edema development and reducing the neurological deficit severity (the severity of cognitive and sensory-motor impairments) in the animals with an acute and chronic cerebral circulation insufficiency**.**

Keywords: DPP-4 inhibitors; sitagliptin; preclinical studies; acute and chronic cerebral circulation insufficiency; traumatic brain injury

Abbreviations: iNOS – inducible nitric oxide synthase; mNSS – modified Neurological Severity Score; NO – nitric oxide (II); HS – hemorrhagic stroke; GLP-1 – glucagon-like peptide-1; iDPP-4 – inhibitors of dipeptidyl peptidase-4; SGLT-2 – sodium-glucose linked transporter-2; BP – blood pressure; CVDs – cerebrovascular disorders; ACCI – acute cerebral circulation insufficiency; CCAs – common carotid arteries; CCCI – chronic cerebral circulation insufficiency; TBI – traumatic brain injury; ED – endothelial dysfunction.

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Церебропротекторное действие комбинации ситаглиптина с аминогуанидином при нарушениях мозгового кровообращения

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Цель. Оценить церебропротекторную активность комбинации ситаглиптина с аминогуанидином у крыс с острым и хроническим нарушением мозгового кровообращения, а также черепно-мозговой травмой.

Материалы и методы. Исследование проведено на крысах-самцах линии Wistar в 3 этапа с использованием модели хронической патологии: хроническое нарушение мозгового кровообращения (ХНМК), а также 2-х моделей острого повреждения головного мозга (ГМ) – острое нарушение мозгового кровообращения (ОНМК) и черепно-мозговая травма (ЧМТ). ХНМК моделировали двусторонним стенозированием общих сонных артерий (на 50%); в качестве ОНМК использовали модель геморрагического инсульта, вызванного внутримозговой инъекцией аутокрови; ЧМТ моделировали механическим повреждением ткани ГМ. Для оценки тяжести течения патологии использовали следующие тесты: «Адгезивный тест», «Открытое поле», «Водный лабиринт Морриса», а также шкалы Garcia и «Combs&D'Alecy». У животных с острым повреждением ГМ в конце эксперимента определяли выраженность отека пораженной гемисферы. В качестве лечения вводили ситаглиптин (10 мг/кг), аминогуанидин (25 мг/кг) или их комбинацию. Полученные данные подвергались статистической обработке.

Результаты. В ходе исследования было установлено, что введение комбинации ситаглиптина с аминогуанидином, в отличие от действия каждого из компонентов по отдельности, оказывало церебропротекторное действие у животных с хроническим или острым вариантом повреждения ГМ, снижая выраженность психоневрологических (когнитивных и сенсорно-моторных) нарушений, а также отёка ГМ.

Заключение. Аминогуанидин как блокатор iNOS усиливает действие ситаглиптина, предотвращая развитие отёка ГМ и уменьшая выраженность неврологического дефицита (выраженность когнитивных и сенсорно-моторных нарушений), у животных с острой или хронической недостаточностью мозгового кровообращения.

Ключевые слова: ингибиторы ДПП-4; ситаглиптин; доклинические исследования; острые и хронические нарушения мозгового кровообращения; черепно-мозговая травма

Список сокращений: iNOS – индуцибельная синтаза оксида азота; mNSS – шкала оценки тяжести неврологической симптоматики; NO – оксид азота (II); ГИ – геморрагический инсульт; ГМ – головной мозг; ГПП-1 – глюкагоноподобный пептид-1; иДПП-4 – ингибиторы дипептидилпептидазы-4; НГЛТ-2 – натрий-глюкозный котранспортер 2-го типа; НМК – нарушения мозгового кровообращения; АД – артериальное давление; ОНМК – острое нарушение мозгового кровообращения; ОСА – общая сонная артерия; ХНМК – хроническое нарушение мозгового кровообращения; ЧМТ – черепно-мозговая травма; ЭД – эндотелиальная дисфункция.

INTRODUCTION

In the Russian Federation, a number of patients suffering from a chronic cerebral circulation insufficiency (CCCI) reaches at least 700 cases per 100 000 people. The CCCI manifests itself as a diffuse and gradually increasing deterioration in the blood supply to the brain tissue, which contributes to the formation of cognitive and sensory-motor disorders. The pharmacotherapy of CCCI is aimed at improving hemoperphysis and metabolism of the nervous tissue, as well as reducing the inflammation [1]. Various types of an acute cerebral circulation insufficiency (ACCI) are widely spread. They are characterized by a pronounced neurological deficit, a high mortality, and require a long-term rehabilitation with the possibility of using neuroprotective drugs with a polytargeted mechanism of action (anti-inflammatory, antioxidant, vasodilating, etc.).

In 2010 and 2015, there were 5.406 and 6.240 million stroke deaths worldwide, respectively. At the same time, a significant mortality – about 60% – is caused by a hemorrhagic stroke (HS), which makes up 10-15% of all types of a cerebral circulation insufficiency (CCI) [2, 3]. Its most significant risk factors are a history of CCI, a high blood pressure (BP), and diabetes mellitus (DM) [4]. The problem of a traumatic brain injury (TBI) is also a serious challenge for modern medicine, especially given a limited number of available and effective treatments that take into account pathogenetic mechanisms (inflammation, edema, etc.) [5].

The consequence of TBI, as well as that of acute acute CCI (ACCI) and chronic CCI (CCCI), is the formation of neurological disorders, different in spectrum and severity, leading to a director disability and a decrease in the quality of life, i.e. a cognitive impairment (problems with memory, attention and learning), sensory, motor and other disorders. DM, which is pandemic in nature, significantly complicates the course severity and worsens the prognosis of acute and chronic brain pathologies associated with both a CCI and TBI [4, 6]. In this regard, there is a need for drugs with a cerebroprotective potential that can improve the course prognosis of various types of brain injuries, which can also be used in DM.

Currently, a wide range of drugs that can improve a person's intellectual and mental abilities to varying degrees, including cases of the brain tissue damage, is available. However, their effectiveness does not fully meet the expectations of specialists, therefore, the search for new compounds and approaches, the use of which would reduce the incidence of dementia, increase the resistance of brain tissues to various adverse factors, facilitate and/or accelerate their recovery after various types of traumatic brain injuries, continues.

Over the past 10 years, the interest of specialists in drugs from the group of incretin mimetics has not decreased. The clinical data indicate a reduction in the risk of occurrence and severity of cardiovascular complications in the patients with type 2 diabetes (myocardial infarction and a stroke) when using primarily analogues of glucagon-like peptide-1 (GLP-1). The open pleiotropic properties of incretin mimetics are only partially associated with a hypoglycemic effect, and their basis is multidirectional effects due to the wide prevalence of the GLP-1 receptor in the body and, accordingly, the consequences of its activation [7, 8]. Dipeptidyl peptidase-4 (iDPP-4) inhibitors, having a moderate antihyperglycemic activity, are ones of the most affordable and sought-after drugs from the group of incretin mimetics, and are being actively studied in the form of new fixed combinations [9].

Aminoguanidine is an inhibitor of the end products glycation formation and an inducible isoform of nitric oxide synthase (iNOS), the activity of which is accompanied by a number of positive effects, but mainly it underlies the pathophysiological processes that make up the essence of most cardiovascular diseases [10]. Given a significant negative role of iNOS in the pathogenesis of a CCI, a decrease in the activity of this enzyme can be an effective way to treat this group of pathologies. Many experimental studies have shown a pronounced protective effect of aminoguanidine [11–14] in various CCIs, accompanied by an iNOS activation, tissue hypoxia and edema.

Initially, aminoguanidine was developed as an antiglycation agent with a target positioning for use in diabetic nephropathy [15]. Despite the termination of the clinical studies due to doubts about their safety and lack of efficacy, the potential of aminoguanidine as a promising molecule for the prevention and treatment of DM and its vascular complications, including combinations with other antihyperglycemic agents, remains undiscovered. As indicated above, aminoguanidine showed a pronounced cerebroprotective effect in various experimental CCI models, cardio- and cerebroprotective potential, not associated with their hypoglycemic effect, was also proven for iDPP-4. Based on this, it seems appropriate to evaluate the possibility of their combined use in preclinical studies in order to correct the consequences of a CCI and in conditions of an acute TBI.

THE AIM of the study was to evaluate a cerebroprotective activity of the sitagliptin and aminoguanidine combination in rats with an acute and chronic CCI, as well as with a TBI.

MATERIALS AND METHODS

Model objects

All experiments were performed in accordance with the legislation of the Russian Federation (GOST R 53434-2009, GOST R 51000.4-2011) and the technical standards of the Eurasian Economic Union for good laboratory practice. The design of the study was approved by the Regional Independent Ethics

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Committee, the registration number IRB 00005839 IORG 0004900 (OHRP), as evidenced by an extract from Protocol No. 132 dated May 20, 2019 of the meeting of the Commission for Expertise of the Study at Volgograd State Medical University.

The work was performed on 150 male Wistar rats (aged 5–6 months, body weight of 300 g, the "Rappolovo" nursery for laboratory animals). After the arrival from the nursery, the animals were quarantined for 14 days in the vivarium of Volgograd State Medical University, where they were kept throughout the experiment at 20±2°C under conditions of 40–60% humidity and an alternating day/night cycle (12/12 h) with an unlimited access to food and water.

All surgical procedures were performed using combined anesthesia: zoletil 20 mg/kg (Zoletil®100, Valdepharm, France) + xylazil 8 mg/kg (Xyla, Interchemie, Netherlands), intraperitoneally. The eyes of the animals were closed during the operation, and blepharogel was applied to them to prevent the cornea from drying out. After the operation, the wounds were irrigated with a solution of chlorhexidine (0.05%); the edges were sutured with a continuous surgical suture.

Pathology modeling

CCCI simulation by stenosis of common carotid arteries [16]

After isolating the common carotid arteries (CCA), the vessels were tied to a nylon thread of a given diameter, and then it was removed so that the vessel could fill the vacated space, which would allow the blood flow to be restored by only 50%. The blood flow velocity was assessed using dopplerography polygraph MP150 with an LDF100C module (Biopac Systems, USA).

Modeling of hemorrhagic stroke by intracerebral injection of autologous blood [17]

An anesthetized animal was fixed in a stereotaxic apparatus (Shenzhen RWD Life Science Co, China) using a tooth holder and ear fixators. The wool cover was shaved off over the parietal bone. After processing the surgical field with a scalpel, a skin incision along the midline above the bregma (8–10 mm long) was made. Using a RESTAR-03 drill (RESTAR, Russia), a cranial foramen 1 mm in diameter was drilled at the level of the bregma (3.5 mm lateral to the midline) in the left coronal suture. Next, 100 µl of blood was taken from the tail vein into a tuberculin syringe without anticoagulant, fixed in a stereotaxic apparatus, and slowly injected through the cranial foramen under the brain membranes using a 29G needle.

Modeling of traumatic brain injury

To simulate an acute TBI, after a similar preparation of the surgical field, according to the stereotaxic coordinates, a hole was drilled in the parietal bone (ø 4 mm) using a drill, after which the brain tissue was injured with a modified mandrin knife.

Determination of pathological changes severity and treatment effectiveness

A neurological deficit in the animals with an acute pathology (a stroke and a traumatic brain injury) was assessed using the following scales: the 9-point "Combs&D'Alecy" scale and the Garcia scale [18, 19]. The "Combs&D'Alecy" scale was used to detect extrapyramidal disorders, including tests to assess a muscle strength, a tenacity-traction and the animals' balance. A lower total score corresponds to more pronounced neurological disorders. According to the Garcia scale, the severity of the following indicators was determined: a muscle tone, a motor activity, basic physiological reflexes, a coordination of movements, and a tactile sensitivity. For each test, the following scores were assigned: 3 points – normal, 2 points – a slight violation of symmetry, 1–0 points – pronounced violations or a lack of movements.

A psychoneurological deficit in the CCCI animals was assessed using the modified Neurological Severity Score (mNSS) [18]. This scale is scored on a variety of parameters, including a muscle strength, motor skills, a sensory function, and a motor coordination. The maximum number of points is 14, the minimum is 0. The higher the total score on the mNSS scale is the severer the neurological deficit is considered.

Sensory-motor disorders (sensitivity and fine motor skills of the forelimbs) were assessed using an Adhesive test [18, 20]. As a foreign object, square pieces of the adhesive tape (5 mm^2, Veropharm, Russia) were quickly glued to the volar surface of the animal' forelimbs. The severity of sensory-motor disorders was judged by the time of detection and removal of the adhesive plaster from the paws by the animals.

Motor disorders were assessed in the Open field test, in which a spontaneous motor activity was judged by the number of sectors crossed during 3 min, and an orienting-exploratory activity was judged by the sum of the stands and peeps into the holes.

The hydration degree of the brain tissues was determined as follows: the cerebral hemispheres were dissected into ipsi- and contralateral (left and right) parts, the affected hemisphere was weighed and dried in a thermostat at 100°C for 24 h to determine the water content procentage according to the formula:

$$W = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%$$

The cognitive functions of the CCCI animals were evaluated in the Morris Water Maze test [18, 21], which is designed to study the formation of the spatial memory. The installation is a water pool, divided into 8 designated zones (4 main and 4 additional; Fig. 1), in the center of one of which there is a platform submerged 1 cm into the water, and on the walls of the installation (at points "N", " E", "S", "W") visual landmarks are fixed.

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The scheme of the experiment is the following: according to the certain scheme, during 4 training days (4 attempts per day), an animal was placed in the water at the distance of 10 cm from the maze wall and the latent period of the flooded platform was recorded (Fig. 1). The test sessions lasted for 1 min with a break between the sets of 30 sec. If the rat did not find the platform within 1 min, it was lured to the platform and left on it for 15 sec.

Study design

The study was performed in 3 series (Fig. 2): in the 1st series, CCCI was modeled by stenosis of the CCAs by 50%, the treatment was started 40 days after the operation and continued for 21 days. In the 2nd and 3nd series of the experiment, the animals were simulated an acute brain injury by the methods of creating TBI or a HS; the treatment was started on the 2nd day after the operation and carried out for 14 days; the severity of symptoms of a neuropsychiatric deficit in all the series was assessed before and after the treatment. Sitagliptin (Januvia, 10 mg/kg/day, *per os*) or aminoguanidine (Sigma, 25 mg/kg/day, i.p.) or their combination was administered as a treatment (dose, regimen, and route of administration for each drug remained unchanged). At the end of the study, the severity of cognitive impairment in the Morris Water Maze test was determined in the CCCI animals, and the severity of brain edema was determined in the animals with an ACCI – after euthanasia.

In each series of the studies, the animals were divided into equal (n=10) groups: intact, ACCI/CCCI+ placebo (0.9% NaCl) – "Placebo", ACCI/CCCI+ sitagliptin – "Sit", ACCI/CCCI+aminoguanidine – "Amg", ACCI/CCCI+sitagliptin+aminoguanidine – "Sit+Amg". The doses of the substances were selected taking into account the literature data [22, 23].

Euthanasia

In accordance with Directive 2010/63/EU of the European Parliament and the Council of the European Union on the protection of animals used for scientific purposes dated September 22, 2010, the animals were euthanized after the completion of the experiment by placing them in a CO_2 chamber.

Statistical processing

Statistical processing of the obtained results was carried out by methods of descriptive and analytical statistics using Prism 6 Software (GraphPad Software Inc., USA). The distribution of quantitative indicators was assessed using the Shapiro-Wilk test. The intergroup differences were assessed using a one-way analysis of variance with the Newman-Keuls post hoc test. The differences were considered significant at p <0.05. The numerical values were presented as the arithmetic mean and the standard error of the arithmetic mean (Me+m).

RESULTS

A partial ligation of the CCAs by 50% caused pronounced symptoms of a psycho-neurological deficit in the animals, which manifested themselves on the 61st day of the experiment in the form of an increase in the neurological deficit score on the mNSS scale (4.2±2.1 *vs* 0.1±0.1; p <0.05), a motor decrease (18.6±7.1 *vs* 49.8±15.5; p <0.05), and an exploratory activity (3.1±0.5 *vs* 7.5±1 .6; p <0.05), the latent period of detection and removal of foreign objects from the palms of the animals forelimbs. In these animals, cognitive impairments have been also noted, since they memorized the location of the flooded platform in the Morris Water maze test much longer (54.9±12.9 *vs* 22.1±7.2; p <0.05, respectively). The animals with partially ligated OSAs and administered with sitagliptin or aminoguanidine for 21 days, had less expressed neuropsychiatric symptoms than those treated with placebo, but the results of the study show that these drugs are not effective enough. When a combination of these drugs was administered to the animals with partially ligated CCAs, the symptoms of a psychoneurological deficit, indicators of motor and orienting-exploratory activities, fine motor skills and a cognitive dysfunction were less pronounced, while the differences with the indicators of the animals that had been injected with placebo were statistically significant (p <0.05). That makes it possible to judge on the presence of cerebroprotective properties of the studied combination (Table 1).

The animals that had been modeled for an acute brain injury resulting from the TBI or a HS showed pronounced disorders that manifested themselves as symptoms of a neurological deficit according to the Combs&D'Alecy and Garcia scales, decreased motor and orienting activities, and impaired fine motor skills of the forelimbs. (Table 2). In these animals, a higher water content in the brain tissue was also recorded. In the animals that had been injected with sitagliptin or aminoguanidine after modeling the TBI or a HS, the indicators of a neuropsychiatric deficit did not significantly differ from those recorded in the placebo group. With the administration of these drugs combination, the symptoms of a psychoneurological deficit, as well as the brain edema degree, were significantly lower compared to the group without any treatment in conditions of both pathologies. The results obtained indicate a significant protective effect of the sitagliptin and aminoguanidine combination in the animals with an acute brain injury (ACCI and TBI).

DISCUSSION

Given the disappointing dynamics of the spread of carbohydrate metabolism disorders and obesity, the burden of DM and, accordingly, its complications will increase. Despite significant advances in pharmacology (development of new classes of antihyperglycemic drugs – sodium glucose cotransporter type 2 (SGLT-2) inhibitors, GLP-1 and GLP-1/GIP agonists), the therapy (development of new algorithms and tactics of patients with diabetes' treatment) and a diagnosis (an early detection, an available screening), the effectiveness of the diabetes treatment and the prevention of its complications remain insufficient.

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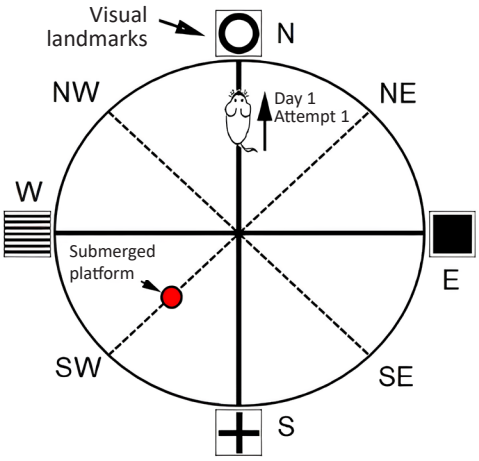

Plan of training

Day	Attempt			
	1	2	3	4
1	N	E	SE	NW
2	SE	N	NW	E
3	NW	SE	E	N
4	E	NW	N	SE

Figure 1 – Layout of installation and scheme of landing sites for animals during training in Morris Water Maze

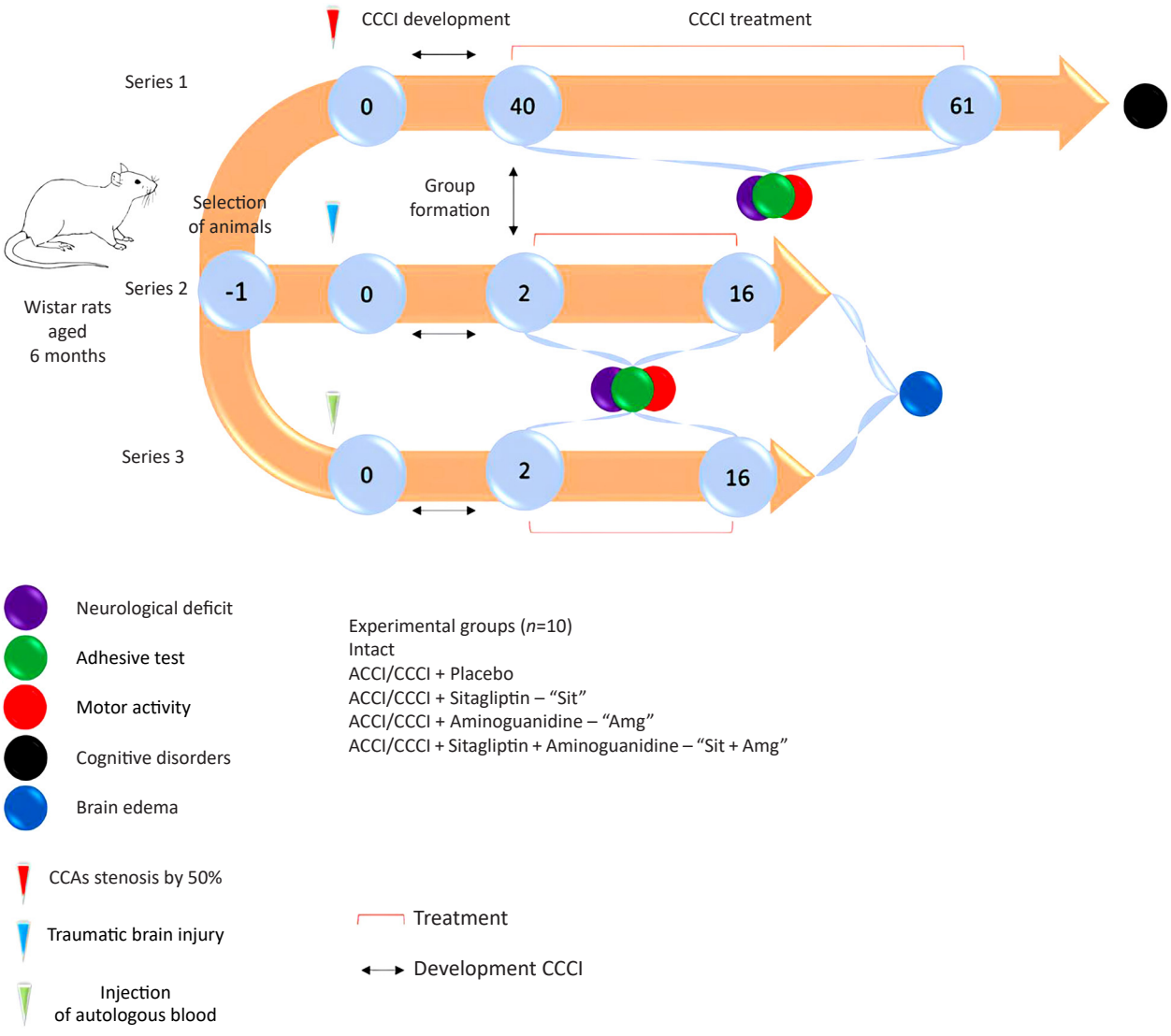

Figure 2 – Study design

Note: CCAs – common carotid arteries, CCCI – chronic cerebral circulation insufficiency, ACCI – acute cerebral circulation insufficiency, Sit – sitagliptin, Amg – aminoguanidine.

Table 1 – Indicators of psychoneurological deficit in CCCI animals

Indicator		Intact	CCCI+			
			Placebo	Sit	Amg	Sit+Amg
mNSS scale, points		0.1±0.1*	4.2±2.1	1.2±0.4*	1.1±1.2	0.5±0.8*
Open field	MA	49.8±15.5*	18.6±7.1	27.3±8	25.6±5.4	35.8±7.8*
	OEA	7.5±1.6*	3.1±0.5	3.4±1.3	3.9±1.6	3.2±1.2
Adhesive test	Average sticker detection time, sec	10.9±5.8*	101.2±41.6	41.7±17.6*	48.4±31.6	33.6±15.8*
	Average sticker removal time, sec	11.2±6.3*	114.2±49.4	43.3±15.7*	53.8±32.2	39.1±11.9*
Morris Water Maze	Day 1, search time, sec	72.3±5.6*	95.8±15.1	89.4±10.7	82.8±5.7	86.8±9.5
	Day 2, search time, sec	59.1±8.2	69.7±19.3	79.4±11.2	69.7±9.5	59.9±7.5
	Day 3, search time, sec	56.3±12.3	57.1±11.1	69.9±10.7	52.1±8.5	45.1±7.9*
	Day 4, search time, sec	32.3±7.3*	51.8±14.3	49.4±10.8	49.8±4.9	37.1±5.2*

Note: MA – motor activity, OEA – orienting-exploratory activity, CCCI – chronic cerebral circulation insufficiency, Amg – aminoguanidine, Sith – sitagliptin, * – differences with the placebo group are significant at p <0.05.

Table 2 – Indicators of psychoneurological deficit in animals with acute brain injury (ACCI and TBI)

Group	Neurological deficit, points		Open field		Adhesive test				Water content in affected hemisphere, %
					Left paw ipsilateral to lesion		Right paw Contralateral to lesion		
	Combs & D'Alecy Scale	Garcia Scale	MA	OEA	Sticker detection time, sec	Sticker removal time, sec	Sticker detection time, sec	Sticker removal time, sec	
Intact	9±0.0*	18±0.0*	42.4±8.2*	25.4±1.6*	15.8±1.1*	23.9±1.4*	14.8±1.3*	19.2±1.2*	76.32±0.03*
TBI+placebo	3±0.1	8.7±0.4	5.1±0.9	5.2±0.3	123.6±15.3	135.9±14.5	180.0±0.0	180.0±0.0	78.58±0.15
TBI+Sit	3.1±0.2	8.7±0.3	4.0±1.2	1.9±0.4	91.1±14.1*	102.1±12.7*	180.0±0.0	180.0±0.0	78.88±0.21
TBI+AMG	3.2±0.2	8.8±0.4	5.2±1.3	1.2±0.2	99.6±12.4	108.9±11.5	180.0±0.0	180.0±0.0	78.19±0.13*
TBI+Sit+ Amg	4.9±0.2*	9.9±0.5	20.5±2.9*	10.5±0.3*	92.9±14.4*	102.7±22.2	180.0±0.0	180.0±0.0	77.83±0.12*
Intact	9±0.0*	18±0.0*	45.2±1.8*	19.4±1.8*	13.9±1.4*	15.8±1.1*	14.8±1.8*	19.2±1.9*	76.06±0.13*
ACCI+ placebo	3±0.3	8.5±0.6	5.5±0.9	3.8±0.4	91.2±11.3	96.8±12.1	180.0±0.0	180±0.0	79.12±0.15
ACCI+Sit	3.9±0.2	9.6±0.3	4.8±0.6	3.2±0.6	80.8±12	83.4±18.2	128.2±10.1*	180±0.0	78.59±0.19*
ACCI+Amg	3.0±0.4	8.7±0.6	5.8±0.3	2.2±0.5	81.8±11	85.9±14.7	135.3±8.1*	180±0.0	78.49±0.29*
ACCI+Sit+ Amg	4.8±0.2*	10.9±0.5*	25.0±1.7*	15.1±2.3*	52.5±11.6*	63.6±11.4*	66±11.8*	75.2±8.1*	78.05±0.24*

Note: TBI – traumatic brain injury, t – time, MA – motor activity, OEA – orienting-exploratory activity, ACCI – acute cerebral circulation insufficiency, Amg – aminoguanidine, Sit – sitagliptin, * – differences with the placebo group are significant at p <0.05.

It is obvious that a compensation of carbohydrate metabolism disorders is the key to the effective prevention of DM complications, but for various reasons, only a small proportion of patients with this diagnosis can reach and maintain the glycemic values set by the doctor. The low availability of modern highly effective antihyperglycemic drugs, as well as the presence of side effects that limit patient adherence to the treatment, are significant obstacles to attempts to manage DM.

The main cause of death in DM remains its cardiovascular complications, the leading cause of which is an endothelial dysfunction (ED) formed under the influence of chronic hyperglycemia and, as a consequence, microcirculation disorders. Even an accidental TBI in DM is much severer, with a higher risk of disability or death [6]. A chronic inflammation and oxidative stress do not only damage the endothelium, exacerbating its dysfunction, but also increase an iNOS expression, which plays a significant negative role under these conditions.

The production of nitric oxide by inducible synthase (iNOS) significantly exceeds that of other isoforms. In addition to the participation of NO in the process of the synthesis and secretion of insulin, microcirculation,

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this molecule is involved in maintaining homeostasis of all body tissues. High concentrations of NO, arising from the activation of iNOS, have a negative effect on the cardiovascular system, increasing an ED. The iNOS activity suppression may play a positive role in relation to the ED and functioning of the insular apparatus [10].

Over the years, various approaches to modulate the iNOS activity – blocking the production of ROS in the upstream direction, the introduction of BH4, the introduction of folic acid for recycling BH2 to BH4, the use of arginase inhibitors, resveratrol, calcium dobesilate, cavnoxin, NOS transcription enhancers (AVE3085 and AVE9488), L-arginine, blockers and activators of various NOS – have been developed [10], but none of them is widely used in medical practice.

Aminoguanidine was developed as a drug for the treatment of diabetes and its complications (nephropathy). This drug combines several potentially valuable properties, i. e., the ability to reduce the iNOS activity and reduce the accumulations of advanced glycation end products. Aminoguanidine was withdrawn from the promotion on the pharmaceutical market for reasons that can be currently revised from the standpoint of modern requirements (changes in indications, additional studies) and opportunities (a molecule optimization, additional studies according to protocols using modern methods of collecting and processing information). Aminoguanidine is also interesting from the point of view of the results of preclinical studies of its cerebroprotective properties in various disorders of cerebral circulation, which is of a high value considering the causes and consequences of diabetes complications.

DPP-4 inhibitors are the first representatives of the class of antihyperglycemic drugs, the mechanism of action of which is associated with the effect on the incretin system, which is involved not only in the regulation of the carbohydrate metabolism, but has also a number of unrelated effects. So, for incretin mimetics to a greater (GLP-1 agonists) or less (iDPP-4) degree, cerebro-, cardio- and endothelioprotective properties have been established [24].

A creation of combined drugs, along with the development of an original drug or its analogue, a new dosage form, a new indication for use, is one of the winning strategies for obtaining a new pharmaceutical product.

Taking into account a wide range of pleiotropic iDPP-4 effects, the list of which is expanding with the accumulation of experience in clinical use, to evaluate a cerebroprotective effect of the sitagliptin and aminoguanidine combination was considered appropriate.

In this study, cerebroprotective effects of the sitagliptin and aminoguanidine combination were established in models of an acute and chronic CCI, and it was found that the protective effect of the combination significantly exceeds the effects of the drugs administered separately, which indicates synergism due to different exposure targets and, accordingly, effects.

CONCLUSION

A combined use of sitagliptin and aminoguanidine has a cerebroprotective effect in a CCI, reproduced by stenosis of the CCAs by 50% and an acute brain injury, reproduced by the application of the TBI or an experimental HS, reducing the severity of neuropsychiatric deficit symptoms, improving cognitive functions and reducing the severity of cerebral brain edema. The sitagliptin and aminoguanidine combination can become the basis for the development of a new promising approach to the treatment of DM and its complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Denis V. Kurkin, Andrey V. Strygin – idea and study planning, graphic material design, approval of the manuscript final version; Tamara M. Andriashvili, Alina A. Sokolova, Nikita S. Bolokhov, Vladislav E. Pustynnikov, Evgeny A. Fomichev – pathology modeling, experimental work; Evgeny I. Morkovin, Anna V. Kasparova, Sarkis S. Polodyants – statistical data processing, graphic material design, text editing; Dmitry A. Bakulin, Yulia V. Gorbunova, Alexandra V. Baskova – collection and analysis of literature data, manuscript writing. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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RESEARCH ARTICLE

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Development of composition and technology for obtaining antimicrobial composition based on mono- and sesquiterpenoids

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The article reflects the results of the essential oils selection for the compositions with antibacterial and antifungal properties. The technology of their production is represented.

The aim of the work was the development of technology and the study structure of new essential oils compositions with antibacterial and antifungal activities.

Material and methods. The following plants have been used in the work: the herb of *Hyssopus ambiguus* (Trautv.) Iljin, the herb of *Thymus crebrifolius* Klokov, the herb of *Thymus marschallianus* Willd, the herb of *Thymus serpyllum* L., and the essential oils obtained from them. The composition of the essential oils was determined by Gas Chromatography Mass Spectrometry. The main physical and chemical parameters of the compositions were evaluated in accordance with the requirements of the Russian State Pharmacopoeia, the XIVth edition. The tests for the antimicrobial activity were carried out using the strains of *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231.

Results. Compositions with an activity against microorganisms *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and fungi *Candida albicans* have been obtained and studied. The composition based on essential oils of *Hyssopus ambiguus* (Trautv.) Iljin and *Thymus marschallianus* Willd. contained 139 components, the main ones of which are eucalyptol (6.51%) and terpinen-4-ol (1.95%). The composition of essential oils of *Hyssopus ambiguus* (Trautv. Iljin) and *Thymus crebrifolius* Klokov contained 137 components (eucalyptol (20.37%), terpinen-4-ol (7.03%), Ţ-muurol (2.28%), γ-terpinene (2.23%), β-myrcene (2.09%), etc.). The composition of the essential oils of *Hyssopus ambiguus* (Trautv.) Iljin and *Thymus serpyllum* L. contained 149 components (the main ones are eucalyptol (7.33%) and α-terpineol (0.9%)).

Conclusion. The technology has been proposed and the structure of the essential oils compositions with antibacterial and antifungal activities has been established.

Keywords: essential oils; isoprenoids; essential oils composition; technology; antibacterial and antifungal activities

Abbreviations: SP – State Pharmacopoeia; RK – Republic of Kazakhstan; RF – Russian Federation; BAS – biologically active substances; MPRMs – medicinal plant raw materials; EOs – essential oils; IgA – immunoglobulin A; PhM – pharmacopoeial monograph; GPhM – general pharmacopoeial monograph.

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Разработка состава и технологии получения антимикробной композиции на основе моно- и сесквитерпеноидов

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В статье отражены результаты подбора эфирных масел для композиций с антибактериальными и противогрибковыми свойствами. Приведена технология их получения.

Цель. Разработка технологии и изучение состава новых композиций эфирных масел, обладающих антибактериальной и противогрибковой активностью.

Материал и методы. В работе использованы: трава иссопа сомнительного (*Hyssopus ambiguus* (Trautv.) Iljin), трава тимьяна частолистого (*Thymus crebrifolius* Klokov), трава тимьяна Маршалла (*Thymus marschallianus* Willd.), трава тимьяна ползучего (*Thymus serpyllum* L.), и полученные из них эфирные масла. Состав эфирных масел определяли методом газовой хроматографии с масс-спектральной детекцией. Основные физико-химические показатели композиций оценивали согласно требованиям Государственной фармакопеи Российской Федерации XIV изд. Испытания на антимикробную активность проводили с применением штаммов *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231.

Результаты. Получены и исследованы композиции, обладающие активностью в отношении микроорганизмов *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* и грибков *Candida albicans*. Композиция на основе эфирных масел иссопа сомнительного и тимьяна Маршалла содержала 139 компонентов, из которых основные – эвкалиптол (6,51%) и терпинен-4-ол (1,95%). Композиция из эфирных масел иссопа сомнительного и тимьяна частолистого содержала 137 компонентов (эвкалиптол (20,37%), терпинен-4-ол (7,03%), Ţ-муурол (2,28%), γ-терпинен (2,23%), β-мирцен (2,09%) и др.). Композиция из эфирных масел иссопа сомнительного и тимьяна ползучего содержала 149 компонентов (основные – эвкалиптол (7,33%) и α-терпинеол (0,9%)).

Заключение. Предложена технология и установлен состав эфирномасличных композиций, обладающих антибактериальной и противогрибковой активностью.

Ключевые слова: эфирные масла; изопреноиды; эфирномасличная композиция; технология; антибактериальная и противогрибковая активность

Список сокращений: ГФ – Государственная фармакопея; РК – Республика Казахстан; РФ – Российская Федерация; БАВ – биологически активные вещества; ЛРС – лекарственное растительное сырье; ЛП – лекарственный препарат; ЭМ – эфирные масла; IgA – иммуноглобулин А; ФС – фармакопейная статья; ОФС – общая фармакопейная статья.

INTRODUCTION

To date, one of the actively developing scientific areas is the development of new medicinal drugs (MDs) intended for the treatment and prevention of the upper respiratory tract infections, using innovative technologies and based on natural biologically active substances (BASs) of the plant origin. The essential oils (EOs), complex multicomponent mixtures consisting mainly of isoprenoids, are known to have a wide range of pharmacological activities, in particular, they are able to effectively inhibit the development of pathogenic microflora, and therefore they are of great interest for the development of new drugs [1–4].

Mono- and sesquiterpenoids are the main part of EOs; oxygen-containing derivatives are the rest. Due to the content of phenols, terpenes, alcohols, aldehydes and flavonoids, EOs are able to have bactericidal and bacteriostatic effects, an antiviral effect, and exhibit an antifungal activity. EOs have an immunomodulatory effect, strengthen cell membranes,

and increase the antioxidant activity of blood plasma [5–8].

Numerous studies by domestic and foreign scientists prove the effectiveness of a number of EOs in relation to viral, bacterial and fungal agents. Antiviral and antimicrobial effects, as well as a restorative effect on the protective barriers of the upper respiratory tract, have biologically active substances of the representatives of the following families: *Adoxaceae, Acoraceae*, *Amaryllidaceae, Asteraceae*, *Betulaceae*, *Fabaceae*, *Ericaceae*, *Hypericaceae, Salicaceae*, *Zingiberaceae*, *Onagraceae*, *Cupressaceae*, *Alliaceae*, *Myrtaceae*, *Parmeliaceae*, *Rosaceae*, *Polemoniaceae*, *Pinaceae*, *Lamiaceae,* etc. [9–12].

The components of the EOs that exhibit antiviral and antibacterial actions are phenolic compounds (thymol, carvacrol, eugenol, etc.), terpene alcohols (linalool, geraniol, and menthol), aldehydes (neral, geranial, and citronellal), ketones (thujone, fenchon, carvone, pinocamphone, camphor, menthon, etc.), esters (anethole, estragol and others) [13–16].

The use of EOs and the drugs based on them in respiratory infections has a great potential and requires further research due to the problem of their increasing antibiotic resistance, because they are able to exert an antimicrobial effect without causing the development of resistance in microorganisms, unlike antibiotics and antiviral chemotherapy drugs [17].

The EOs inhalation is one of the effective methods for preventing colds [18-21]. They do not cause any resistance; have expectorant and secretolytic effects; increase the production of immunoglobulin A (IgA), which helps to increase immunity; favorably affect the symptoms of SARS; increase the activity of the ciliary epithelium of the nasal cavity [22].

In order to develop a new medicinal product with antimicrobial properties, combinations of the EOs obtained from MPRMs of the *Lamiaceae* plants growing on the territory of the Republic of Kazakhstan (RK), i. e, *Hyssopus ambiguous* (Trautv.) Iljin, *Thymus marschallianus* Willd., *Thymus serpyllum L.* and *Thymus crebrifolius* Klok. were studied.

THE AIM of the work was the development of technology and the study structure of the new essential oils compositions with antibacterial and antifungal activities.

MATERIALS AND METHODS

Objects of study

The objects of the study were the EOs compositions, made up on the basis of the results of microbiological studies in compliance with the rules for compiling EOs compositions in aromatherapy. The samples of the MPRMs were collected during the flowering period. It is during this period that the EOs of these plants have the most suitable qualitative and quantitative components composition. In all the cases, the areal part was used.

The confirmation of the species affiliation of producing plants was carried out with the participation of Professor of the Department of Botany M.Yu. Ishmuratova; the plant samples were deposited in the herbarium of the Faculty of Biology and Geography of Karaganda University named after E.A. Buketov (Karaganda, RK). Previously, MPRMs passed a radiation control and a test for heavy metals. When studying these species, for the first time, a morphological and histochemical study of the herb of *Hyssopus ambiguus* (Trautv.) Iljin) was carried out [23].

Methods for obtaining EOs

EOs were obtained in a setup consisting of a reaction round-bottomed flask with a capacity of 1 dm^3, a bent steam pipe, a refrigerator, a receiver with a drain cock, and a drain pipe. About 15-20 g of raw materials were placed in a flask, 300 ml of water was added, the flask was connected through a thin section to a steam pipe and filled with water through a tap using a hose with a funnel. The contents of the flask were heated and boiled at the intensity at which the distillate flow rate was 60–65 drops per 1 min for 2 h. The EOs volume was measured 5 min after the end of the distillation. The percentage content was determined by the volumetric method. For each name of the medicinal plant, the production of EOs was carried out separately and until at least 10 ml of each studied object was obtained, that was sufficient to study the physical and chemical properties and the production of samples of the studied compositions. The output of EOs for each of the used types of plant materials is presented in Table 2.

The EOs samples were dissolved in n-hexane (50 ml); the solution was kept with anhydrous magnesium sulfate for 1 h, then filtered through filter paper and evaporated under vacuum on a Stegler XD-52AA rotary evaporator (Russia) until the organic solvent was completely removed.

Determination of the component composition of essential oils compositions

The following essential oils compositions were used in the work: composition 1 “hyssop dubious+Marshall’s thyme”, composition 2 “hyssop dubious+Mother-of-thyme” and composition 3 “hyssop dubious+creeping thyme”.

The component of the EOs compositions was determined by GC-MS according to the requirements of the State Pharmacopoeia of the Republic of Kazakhstan

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(SP RK, Vol. I (2.2.28))[1]. For the analysis, an Agilent-7890A chromatograph (Agilent Technologies, USA) with a capillary column HP-5 ms 30 m×0.25 mm (film thickness 0.25 µm) and in combination with a selective mass spectral detector 5975C was used. The results obtained were processed using the Agilent ChemStation software and the EOs components were identified using the NIST-2017 mass spectrum library.

The EOs in the amount of 150 µl was dissolved in 800 µl of the solvent (chloroform for composition 1 and 70% ethyl alcohol for compositions 2 and 3) and stirred until the oil was completely dissolved. The analysis was carried out using a temperature program: starting from +70°C for 2 min, then raising the temperature at the rate of 20°C/min to +270°C (holding for 30 min). The carrier gas was helium. The injector temperature was +250°C; the detector temperature was +230°C. Mass spectra were recorded using an ionization energy of 70 eV and a separation temperature of +280°C; the acquisition mass range was M/z 10–650.

Study of antimicrobial activity of essential oils compositions

The analysis of the EOs antimicrobial activity, compositions and results was carried out at the Department of Biomedicine in the microbiological laboratory of Medical University of Karaganda (RK). The following materials were used in the work: Chistovich's nutrient media, meat-peptone agar, Endo's medium, Sabouraud's medium, blood agar: museum culture strains of *Staphylococcus aureus, Bacillus subtilis, Ecsherichia coli, Candida albicans;* the disks manufactured by Bioanalyse Limited (Turkey) impregnated with benzylpenicillin sodium salt (1 U/disk), ampicillin trihydrate (25 µg/disk), nystatin (100 µg/disk) and fluconazole (10 µg/disk) to determine the sensitivity of microorganisms to antibiotics; a ruler-template for measuring the zones size of the growth inhibition of microorganisms (PW096), size – 370×65 mm (HiMedia Laboratories Pvt. Limited, India); a densimeter to determine the concentration of culture in saline.

An antimicrobial activity was determined in accordance with the requirements of the SPh of the Republic of Kazakhstan according to the method for determining the sensitivity to antibiotics by the microbiological method, i.e., the agar diffusion method [2].

For the cultivation of *Staphylococcus aureus*, Chistovich's medium was used; for *Escherihia coli*, it was Endo's medium; for *Bacillus subtilis*, it was meat-peptone broth; for *Candida albicans* - Saburo's medium was used. According to the methods used, daily cultures diluted in saline were used. Filter paper discs impregnated with an undiluted essential oil composition were placed on one segment of the Petri dish; the discs with the control preparation (benzylpenicillin sodium salt, nystatin) were placed on the other. Taking into account that medicinal products containing the studied EOs in their composition are not represented on the market of the RK, it was decided to use a cosmetic product with antimicrobial properties as a comparison drug, i. e, the "Breathe"/"Dyshi" spray (ser. 061022, manufacturer JSC "Akvion", Russia).

Petri dishes with bacterial cultures were placed in a thermostat for 24 h and incubated; for bacteria, the temperature regime was +36(±1)°C, for fungi of the genus *Candida* – +28°C. The zones of the microorganism growth absence were determined with a special ruler-template. The diameters of the growth inhibition zones less than 10 mm and a continuous growth in the cup were evaluated as the absence of the antimicrobial activity, 10–15 mm – as a weak activity, 15–20 mm – as a moderately pronounced activity, more than 20 mm – a pronounced one.

Standardization of resulting essential oils compositions

The above essential oils compositions were tested in accordance with the requirements of the Russian State Pharmacopoeia XIV edition (hereinafter SP RF XIV ed.), GPhM.1.5.2.0001.15 Essential oils[3].

Description. The essential oils compositions were placed in a transparent glass cylinder with a diameter of 3 cm, the mobility of the liquid and their color were determined in the transmitted diffused daylight. The odor determination was as follows: about 0.1 ml (2 drops) of the EOs composition was applied to a strip of filter paper 12 cm long and 5 cm wide so that the oil did not wet the edges of the paper, then the odor was evaluated every 15 minutes.

Authenticity. Its determination was carried out by high-performance gas chromatography in accordance with the requirements of GPhM.1.2.1.2.0004.15 Gas chromatography (see "Determination of the component composition of essential oils compositions"). To establish the authenticity of essential oils compositions, relative retention times of the predominant and specific components were used: for composition 1, eucalyptol and terpinen-4-ol; for composition 2, eucalyptol, terpinen-4-ol, Ƭ-muurolol, ɤ-terpinene, β-myrcene; for composition 3, eucalyptol and α-terpineol.

[1] State Pharmacopoeia of the Republic of Kazakhstan. Vol. 1; Almaty: Zhibek Zholy Publishing House; 2008, 592 p. Available from: https://pharmacopoeia.ru/wp-content/uploads/2017/01/Gosudarstvennaya-farmakopeya-Respubliki-Kazahstan-PDF.pdf

[2] Ibid.

[3] Russian State Pharmacopoeia XIV edition. T. 1–4; Moscow: FEMB, 2018. Available from: https://femb.ru/record/pharmacopea14

Solubility. The essential oils compositions obtained are insoluble in water. The solubility of the essential oils compositions in hexane, chloroform and dimestyl sulfoxide were also tested.

To determine the solubility of an EOs composition in 70% ethyl alcohol, 1 ml of the essential oil was placed in a 25 ml test tube with a friction-fitted lid. The determination was carried out at the temperature of 20±2°C. Until the oil was completely dissolved, alcohol was added in portions of 0.1 ml with frequent vigorous stirring. The volume of the alcohol consumed to obtain a clear solution was recorded. Then the alcohol was added in portions of 0.5 ml with vigorous stirring until the total volume of alcohol added was 20 ml.

If the solution became cloudy or opalescent from the addition of 20 ml of the solvent, then its volume was recorded at the point of appearance of the first signs of opalescence, and the volume of alcohol at which the turbidity or opalescence disappeared. If a clear solution did not form after the addition of 20 ml of alcohol, the test was repeated using 90% ethyl alcohol.

Ethyl alcohol. 1) 2 drops of the EOs composition were added to a few drops of water placed on a watch glass. The composition passed the test if there was no noticeable haze around the EOs droplets on the black background.

2) 1 ml of the EOs composition was poured into a test tube, closed with a loose piece of cotton wool, in the middle of which a crystal of basic fuchsin was placed, then heated to a boil in a water bath. The composition passed the test if the cotton wool did not turn purple-pink.

Fatty and mineral oils. 1) 1 ml of the EOs composition was shaken in a 20 ml test tube with 10 ml of 96% ethyl alcohol. The composition passed the test if cloudiness and oily droplets were not observed.

2) 0.05 ml of the tested essential oils composition was placed on filter paper. The oil stain completely evaporated from the paper within 24 h without a trace.

Essential oil residue after evaporation. About 5 g of the EOs composition was placed in a pre-weighed cup 8 cm in diameter. The cup had been heated in a boiling water bath until the oil completely evaporated, then cooled in a desiccator over anhydrous calcium chloride and weighed. The water level in the bath throughout the experiment was 1–20 mm below the bottom of the evaporating dish.

Water. 0.5 ml of the EO was mixed with 10 ml of petroleum ether. The composition passed the test if no haze was observed.

Density. The test was carried out using an automatic viscometer brand SVM 3000 Stabinger (Austria) in accordance with GPhM.1.2.1.0014.15 Density.

Optical rotation. The test was carried out on an AP-300 polarimeter from ATAGO CO, LTD (Japan) in accordance with GPhM.1.2.1.0018.15 Polarimetry.

Refractive index. The test was carried out on an IRF-454 B2M refractometer manufactured by OAO KOMZ (Tatarastan) in accordance with GPhM.1.2.1.0017.15 Refractometry.

Acid number. The number of milligrams of potassium hydroxide, which is necessary to neutralize the free acids in 1 g of the essential oil, was determined in 2 g (accurately weighed) of the oil dissolved in 5 ml of 95% ethyl alcohol, previously neutralized with phenolphthalein.

Quantitation. The GC-MS method was used to determine the contents of the main components of essential oils compositions: for composition 1, eucalyptol and terpinen-4-ol; for composition 2, eucalyptol, terpinen-4-ol, Ţ-muurolol, ɤ-terpinene, *β*-myrcene; for composition 3, eucalyptol and *α*-terpineol.

Statistical processing. Statistical processing of the results was carried out using the STATISTICA 12.6 program (StatSoft, USA). Variation series were tested for a normal distribution using the Kolmogorov-Smirnov test, the significance level in this study was taken as p=0.05. For all the groups, the mean and standard error of the mean were calculated. Student's t-test was used to assess intergroup differences.

RESULTS AND DISCUSSION

EOs selected as components of essential oils compositions were tested for an antimicrobial activity.

Screening of the antimicrobial activity of EOs of *Hyssopus ambiguus* (Trautv.) Iljin, *Thymus marschallianus* Willd., *Thymus crebrifolius* Klokov and *Thymus serpyllum* L. made it possible to obtain the following data presented in Table 3. To confirm the presence or absence of growth of microorganisms, Petri dishes with crops were viewed in the transmitted light.

As can be seen from the data in Table 3, the most pronounced antibacterial activity was shown by *Hyssopus ambiguous* (Trautv.) Iljin EOs, while the analysis of *Thymus marschallianus* Willd EOs, *Thymus crebrifolius* Klokov and *Thymus serpyllum* L. showed a higher antifungal activity. Taking into account the data obtained, it was decided to combine the EOs of the investigated thyme species (in all compositions, the EOs of *Hyssopus ambiguous* (Trautv.) Iljin was present). Thus, three different essential oils compositions have been obtained: composition 1 "*Hyssopus ambiguous* (Trautv.) Iljin+*Thymus marschallianus* Willd", composition 2 "*Hyssopus ambiguous (*Trautv.*)* Iljin+*Thymus crebrifolius* Klokov" and composition 3 "*Hyssopus ambiguous (*Trautv.*)* Iljin+*Thymus serpyllum* L.)".

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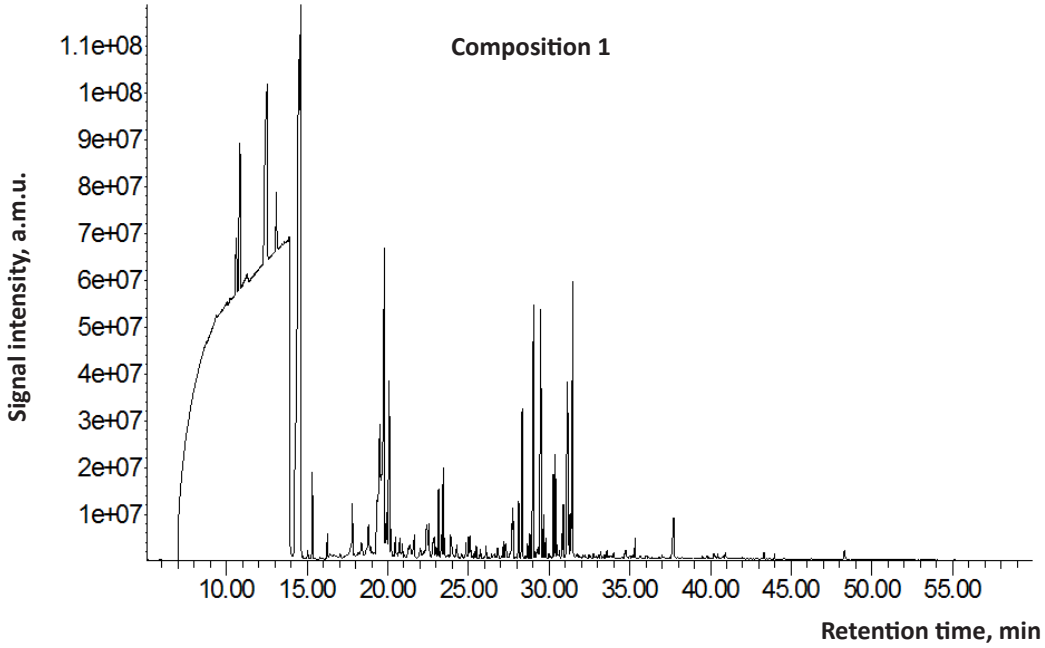

Figure 1 – Chromatogram of essential oils composition 1

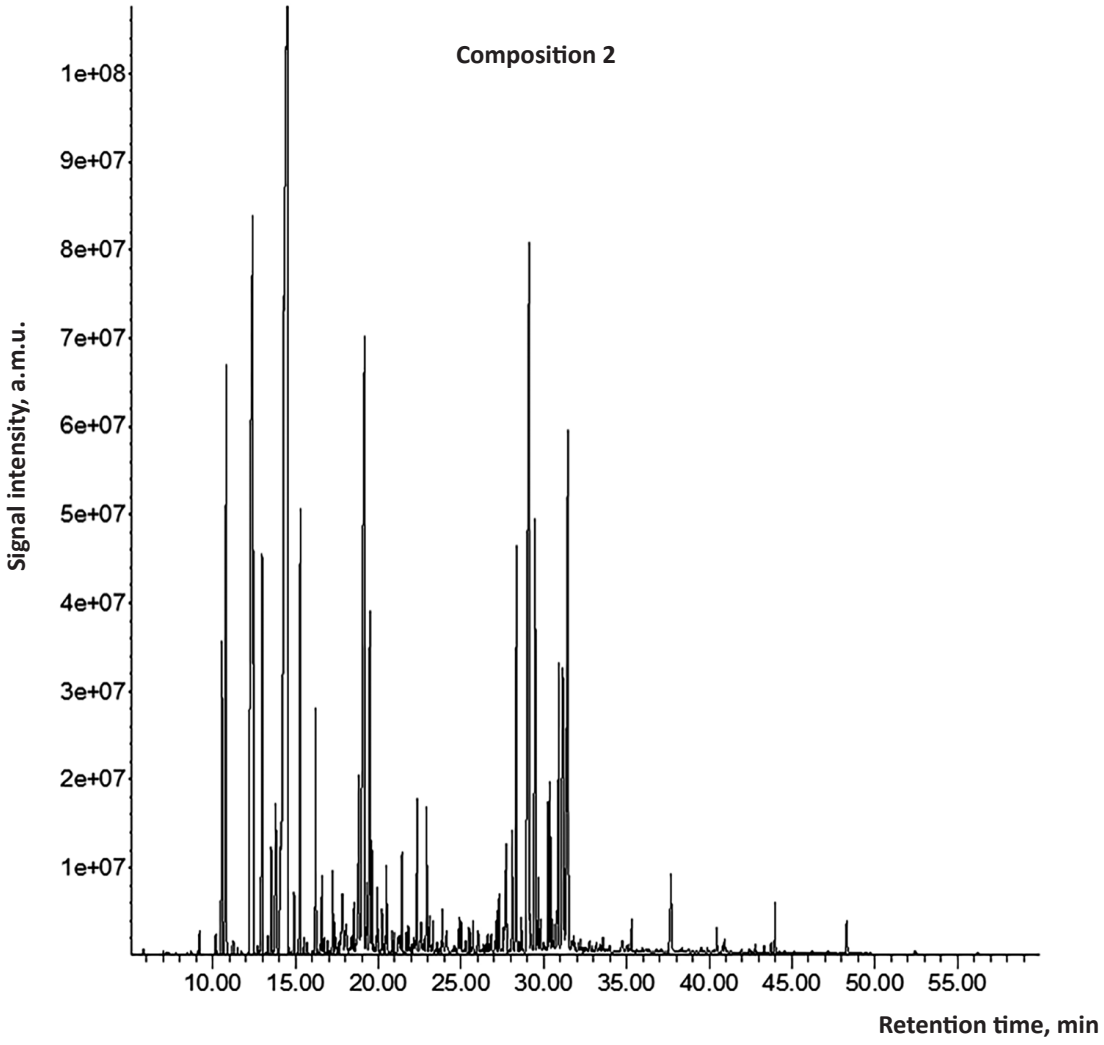

Figure 2 – Chromatogram of essential oils composition 2

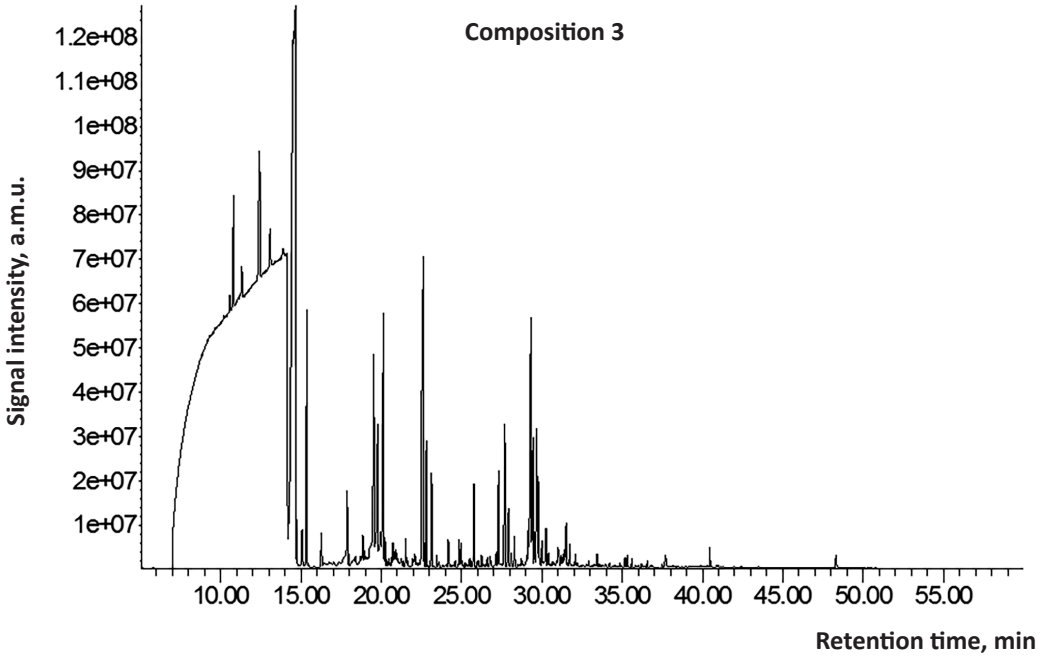

Figure 3 – Chromatogram of essential oils composition 3

Table 1 – Characteristics of MPRMs used to obtain EOs

Name of the producing plant (Russian and Latin)	Gathering place	Geographical coordinates	Collection time, development phase
Hyssopus ambiguous (Trautv.) Iljin	Sorting stlm., Karaganda region, RK	49.970554°N 73.2226789° E	September 2021, flowering phase
Hyssopus ambiguous (Trautv.) Iljin	Karkalinsk suburbs Karaganda region, RK	49.412951° N, 75.477275° E	August 2021, flowering phase
Thymus marschallianus Willd.		49.412951° N, 75.477275° E	June 2020
Thymus serpyllum L.		49.412951° N, 75.477275 E	Flowering phase
Thymus crebrifolius Klok.	Surroundings of the Ulytau mountains, Karaganda region, RK	48.652828° N, 67.025743° E	June 2020

Note: MPRMs – medicinal plant raw materials, EOs – essential oils, RK – Republic of Kazakhstan, stlm. – settlement, N – north latitude, E – east longitude.

Table 2 – Output of EMs in used MPRMs

Name of producing plant (Russian)	Amount (g) of MPRMs to obtain 1 ml of EO	EOs output, %
Hyssop dubious (Karkaralinsk)	65.5	0.40
Hyssop dubious (Sorting)	147	0.60
Marshall's thyme (Karkaralinsk)	197.5	0.50
Mother-of-thyme (Ulytau)	79.5	1.13
Creeping thyme (Karkaralinsk)	201.0	0.40

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Table 3 – Antimicrobial activity of EOs collected on territory of Karaganda region

Name of the producing plant (Russian)	Microorganisms growth inhibition zone, mm			
	Staphylococcus aureus ATCC 6538	*Bacillus subtilis* ATCC 6633	*Escherihia coli* ATCC 25922	*Candida albicans* ATCC 10231
Hyssopus ambiguous (Trautv.) Iljin (Karkaralinsk)	33.0±7.0	33.7±4.9	12.5±2.1	9.7±2.1
Hyssopus ambiguous (Trautv.) Iljin (Sorting)	39.0±3.0	25.5±2.6	10.0±3.0	7.5±0.6
Thymus marschallianus Willd (Karkaralinsk)	7.8±1.5	9.0±3.0	10.7±5.0	22.7±10.8
Thymus crebrifolius Klokov (Ulytau)	7.0±0.8	8.5±2.1	10.2±3.2	9.8±4.3
Thymus serpyllum L. (Karkaralinsk)	13.3±1.7	14.5±0.7	11.0±2.8	13.0±4.1

Table 4 – Main components in essential oils composition 1

Retention index	Retention time, min	Component	Content, %
1022±5 (394)	14.4452	Eucalyptol	6.51
1164±5 (512)	18.9980	Terpinen-4-ol	1.95
1175±5 (540)	19.4381	α-Terpineol	0.61
1086±3 (646)	16.5737	Linalool	0.29
1592±4 (15)	30.3548	β-Oplopenone	0.22
1050±4 (484)	17.2230	ɤ-terpinene	0.21
1472±6 (181)	27.1513	ɤ-Tsuurolen	0.13
1270±5 (28)	22.2448	ρ-Cymen-7-ol	0.12
1494±5 (178)	27.7718	α-Muurolen	0.12
1376±4 (377)	24.5755	Kopaen	0.13
1477±5 (364)	27.2812	Germacren D	0.08
1531±5 (59)	28.7747	α-Kalakorn	0.06
1382±5 (186)	24.8280	(-)-β-Bourbonene	0.05
1419±5 (656)	25.7227	caryophyllene	0.04
1372±4 (94)	25.2753	Methyleugenol	0.02
1451±5 (468)	26.5884	Humulen	0.02
1447±8 (2)	30.8455	Aromadendren	0.01

Table 5 – Main components of essential oils composition 2

Retention index	Retention time, min	Component	Content, %
1022±5 (394)	14.4452	Eucalyptol	20.34
1164±5 (512)	18.9980	Terpinen-4-ol	7.03
1632±5 (119)	31.1125	Ʈ-muurolol	2.28
1050±4 (484)	17.2230	ɤ-terpinene	2.23
983±3 (580)	12.9299	β-Myrcene	2.09
1592±4 (15)	30.3548	β-Oplopenone	0.70
1586±11 (64)	30.2250	Ledol	0.65
1025±7 (52)	14.0555	o-Cymen	0.63
1494±5 (178)	27.7718	α-Muurolen	0.41
1131±9 (76)	17.9879	trans-verbenol	0.33
1086±3 (646)	16.5737	Linalool	0.31
1477±5 (364)	27.2812	Germacren D	0.27
1419±5 (656)	25.7227	caryophyllene	0.22
1164±N/A (1)	18.5146	Pinocarvone	0.33
1472±6 (181)	27.1513	ɤ-Muurolen	0.16
1104±5 (66)	17.3818	α-Campholenal	0.14
1460±6 (178)	26.7760	Alloaromadendren	0.14
998±4 (356)	13.2979	α-Phelandrene	0.12
1241±4 (13)	21.6748	Citral	0.12
860±6 (210)	9.1924	ρ-Xylene	0.10
1270±5 (28)	22.2448	ρ-Cymen-7-ol	0.10
1382±5 (186)	24.8280	(-)-β-Bourbonene	0.10
1222±10 (18)	20.9317	D-Carvone	0.09
1454±6 (7)	27.5626	cis-Muurola-4(15),5-diene	0.08
1372±4 (94)	25.2753	Methyleugenol	0.07
946±5 (423)	11.2560	camphene	0.06
1185±7 (2)	19.7194	(-)-trans-Isopiperithenol	0.05
1376±4 (377)	24.5755	Kopaen	0.05
1038±3 (356)	14.5678	trans-β-ocimene	0.04
1531±5 (59)	28.7747	α-Kalakorn	0.04
1211±3 (146)	20.4917	Citronellol	0.035
1447±8 (2)	30.8455	Aromadendren	0.03
1089±5 (52)	17.0787	thuyon	0.02

Table 6 – Main components of essential oils composition 3

Retention index	Retention time, min	Component	Content, %
1022±5 (394)	14.4452	Eucalyptol	7.33
1175±5 (540)	19.4381	α-Terpineol	0.90
1164±5 (512)	18.9980	Terpinen-4-ol	0.54
1086±3 (646)	16.5737	Linalool	0.35
1477±5 (364)	27.2812	Germacren D	0.26
1419±5 (656)	25.7227	caryophyllene	0.22
1500±5 (196)	27.9160	β-Bisabolene	0.17
1037±4 (15)	14.8997	β-ocimene	0.15
1038±3 (356)	14.5678	trans-β-ocimene	0.11
1401±8 (51)	29.7487	Longifolen-(V4)	0.09
1577±N/A (1)	30.9898	(-)-Spatulenol	0.07
1335±8 (207)	24.0486	Eugenol	0.06
1472±6 (181)	27.1513	ɤ-Muurolen	0.06
1632±5 (119)	31.1125	Ʈ-Muurol	0.06
1270±5 (28)	22.2448	ρ-Cymen-7-ol	0.05
1222±10 (18)	20.9317	D-Carvone	0.04
1447±8 (2)	30.8455	Aromadendren	0.04
1376±4 (377)	24.5755	Kopaen	0.03
1460±6 (178)	26.7760	Alloaromadendren	0.03
1170±N/A (1)	20.5492	D-Verbenone	0.02
1370±6 (83)	24.4672	Ulangen	0.02
1372±4 (94)	25.2753	Methyleugenol	0.02
1401±8 (51)	29.7487	Longifolene	0.01

Table 7 – Antimicrobial activity of essential oils compositions

Name of the composition / comparison preparation	Zone of growth inhibition of microorganisms, mm			
	Staphylococcus aureus ATCC 6538	*Bacillus subtilis* ATCC 6633	*Escherihia coli* ATCC 25922	*Candida albicans* ATCC 10231
Composition 1	28.7±2.3	21.7±2.1	9.7±2.3	16.0±10.4
Composition 2	27.7±4.9	25.0±14.8	9.0±1.0	18.0±10.1
Composition 3	27.5±0.7	19.5±3.5	9.5±2.1	10.0±1.4
“Breathe” / “Dyshi” spray	7.4±0.9	8.5±3.0	16.25±2.2	7.7±0.6
Benzylpenicillin sodium salt	26.2±5.2	31.2±8.5	11.0±1.7	–
Ampicillin trihydrate	27.0±2.1	30.2±23.0	18.2±2.8	–
Nystatin	–	–	–	19.8±3.9

Note: "–" – There is no growth inhibition zone.

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Table 8 - Indicators of standardization of essential oils compositions

Quality indicator	GPhM.1.5.2.0001.15 Essential oils	Test results		
		Composition 1	Composition 2	Composition 3
Description	Colorless or tinted transparent movable liquid, often yellowish in color, with a characteristic odor; lighter than water.	Mobile liquid with a characteristic odor, light orange in color; lighter than water.	Mobile liquid with a characteristic odor, light orange in color; lighter than water.	Mobile liquid with a characteristic odor, light orange in color; lighter than water.
Authenticity	The chromatogram of the test solutions of the compositions should contain peaks of essential oil components to be determined.	The relative retention times of the peaks on the chromatogram of the test solution corresponded to those of eucalyptol and terpinen-4-ol.	The relative retention times of the peaks on the chromatogram of the test solution corresponded to those of eucalyptol, terpinen-4-ol, Ţ-muurolol, ɤ-terpinene and β-myrcene.	The relative retention times of the peaks in the chromatogram of the test solution corresponded to those of eucalyptol and α-terpineol.
Solubility	Slightly soluble, very little soluble or practically insoluble in water; easily soluble or soluble in alcohol of various concentrations, ether and other organic solvents.	Insoluble in water. With ethyl alcohol it formed a white precipitate, which disappeared when 18 ml of 70% ethyl alcohol was added. When the essential oil composition was dissolved in hexane, fatty drops formed at the bottom, it was completely dissolved in chloroform, and when dissolved in dimethyl sulfoxide, a gelatinous precipitate appeared.	Not soluble in water. Completely dissolved in 70% ethyl alcohol already with the addition of 1 ml of alcohol. When dissolved in hexane, a cloudy solution was formed; when dissolved in chloroform, the oily solution was formed; when dissolved in dimethyl sulfoxide, a white precipitate was formed.	Not soluble in water. Completely dissolved in 70% ethyl alcohol already with the addition of 1 ml of ethyl alcohol. When dissolved in hexane, oily droplets formed at the bottom; with chloroform, an oily solution; when dissolved in dimethyl sulfoxide – a white precipitate.
Ethyl alcohol	Method 1 – there should be no turbidity around the drops of the essential oil composition Method 2 – there should be no purple-pink staining of cotton wool.	It does not give haze by Method 1 and does not give purple-pink staining by Method 2.		
Fatty and mineral oils in essential oils	There should be no turbidity of the solution and the formation of greasy drops.	Turbidity was not observed, fatty drops were not formed.	Turbidity was not observed, fatty drops were not formed.	Turbidity was not observed, fatty drops were not formed.
Residue of the essential oil after evaporation.	Norms have not been established.	1.35%	1.01%	1.60%
Density	Norms have not been established.	0.900 g/cm³	0.901 g/cm³	0.910 g/cm³
Optical rotation	Standards have not been established.	+0.01°	+0.01°	+0.01°
Refractive index	No standards have been set.	1.3800	1.5400	1.4800
Acid number	It should not exceed 4	1.02	1.03	1.00
Quantification	Norms have not been established.	Eucalyptol content – 6.51%; terpinen-4-ol – 1.95%	Eucalyptol content – 20.34%; terpinen-4-ol – 7.03%, Ţ-muurolol – 2.28%, ɤ-terpinene – 2.23% and β-myrcene – 2.09%.	Eucalyptol content – 7.33%; α-terpineol – 0.90%.

An important factor in the preparation of essential oils compositions was the synergism of the EOs included in the composition. The *Hyssopus ambiguous* (Trautv.) Iljin oil, which has a medium volatility, is combined with the thymes EOs, which have a high volatility. When developing the compositions, attention has also been paid to the smell of the final composition, because *Hyssopus ambiguous* (Trautv.) Iljin EOs in its pure form has a rather pungent odor due to a high content of eucalyptol, while the EOs of the thyme species collected had a less pungent odor, which in total could correct the final smell of the composition [24]. The odors of the obtained essential oils compositions were evaluated subjectively. All the presented EOs compositions were mobile liquids from light yellow to light orange color with a pleasant characteristic odor.

The developed compositions were tested for an antimicrobial activity. According to the results of the study, only the samples that showed a pronounced activity against at least one of the studied strains of microorganisms were selected.

After screening for antimicrobial and antifungal activities, which made it possible to make a choice in favor of the most promising composition, their component composition was studied by GC-MS.

Fig. 1 shows the chromatogram of composition 1.

Table 4 shows the main components found in essential oils composition 1.

The predominant components of composition 1 were eucalyptol, terpinen-4-ol and α-terpineol.

Fig. 2 shows the chromatogram of essential oils composition 2.

In composition 2, 137 components were found, among which eucalyptol, terpinen-4-ol, Ţ-muurolol, ɣ-terpinene, and β-myrcene predominated.

Table 5 shows the main components of essential oils composition 2.

Figure 3 shows the chromatogram of essential oils composition 3.

In composition 3, 149 components were found, among which eucalyptol and α-terpineol predominated.

Table 6 shows the main components of essential oils composition 3.

The study results of the antimicrobial activity of the developed compositions are presented in Table 7.

As can be seen from the data presented in the table, the EOs compositions developed had pronounced antibacterial and significant antifungal activities.

The carried out analysis of the chemical composition made it possible to characterize the dominant components:

Eucalyptol (1,8-cineol), a monoterpenoid, which is a colorless liquid, insoluble in water. By its structure, it is a cyclic ether. It is part of the EOs of a number of plants, for example, many species of eucalyptus. Eucalyptol shows a pronounced activity against *Enterococcus faecalis* ATCC 29212; a moderate activity against *Pseudomonas aeruginosa* ATCC 27853. Eucalyptol is highly active against *Botrytis cinerea, Colletotrichum acutatum, Aspergillus flavus* ATCC 22546; moreover, the level of its antimicrobial activity significantly depends on the components of the EO which are in synergy with eucalyptol [25, 26].

Terpinen-4-ol[4] is a monoterpenoid, insoluble in water. It has a sharp citrus scent. Terpinen-4-ol has strong antibacterial and antifungal properties. The literature studies have shown that terpinen-4-ol induces antitumor effects by selectively causing the death of necrotic melanoma cells. Terpinen-4-ol is highly active against biofilms formed by *Streptococcus mutans, Lactobacillus acidophilus, Porphyromonas gingivalis, and Fusobacterium nucleatum* [26].

Ţ-Muurol is a sesquiterpenoid that is practically insoluble in water. It has a slight spicy smell and a herbal taste. It exhibits an antifungal activity against *Trametes versicolor* BCRC 35253, *Gloeophyllum trabeum* BCRC 31614, *Laetiporus sulphureus* BCRC 35305, *Lenzites betulinus* BCRC 35296 [26].

ɣ-Terpinene is a branched unsaturated hydrocarbon, it is an isoprenoid. It is present in all eukaryotic organisms. It has a "herbal" smell and a bitter taste. It has antioxidant, anti-inflammatory and antiproliferative properties [26].

β-Myrcene is an acyclic monoterpene, an important component of the EOs of a number of plants. It has a pleasant smell and taste. It has analgesic, anti-inflammatory, antibacterial and antimutagenic activities [26, 27].

α-Terpineol is a monoterpene alcohol that smells like lilac. It has anti-inflammatory, antitumor properties. It is active against the strains of *Serratia liquefaciens, Cordulephya divergens, Listeria innocua* and *Salmonella Typhimurium,* and has an antioxidant activity [26, 28].

As a result of the study, compositions have been determined and optimal technological methods for the manufacture of essential oils compositions from the medicinal plant raw materials, common in the RK, the plants with antimicrobial properties, have been proposed. In the course of testing the obtained compositions, it was found that the developed compositions correspond to the basic physicochemical and technological requirements for essential oils of the SP RF XIV ed. The results are presented in Table 8.

CONCLUSION

In the course of complex studies, the samples of the EOs obtained from the medicinal plant raw materials, representatives of the *Lamiaceae* family of the RK, screening of their antimicrobial activity was carried out. Taking into account the chemical composition and activity of the obtained samples, 3 essential oil compositions with pronounced

[4] Merck KGaA, Darmstadt. Available from: https://www.sigmaaldrich.com/KZ/en/product/aldrich/223190.

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antibacterial and significant antifungal effects, have been developed.

The developed essential oils compositions have a number of features, in particular:

1. A complex chemical composition, including isoprenoids of a plant origin; it prevents the development of resistance in microorganisms and fungi, and may be an advantage of the developed compositions over synthetic analogues of antibacterial and antifungal activities.

2. The revealed chemical structure of essential oils compositions provides a wide range and severity of antibacterial and antifungal actions compared to the reference drugs - benzylpenicillin sodium salt, ampicillin trihydrate, nystatin and essential oils composition – the "Breathe"/"Dyshi" (spray). The developed compositions, as simultaneously possessing antibacterial and antimicrobial activities, can be considered as promising objects for the creation of new drugs based on them, intended for the treatment and prevention of diseases of the upper respiratory tract. Essential oils compositions can be administered by passive inhalation, which is characterized by ease of use and safety.

3. The development and further study of compositions based on the EOs from the MPRMs growing in the territory of the Republic of Kazakhstan allows the development of localized production of high-quality and relatively inexpensive therapeutic and prophylactic agents that can effectively fight infectious diseases of the upper respiratory tract.

The conducted microbiological and technological studies proved that the obtained essential oils compositions met the quality standards (SP RF XIV ed., SP RK) and the generally accepted rules for compiling essential oils compositions in aromatherapy. The resulting compositions can be both an alternative and a supplement to currently used antimicrobial and antifungal drugs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Ekaterina V. Lakomkina – collection of medicinal plant materials, production of essential oils, screening of essential oils for antimicrobial activity, structuring of essential oils compositions and their subsequent screening for antimicrobial activity, GC-MS of the obtained compositions, development of the concept of the article, article writing, literature sources collection; Gayane A. Atazhanova – management of the work on the technological part of the study, approval of the text for the publication, approval of the final version of the publication; Saule B. Akhmetova – management of the work on the microbiological part of the study, approval of the text for the publication, approval of the final version of the publication; Ifrat N. Zilfikarov – consulting, editing and approval of the text for the publication, approval of the final version of the publication.

All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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Quantitative determination of total flavonoids in *Glycyrrhiza Glabra* L. herbs

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Licorice herb (*Glycyrrhiza glabra* L.) is a promising herbal raw material, which can be comprehensively used to develop drugs with an anti-inflammatory action.

The aim of the article was to development a quantitative determination method of total flavonoids in *Glycyrrhiza glabra* L. herbs.

Materials and methods. The subjects of research were 5 samples of licorice herb harvested in summer in various places of growing and cultivation. Pinostrobin was used as a standard sample. The registration of the electronic spectra was carried out with a spectrophotometer (Analytik Jena AG, Germany) by differential spectrophotometry, 96% ethanol was used as a solvent.

Results. The methods for quantitative determination of total flavonoids in *Glycyrrhiza glabra* L. was carried out at an analytical wavelength of 310 nm equivalent to pinocembrin. The optimum parameters for the extraction of total flavonoids from *Glycyrrhiza glabra* L. were as follows: the extractant – 90% ethanol; the «raw material-extractant» ratio was 1:50; the extraction time was 60 min; the degree of atomization was 2 mm. The content of total flavonoids for the *Glyccyrhiza glabra* L. herb has been determined, it varies from 0.39±0.002 to 3.41±0.015% with the humidity of the vegetative raw material from 9.97±0.003 to 10.03±0.003% depending on the place of the vegetation, cultivation and year of the raw material collection. The error of the single determination with a 95% confidence level was ±0.73.

Conclusion. The developed methods for the quantitative determination of total flavonoids in *Glycyrrhiza glabra* L. herbs can be used to solve the issues of standardization of these medicinal plant raw materials.

Keywords: licorice; *Glycyrrhiza glabra* L.; herb; flavonoids; pinocembrin; standardization; spectrophotometry

Abbreviations: SS – standard sample; PhM – pharmacopoeial monograph; GPhMK – general pharmacopoeial monograph; BAC – biological active compounds; HPLC – High Performance Liquid Chromatography.

Методика количественного определения суммы флавоноидов в траве солодки голой

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Трава солодки голой (*Glycyrrhiza glabra* L.) является перспективным растительным сырьём, которое может быть комплексно использовано для разработки лекарственных препаратов с противовоспалительным действием.

Цель. Разработка методики количественного определения суммы флавоноидов в траве солодки голой.

Материал и методы. Объектами исследования являлись 5 образцов травы солодки голой, заготовленных в летний период времени в различных местах произрастания и культивирования. В качестве стандартного образца использовали пиностробин. Регистрацию УФ-спектров проводили с помощью спектрофотометра «Specord 40»

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(Analytik Jena AG, Германия) методом дифференциальной спектрофотометрии. В качестве растворителя использовали спирт этиловый 96%.

Результаты. Количественное определение суммы флавоноидов в траве солодки голой проводили при аналитической длине волны 310 нм в пересчёте на пиноцембрин. Установлены оптимальные параметры экстрагирования суммы флавоноидов из травы солодки голой: экстрагент – спирт этиловый 90%; соотношение «сырье-экстрагент» – 1:50; время экстракции – 60 мин; степень измельчения сырья – 2 мм. Определено содержание суммы флавоноидов для травы солодки голой, которое варьирует от 0,39±0,002 до 3,41±0,015% с учётом влажности растительного сырья от 9,97±0,003 до 10,03±0,003% в зависимости от места произрастания, культивирования и года сбора растительного сырья. Погрешность единичного определения с доверительной вероятностью 95% составляла ±0,73%.

Заключение. Разработанная методика количественного определения флавоноидов в траве солодки голой может быть использована для решения вопросов стандартизации указанного лекарственного растительного сырья.

Ключевые слова: солодка голая; *Glycyrrhiza glabra* L.; трава; флавоноиды; пиноцембрин; стандартизация; спектрофотометрия

Список сокращений: ЛП – лекарственный препарат; СО – стандартный образец; ФС – фармакопейная статья; БАВ – биологически активные вещества; ВЭЖХ – высокоэффективная жидкостная хроматография.

INTRODUCTION

Glycyrrhiza glabra L. (*Fabaceae* family) is one of the most researched and well-studied plants in the world. This is a perennial herbaceous plant with erect and sparsely branched stems, reaching a height of 50–100 cm (in exceptional cases up to 200 cm). The underground organs are well developed; its roots penetrate into the soil to a depth of 6–8 m and form a thick network, which helps maintain the licorice population. The leaves are alternate, compound, unpaired. The flowers are aggregated in loose racemes on long pedicels. The plant is common in many regions, even though it belongs to the Mediterranean species. In the valleys of the major rivers of Central Asia, Uzbekistan and Kazakhstan, licorice forms thickets[1,2].

The roots of this plant are widely used in officinal medicine. On the basis of isolated biologically active compounds (BAC) from the roots, many multicomponent drugs have been developed. In the course of studies, a group of scientists from Samara State Medical University and the All-Russian Institute of Medicinal and Aromatic Plants have also developed the state standard sample (SS) of glycyram (PhM-42-0034-00), with the specification of chemical structure, and studied physical and chemical properties of glycyrrhizic acid [1–4].

Licorice roots are valuable pharmacopoeial raw materials that are widely used for the production of drugs with an expectorant effect and an anti-inflammatory activity. This raw material is particularly popular not only in Russia but also abroad [5–7]. In recent decades, scientists have paid attention to various species of the genus *Glycyrrhiza* L. as promising sources of BACs used to create phytopreparations. Along with licorice roots, one of the promising sources is also the aerial part, i.e., the licorice herb [8, 9].

According to literature data, it is known that the above-ground part of licorice contains flavonoids, polysaccharides, tannins, triterpenoids, vitamins, etc. It is known that the flavonoid composition of licorice herb is represented by pinocembrin, glabranin, prunetin, astragalin and vitexin [9]. It should be concluded that the above-ground part of licorice is rich in a high content of BAC, among which flavonoids are of the greatest interest in terms of creating medicines.

Flavanones prevail in the herb of this plant. The dominant among them is pinocembrin (5,7- dihydroxyflavanone) the efficacy of which has been proven in preclinical practice. A number of *in vitro* and *in vivo* studies have shown that pinocembrin improved the regional cerebral blood flow of non-pedigreed rats and reduced postischemic neurovascular block damage. It follows that pinocembrin has a neuroprotective activity [10–14]. It has also a powerful antifibrotic effect, which explains its antioxidant properties. Pinocembrin alleviated bleomycin-induced skin fibrosis and a fibrosis-related protein expression in keloid tissues in xenografted mice [15, 16].

Pinocembrin had a protective effect against gentamicin-induced nephrotoxicity, which may be partially related to its antioxidant and anti-apoptotic effects, subsequently leading to the improved renal function [17]. This flavanone showed a pronounced

[1] Budantsev AL. Plant resources of Russia. Wild flowering plants, their component composition and biological activity. Vol. 3: *Fabaceae – Apiaceae*: A.L. Budantsev ed. Moscow: Association of Scientific Editions KMK; 2009. 599 p. Russian

[2] Maevsky PF. Flora of the middle belt of the European part of Russia. 11th ed. M.: KMC Scientific Publishers Association; 2014. 635 p. Russian

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activity against Gram-positive bacteria, while its methyl ester pinostrobin (5-hydroxy-7-methoxyflavanone) was less pronounced against *Esherichia coli* due to its chemical structure determining the lipophilic properties of the substance and its affinity to the lipid membrane of gram-negative bacteria [18–20]. In an *in vitro* study, pinocembrin showed a photoprotective efficacy [20, 21] and inhibited an allergic airway inflammation [22].

In one study, pinocembrin showed a more pronounced anti-inflammatory activity than the non-selective cyclooxygenase inhibitor indomethacin. At the same time, the anti-inflammatory drugs of comparison were amidopyrine, hydrocortisone, and indomethacin. A formalin-induced inflammation in mice was reduced by 40.3% at the pinocembrin dose of 25 mg/kg and by 43.4% at the 50 mg/kg dose. In methylation and ethylation of pinocembrin, at position C_8, the formation of its derivatives led to a decrease in the anti-inflammatory activity [23].

In view of the above, a wide range of therapeutic uses of the licorice herb seems appropriate from the position of complex processing of raw materials to study the properties of water-alcoholic extracts and preparations based on the herb of this plant. This will expand the range of ideas about the pharmacological activity of flavonoids and substances of the above-ground part of *G. glabra* and evaluate the possibility of using this object in the creation of domestic drugs.

Based on the sum of flavonoids from the above-ground part of licorice, foreign scientists developed a drug called “Glacembrine” with anti-inflammatory and analgesic effects [24]. Glacembrin has undergone a number of clinical trials according to the following parameters: an acute toxicity, an anti-inflammatory activity. The results showed that “Glacembrin” did not differ from the similar indicator of pinocembrin, and the anti-inflammatory activity of the drug was higher than in the case of pinocembrin. In addition, “Glacembrin” has a pain-relieving effect on the model of acetic cortex in mice [23].

All of the above-mentioned has opened new opportunities for the use of the licorice herb in the pharmaceutical industry as a raw material for the manufacture of standardized drugs with anti-inflammatory and other effects. In general, the literary analysis showed an insufficient degree of study and elaboration of the licorice herb standardization. Attention should be paid to the developed methods of the quantitative determination of total flavonoids in the above- ground part of licorice by direct spectrophotometry equivalent to pinocembrin, proposed by Uzbek scientists [25]. In our opinion, this technique can give overestimated results of determination since other phenolic compounds also contribute to the optical density at the analytical wavelength of 290 nm. In addition, a multiple extraction (3 times) of raw materials cannot be always justified, because these extraction conditions can increase the error of the analysis method.

In this regard, the research is relevant in terms of improving the methods for a quantitative determination of total flavonoids in the *Glycyrrhiza glabra* L. herb.

THE AIM of the article was to work out methods for quantitative determination of total flavonoids in *Glycyrrhiza glabra* L.

MATERIALS AND METHODS

The objects of the study were five samples of the *Glycyrrhiza glabra* L. herb. They were prepared: No. 1 – in the Samara region (Kinelsky district, Alekseevka village), August 2021; No. 2 – in Samara city, the Botanical Garden of Samara State Medical University, August 2021; No. 3 – in the Orenburg region, (Sakmarsky district, Tatarskaya Kargala village), July 2017; No. 4 – in the Republic of Kazakhstan, Derzhavinsk city, June 2018; No. 5 – in the Samara region (Bolshechernigovsky district, Bolshaya Chernigovka village), August 2019. The moisture content of the plant raw materials was determined in accordance with the requirements of the Russian State Pharmacopoeia XIV edition GPhM.1.5.3.0007.15 «Determination of moisture content of medicinal plant raw materials and medicinal plant preparations»[3].

The used methods of spectrophotometry was performed in accordance with the requirements of the Russian State Pharmacopoeia XIV edition GPhM.1.2.1.1.0003.15 «Spectrophotometry in ultraviolet and visible regions». Spectral characteristics of aqueous-alcohol extracts were evaluated on a spectrophotometer «Specord 40» (Analytik Jena AG, Germany) in cuvettes with a layer thickness of 10 mm, 96% ethanol was used as a solvent.

The solution of pinostrobin prepared in 96% ethanol was used as a SS (Fig. 1). Pinostrobin standard

[3] Russian State Pharmacopoeia. XIV ed. Vol. 1–4. Moscow; 2018. Available from: http://http://femb.ru/femb/ pharmacopea.php

sample corresponded to the requirements of the pharmacopoeial article (PhM 42-0073-01) and was provided to the Scientific and Educational Center «Pharmacy» of Samara State Medical University to determine by high-performance liquid chromatography (HPLC) the degree of purity, which was not less than 98.0%. Aqueous-alcoholic extracts were prepared using 96% ethanol (brand name: chemical clean, Hippocrates, Russia). The necessary concentrations of alcohol (50, 60, 70, 80, 90%) were obtained by diluting 96% ethanol according to Table 5 of Appendix to the Russian State Pharmacopoeia XIV edition.

Preparation of working solutions for analysis by UV-spectrophotometry

The analytical sample of raw materials was crushed to the particle sizes, passing through a sieve with holes of 2 mm in diameter. About 1 g of the crushed raw material (exact weight) was placed in a conical heat-resistant Erlenmeyer flask with a 100 ml slit, 50 ml of 90% ethanol was added. The flask was closed with a stopper and weighed on Sarto GOSM laboratory scales (Russia) with an accuracy of ±0,001. The flask was attached to a reflux condenser and heated in a boiling water bath (moderate boiling) for 60 min. Then it was cooled for 30 min, closed with the same cap, weighed again and the missing extractant was replenished to the original volume. The extraction was filtered through a paper filter (red band).

To prepare test solution pinostrobin for the UV-spectrophotometry, about 0.02 g (exact weight) of the substance was weighed, placed in a 50 ml volumetric flask, dissolved in 30 ml of 96% ethanol and heated in a water bath. Use of 96% ethanol provided the best dissolution of the reference standard of pinostrobin. After cooling the contents of the flask to room temperature, its volume was brought to the mark with 96% ethanol (pinostrobin standard solution A). Then 1 ml of pinostrobin standard solution A was placed into a 25 ml volumetric flask, 2 ml of aluminum trichloride 3% alcohol solution was added and the solution volume was brought to the mark with 96% ethanol (test solution B of pinostrobin).

The reference solution was prepared as follows: 1 ml of pinostrobin A solution was placed in a 25 ml volumetric flask and the volume of the solution was brought to the mark with 96% ethanol (pinostrobin B reference solution). The optical density of test solution B of pinostrobin was measured on a spectrophotometer at 310 nm against the background of reference solution B of reference standard of pinostrobin.

Quantitative determination of total flavonoids in water-alcoholic extraction of licorice herb

About 1 ml of the obtained extraction was placed in a 50 ml volumetric flask, 2 ml of 3% alcohol solution of aluminum (III) chloride was added, the volume of solution was brought to the mark with 96% ethanol (test solution A), stirred and left for 40 min to form a flavonoid complex with aluminum. The solution obtained as follows was used as a reference solution: 1 ml of the extraction (1:50) was placed in a 50 ml measuring flask and the volume of the solution was brought to the mark with 96% ethanol (comparison solution A). Then the optical density of the test solution A was measured on a spectrophotometer at 310 nm against the background of the reference A.

The content of total flavonoids equivalent to pinocembrin and absolutely dry raw materials in percentage (X, %), is calculated by the formula:

$$x = \frac{D * m_0 * 50 * 50 * 1.05 * 100}{D_0 * m * 50 * 25 * (100 - W)},$$

where D – the optical density of the test solution; D_0 – the optical density of the pinostrobin SS; m – the mass of raw materials, g; m_0 – the mass loss in drying, %; 1.05 – the conversion factor.

In the absence of a pinostrobin standard sample, it is advisable to use the theoretical value of the specific absorption index, 680:

$$x = \frac{D * 50 * 25 * 100}{m * 680 * (100 - W)},$$

where D – the optical density of the test solution; m – the mass of raw materials, g; 680 – specific absorbance ($E_{1\,cm}^{1\%}$)of standard sample of pinocembrin at 310 nm; W – loss in weight during drying, %.

Validation of analytical methods

Validation of the developed methods was carries out according to the following indicators: specificity, linearity, precision (repeatability), intralaboratory precision, accuracy in accordance with the requirements of the Russian State Pharmacopoeia XIV edition. Microsoft Excel 2013 Software was used for calculations.

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RESULTS AND DISCUSSION

In the course of the experiment, water-alcoholic extracts of the licorice herb were obtained and their UV-spectra were studied. In the herb of this plant, flavanones prevail, the dominant flavonoid is pinocembrin (Fig. 1), which has a maximum absorption at a wavelength of ±2 nm (Fig. 2).

In our opinion, it is pinocembrin that mainly determines the character of the absorption curve of the water-alcohol extract from the licorice herb (Fig. 3). This conclusion is consistent with the literature data [7]. Since pinocembrin is not registered in the Russian Federation as a standard sample, the possibility of using pinostrobin (PhM 42-0073-01), close in chemical structure to pinocembrin as SS, was studied. It was determined that pinotembrine and pinostrobine have a maximum absorption at a wavelength of 290 nm (direct spectrophotometry) (Fig. 2 and 4). Taking into account the fact that in the case of the flavonoid sum content by direct spectrophotometry there is an overestimation of the experimental results, the possibility of using differential spectrophotometry with aluminum trichloride as a complexing reagent was studied.

It was found that the addition of aluminum (III) chloride to the test solution and the pinocembrin and pinostrobin solutions resulted in a bathochromic shift in the long-wave absorption spectrum (Fig. 2–4). At the same time, it was determined that differential spectrophotometry by the short-wave absorption maximum of pinostrobin and pinocembrin solutions are at wavelength 310 nm (Fig. 5 and 6), while in the long-wave region of the spectrum their absorption maxima do not coincide, which allows us to recommend 310 nm as an analytical wavelength. When using pinostrobin SS, the content of the flavonoid sum to pinocembrin was recalculated by introducing a coefficient into the formula for the calculation.

In the absence of pinostrobin SS, the theoretical value of pinostrobin specific absorption established experimentally, was used.

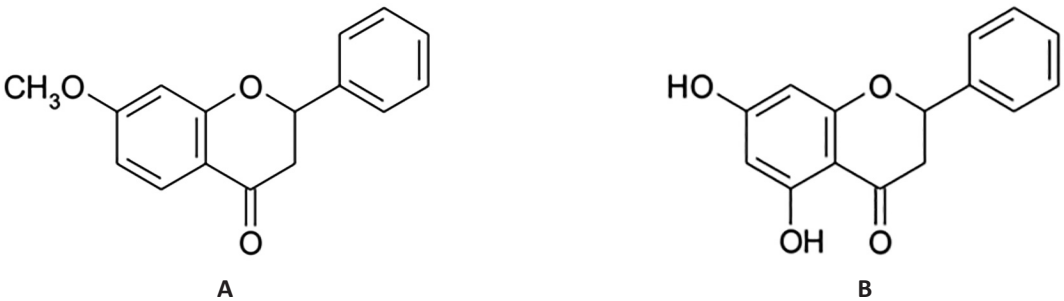
It was found that the total extraction of flavonoids from the licorice herb is achieved with 90% ethanol. The next step was to conduct an experiment to determine the optimal «raw material- extractant» ratio (1:50). Then the time parameters of the extraction, during which there was a maximum extraction of flavonoids from the raw materials were established − 60 min. The final step was to determine the degree of grinding of the raw material (2 mm), contributing to the full extraction of flavonoids by the extractant (Table 1).

The methods specificity was determined by the correspondence of the absorption maxima of the *Glycyrrhiza glabra* L. herb flavonoid complex and the solution of the standard pinostrobin sample with aluminum trichloride and the differential peak of the standard pinostrobin sample.

The methods linearity was determined for a series of pinostrobin solutions with concentrations ranging from 0.016 to 0.16 mg/ml (0.016; 0.032; 0.08; 0.16). Based on the data obtained, the dependence of the optical density values of pinostrobin solutions with aluminum trichloride on pinostrobin concentration was plotted, and then a linear regression equation was calculated (Fig. 8).

In studying the linear dependence of $y=bx+a$, the correlation coefficient was 0.9981, therefore, pinostrobin SS can be used to analyze the amount of flavonoids in the licorice herb in the indicated concentration range (Fig. 8).

The precision of the methods (a repeatability level) was estimated by analyzing the test sample of plant material in 11-fold repetition. The error of single determination of the amount of flavonoids in licorice herb with a 95% confidence level is ±0.73% (Table 2).



A B

Figure 1 – Structural formulas of pinostrobin (A) and pinocembrin (B)

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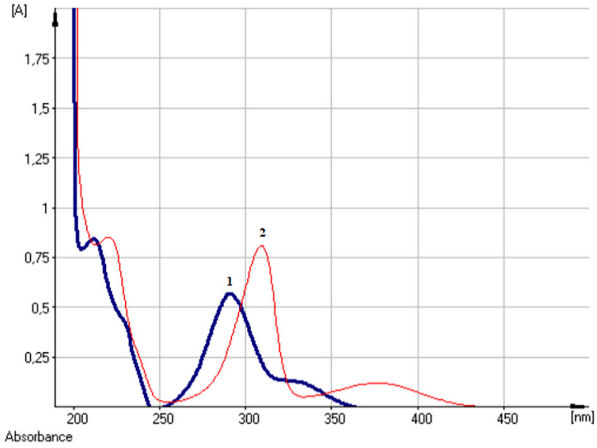

Figure 2 – Electronic spectra of solutions of water-alcohol solutions of pinocembrin
Note: 1 – pinocembrin solution (direct spectrophotometry),
2 – pinocembrin solution with addition of aluminum trichloride.

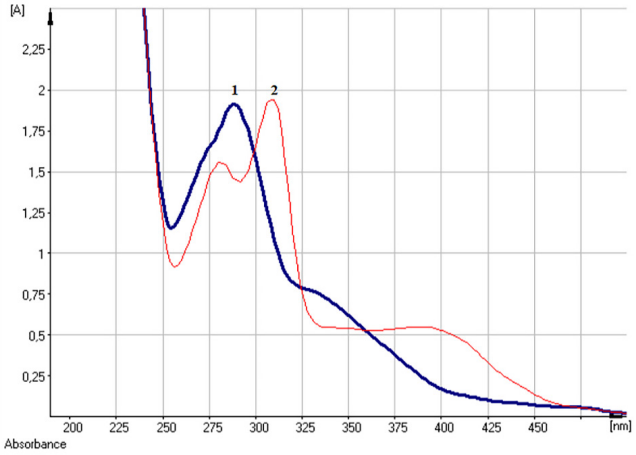

Figure 3 – Electronic spectra of water-alcohol extraction from *Glycyrrhiza glabra* L. herbs
Note: 1 – extraction solution (direct spectrophotometry),
2 – extraction solution with addition of aluminum trichloride.

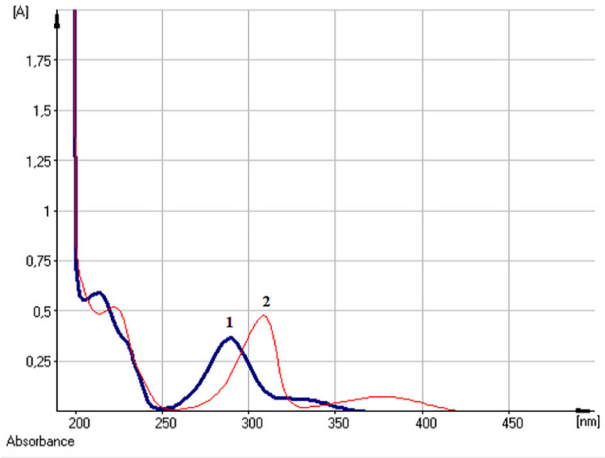

Figure 4 – Electronic spectra of pinostrobin solution
Note: 1 – pinostrobin solution (direct spectrophotometry),
2 – pinostrobin solution with addition of aluminum (III) chloride.

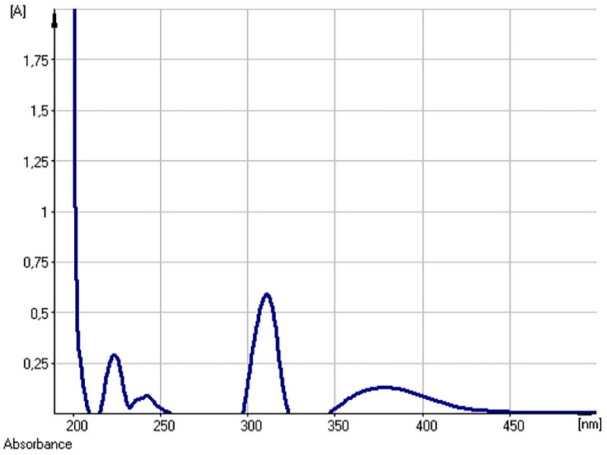

Figure 5 – Differential spectrum of pinocembrin solution

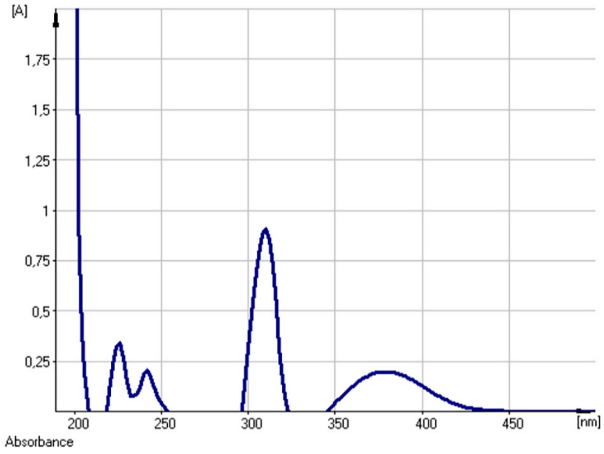

Figure 6 – Differential spectrum of pinostrobin solution

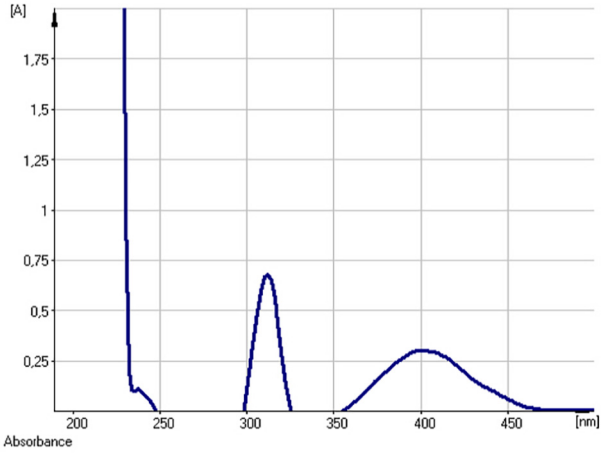

Figure 7 – Differential spectrum of water-alcohol extraction from *Glycyrrhiza glabra* L. herbs

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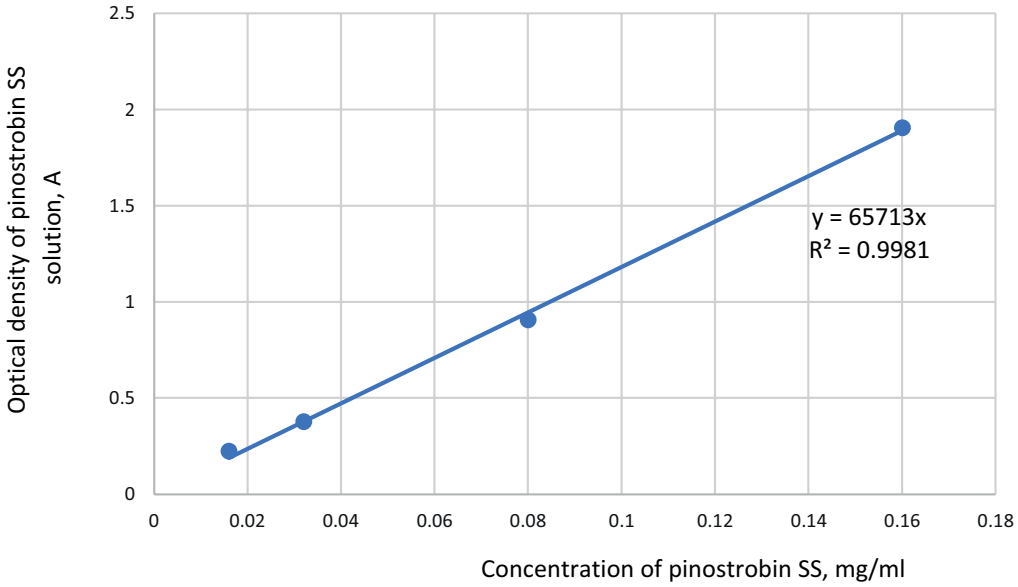

Figure 8 – Dependence of optical density values of pinostrobin solutions with aluminum (III) chloride on pinostrobin concentration (differential version)

Table 1 – Optimal extraction rates of total flavonoids from *Glycyrrhiza glabra* L. herbs at wavelength of 310 nm

Concentration of ethyl alcohol, %	«Raw materials-extractant» ratio	Extraction time, min	Degree of atomization, mm	Total flavonoids content equivalent to per pinocembrin and absolutely dry raw material, %
I. Extractor				
50	1:50	60	2	1.50±0.006
60				1.19±0.005
70				1.21±0.031
80				1.86±0.005
90				**2.28±0.001**
96				1.59±0.007
II. Extraction time				
90	1:50	30	2	2.02±0.008
		45		2.31±0.001
		60		**2.76±0.012**
		120		2.39±0.010
III. Degree of atomization				
90	1:50	60	1	3.10±0.013
			2	**3.41±0.015**
			3	2.52±0.011
IV. «Raw materials-extractant» ratio				
90	1:30	60	2	2.18±0.009
	1:50			**3.41±0.015**
	1:100			3.21±0.014

Table 2 – Precision estimation results of quantitative determination methods of total flavonoids in *Glycyrrhiza glabra* L. herbs (a repeatability level)

X, %	Metrological characteristics
3.40	
3.41	N=11
3.42	f=10
3.42	$\overline{X}$=3.41
3.45	SD=0.03690
3.46	RSD=1.0813%
3.39	$S_{\overline{X}}$=0.01113
3.47	P, %=95
3.35	t (P, t)=2.23
3.40	$\Delta\overline{X}$=0.02481%
3.37	E=0.73%

Table 3 – Validation of the laboratory precision of the methods for determining total flavonoids in *Glycyrrhiza glabra* L. herb

X, %	X, %	Metrological characteristics	
Analyst 1	Analyst 2	Analyst 1	Analyst 2
3.40	3.39	$\overline{X}$=3.43	$\overline{X}$=3.42
3.42	3.41	SD=0.02229	SD=0.02160
3.42	3.42	RSD=0.2652%	RSD=0.2579%
3.44	3.43	$S_{\overline{X}}$=0.009098	$S_{\overline{X}}$=0.008819
3.45	3.44	P, %=95	P, %=95
3.46	3.45	t (P, t)=2.23 (table)	t (P, t)=2.23 (table)
		E=0.59%	E=0.57%
		$\Delta\overline{X}$=0.02%	$\Delta\overline{X}$=0.019%

Notes: $t_{calculated}$=0.66<t (95%;6); $F_{calculated}$=1.06<F (95%;5;5) – differences between the results obtained are random.

Table 4 – Assessment results of the correctness of quantitative determination methods of total flavonoids in *Glycyrrhiza glabra* L. herbs

Injected pinostrobin, mg/ml	Found, mg/ml	Openness, %	Metrological characteristics
7.57	7.49	98.94	
7.57	7.52	99.34	$\overline{X}$=99.68%
7.57	7.56	99.87	SD=0.36062
15.15	15.08	99.54	RSD=0.3618%
15.15	15.11	99.74	$S_{\overline{X}}$=0.120206
15.15	15.16	100.07	P, % =95
22.73	22.66	99.69	t (P, t)=2.23
22.73	22.70	99.87	E=0.27%
22.73	22.74	100.04	$\Delta\overline{X}$=0.27%

Table 5 – Content of total flavonoids in *Glycyrrhiza glabra* L. samples of licorice (in %) equivalent to pinocembrin

No.	Characteristics of the raw material	Moisture content in raw plant materials, %	Content of total flavonoids in absolutely dry raw materials (%) equivalent pinocembrin
1.	Samara region (Kinelsky district, Alekseevka village), August 2021	9.97±0.003	3.38±0.015
2.	Samara city, Botanical Garden of Samara University, August 2021	9.96±0.003	0.48±0.002
3.	Orenburg region, (Sakmarsky district, Tatarskaya Kargala village), July 2017	9.99±0.003	0.39±0.002
4.	Republic of Kazakhstan, Derzhavinsk city, June 2018	10.01±0.002	1.34±0.006
5.	Samara region (Bolshechernigovsky district, Bolshaya Chernigovka village), August 2019	10.03±0.003	1.19±0.005

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To assess in-laboratory precision, the analysis of the test sample was performed by another analyst on the same equipment on other days (Table 3). For each sample, the studies were carried out in six replications. Table 3 shows that the calculated value of Fisher's F-criterion 1.06 is less than the tabulated value of 5.05. Consequently, the variance of the analysis results of both chemistries are statistically equivalent and the differences between the obtained values not significant. Thus, the developed technique meets the validation requirements for the in-laboratory precision.

The correctness of the methods was determined by the standard addition methods. Pinostrobin solutions with the known concentration (25, 50 and 75%) were added to the aliquot of the test sample. The average opening percentage was 99.68±0.27% (Table 4). Three determinations were performed for each concentration. The error determined for the samples with additives of the SS was within the error of a single determination, indicating the absence of a systematic error (Table 4).

It was found that the average content of flavonoids in the studied sample of raw materials was 3.41±0.015% (the relative error of determination was ±0,73%).

Thus, based on the results of the validation evaluation of the experimental results, it can be concluded that this methods is suitable for the quantitative assessment of total flavonoids in terms of pinocembrin.

Using this methods, 5 samples of the licorice herb harvested in summer in different locations were analyzed (Table 5). It was determined that the content of total flavonoids in the analyzed samples varies from 0.39±0.002 to 3.41±0.015% with the moisture content of plant raw materials from 9.97±0.003 to 10.03±0.003% depending on the place of growth and the year of collection of plant raw materials (Table 5).

CONCLUSION

Thus, as a result of this study, the methods of quantitative determination of total flavonoids in the *Glycyrrhiza glabra* L. herb by differential spectrophotometry using a SS of pinostrobin at an analytical wavelength of 310 nm has been developed.

A validation assessment of the developed methods by the indicators of specificity, linearity, precision (a repeatability level), in-laboratory precision, correctness has been carried out. Based on the results of the validation assessment of the experimental results, these methods can be suitable for the quantitative assessment of total flavonoids equivalent to pinocembrin.

The results of the study can be used to create herbal medicines based on the licorice herb with a neuroprotective activity, antifibrotic effects, an antimicrobial action against *Esherichia coli* and a photoprotective efficacy.

The results of the study can be used in the development of a draft regulatory documentation for a promising type of raw materials «Licorice naked herb.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Olga A. Belova – collecting plant materials for analysis, experiment conducting, analyzing and interpreting the data obtained, preparing a draft manuscript, analyzing the literature, writing and preparing the manuscript for publication; Vladimir A. Kurkin – final approval of the manuscript for publication, results processing, verification of critical intellectual content; Maxim V. Egorov – participation in the development of the study concept and design, critical analysis of the study results.

All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Analysis of cytokine response characteristics and immunopathogenetic effects of double-stranded sodium salt RNA-based drug for postexposure prophylaxis against novel coronavirus infection: double-blind, placebo-controlled trial

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The aim of the work was to study cytokine response characteristics in the group of persons contacted by a novel coronavirus infection depending on the development of the disease over the next 14 days. Herewith, for the immunocorrection with a preparation based on RNA double-stranded sodium salt (RADAMIN®VIRO) causing a secondary reduction in the risk of COVID-19 in the analyzed group, potential targets had been selected.

Materials and methods. A double-blind, placebo-controlled study of the drug based on RNA double-stranded sodium salt therapeutic effects was conducted in a group of patients who had been in contact with the persons having a confirmed diagnosis of COVID-19. The method of enzyme immunoassay in dynamics was used to determine the content of interferons alpha and beta (IFNα and IFNβ, respectively), interleukin-1β and -10 (IL1β and IL-10, respectively) in the blood serum and saliva in the contact persons, with a retrospective assessment of changes depending on the administration of the drug or placebo, as well as the development of COVID-19.

Results. In the course of the presented study, it was demonstrated that the established content of IFNα (less than 28 pg/ml) and IFNβ (less than 12 pg/ml) in saliva on the 1st–2nd contact days is a predictor of an increased risk of developing COVID-19. Herewith, the increase degree in these immunoregulatory peptides in the interval of 2–3 contact days is important: IFNα and IFNβ allows leveling the negative prognosis in patients by 250% or more. The lowest rates (p <0.001) of INFα on the 1st–2nd contact days, as well as an increase of less than 21% by the 3rd day, were observed in persons with a waist circumference of more than 80/94 cm (women/men). The incidence in this group was higher and amounted to 85% (16 out of 20 people). The predictor role of IL-1β and IL-10 in the blood and saliva in relation to the start of the infectious process was not revealed. The administration of drug based on RNA double-stranded sodium salt to the contact patients made it possible to correct the interferon response in the form of an increase in the content of IFNα and IFNβ, as well as to reduce the incidence in comparison with the placebo group.

Conclusion. Differences in the interferon regulation upon contact with SARS-CoV-2 in the form of lower INF α and β levels, as well as a slightly pronounced growth dynamics in the interval of the first 3 days, influenced the increased risk of developing COVID-19. RADAMIN®VIRO can be recommended as a means of post-exposure prophylaxis of COVID-19 for both medical institutions and for caregivers and / or contacts with COVID-19 patients.

Keywords: coronavirus; COVID-19; RNA double-stranded sodium salt; RADAMIN®VIRO; prevention; interferon inducer

Abbreviations: COVID-19 – an infectious disease caused by the SARS-CoV-2 virus; EAH – essential arterial hypertension; II – interferon inducer; ARI – acute respiratory disease; PCR – polymerase chain reaction; IWRS – Interactive Web Randomization System; eIRC – electronic individual registration card; IFNα – interferon alpha; IFNβ – interferon beta; IL1β – interleukin-1β; IL-10 – interleukin-10; NAAT – nucleic acid amplification test.

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RESEARCH ARTICLE

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Анализ особенностей цитокинового ответа и иммунопатогенетических эффектов при применении препарата на основе РНК двуспиральной натриевой соли для постконтактной профилактики против новой коронавирусной инфекции: двойное слепое плацебо-контролируемое исследование

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Цель. Изучение особенностей цитокинового ответа в группе лиц контактных по новой коронавирусной инфекции в зависимости от развития заболевания в течение последующих 14 дней с выделением мишеней для иммунокоррекции препаратом на основе РНК двуспиральной натриевой соли (препарат РАДАМИН®ВИРО) с вторичным снижением риска COVID-19 в анализируемой группе.

Материалы и методы. Проведено двойное слепое плацебо контролируемое исследование терапевтических эффектов препарата на основе РНК двуспиральной натриевой соли в группе пациентов, контактировавших с лицами, имеющими подтверждённый диагноз COVID-19. Методом иммуноферментного анализа в динамике определяли в сыворотке крови и слюне содержание интерферона альфа и бета (IFNα и IFNβ соответственно), интерлейкина-1β и -10 (IL1β и IL-10 соответственно) у контактных лиц с ретроспективной оценкой изменения в зависимости от введения препарата или плацебо, а также развития COVID-19.

Результаты. В ходе представленного исследования продемонстрировано, что установленное содержание IFNα (менее 28 пг/мл) и IFNβ (менее 12 пг/мл) в слюне на 1–2 сут контакта выступает предиктором повышения риска развития COVID-19. При этом имеет значение степень увеличения данных иммунорегуляторных пептидов в интервале 2–3 дней контакта: IFNα и IFNβ на 250% и более позволяет нивелировать негативный прогноз у пациентов. Наиболее низкие показатели (p <0,001) INFα на 1–2 сут контакта, а также рост к 3 дню менее 21% наблюдались у лиц с окружностью талии более 80/94 см (женщины/мужчины). Заболеваемость в данной группе была выше и составила 85% (16 из 20 человек). Предикторной роли IL-1β и IL-10 в крови и слюне в отношении старта инфекционного процесса не выявлено. Введение препарата на основе РНК двуспиральной натриевой соли контактным пациентам позволило скорректировать интерфероновый ответ в виде повышения содержания IFNα и IFNβ, а также снизить заболеваемость в сравнении с группой плацебо.

Заключение. Отличия интерфероновой регуляции при контакте с SARS-CoV-2 в виде более низких уровней INF α и β, а также мало выраженная динамика роста в интервале первых 3-х дней влияли на повышение риска развития COVID-19. РАДАМИН®ВИРО может быть рекомендован в качестве средства постконтактной профилактики COVID-19 как для медицинских учреждений, так и у лиц, осуществляющих уход и/или контактировавших с больными COVID-19.

Ключевые слова: коронавирус; COVID-19; РНК двуспиральной натриевая соль; РАДАМИН®ВИРО; профилактика; индуктор интерферонов

Список сокращений: COVID-19 – инфекционное заболевание, вызываемое вирусом SARS-CoV-2; ЭАГ – эссенциальная артериальная гипертензия; ИИ – индуктор интерферонов; ОРЗ – острое респираторное заболевание; ПЦР – полимеразная цепная реакция; IWRS – система интерактивной интернет-рандомизации; э-ИРК – электронная индивидуальная регистрационная карта; IFNα – интерферон альфа; IFNβ – интерферон бета; IL1β – интерлейкин-1β; IL-10 – интерлейкин-10; МАНК – метод амплификации нуклеиновых кислот.

INTRODUCTION

In the context of the COVID-19 pandemic (the causative agent is the SARS-CoV-2 virus), taking into account the frequent occurrence of genovariants and their high contagiousness, the importance of preventive measures increases, and a post-exposure prophylaxis is a factor in preventing the spread of the disease. Drugs that can be used for this purpose should slow down the spread of the epidemic and/or pandemic and reduce the viral load on the body in the event of infection [1].

The course of a novel coronavirus infection has a variety of clinical manifestations: from severe pulmonary

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lesions to a mild and/or asymptomatic course, which may be directly related to the individual response of the body to SARS-CoV-2, where regulatory peptides (cytokines) have a significant impact on the development and course of the diseases [2].

The interferon-mediated antiviral response has a direct relationship with a viral load, which depends on the infecting dose and the immune control degree of replication. The antiviral effects of the drug based on RNA double-stranded sodium salt are realized by stimulating the synthesis of type I interferons, as well as by changing the activity of the spectrum of toll-like receptors with a secondary immunomodulatory effect, which is realized through the links of both innate and adaptive immunity [3]. In the early stages of the infection, despite the inhibition of the endogenous HCoV IFN production, the use of an interferon inducer (II) based on RNA double-stranded sodium salt can act as a factor determining the control of the SARS-CoV-2 replication [4].

With regard to quarantine measures, their effect is not so high, because the data had been limited to case tracking and reporting, the isolation of critically ill patients in hospital, mild cases at home, and contact tracing. To reduce contact and transmission among the population, many countries have implemented social distancing but a high contribution of asymptomatic spread has shown the insufficiency of these measures [5, 6]. At the same time, the shortcomings of short-term measures have been noted; they are associated with the risk of a resumption of the rise in the incidence due to the late introduction of quarantine or its early cancellation [7, 8].

The studies have shown that the forecast for the spread of morbidity can be regarded as unfavorable. Global trends in globalization and urbanization reducing the effectiveness of administrative measures will not play a leading role and will not have a significant impact on the spread of epidemics and pandemics [9, 10].

Pharmaceutical companies around the world are searching for new highly effective innovative molecules characterized by both specific and nonspecific activities against pathogens of socially significant infectious diseases for a therapeutic and prophylactic use [11–13].

The COVID-19 pandemic has spurred the search for effective antivirals for both treatment and prevention of infectious diseases, and has shortened the timeline for introducing antivirals into treatment regimens. In the context of the pandemic, in clinical practice, the approval for the use of drugs for the treatment and prevention of COVID-19 was carried out according to an accelerated scheme for chemotherapeutic and biological drugs with constant and careful monitoring of their efficacy and safety [14, 15].

Currently, there are signs of the COVID-19 pandemic subsiding, which is a good opportunity to prepare effective therapeutic methods and replenish the “arsenal” with new effective and safe drugs [16].

It should be noted that even with a large coverage of the vaccinated and those who have already had COVID-19, the risk of the infection remains quite high, which is associated with the emergence of new SARS-CoV-2 sublines. One of such examples is the sub-variant of the SARS-CoV-2 virus (related to the XBB.1.16 line) – Arcturus, which has a greater virulence and resistance to the established immunity, as evidenced by a recently published study by Indian scientists [17].

With regard to preventive measures, it should be noted that a feature of the COVID-19 course is the presence of asymptomatic carriers and/or mild cases with symptoms of seasonal SARS, which, in turn, revealed the insufficiency of isolation and social distancing measures to control the pandemic [18, 19].

Taking into account the fact that families have members whose professional activities are associated with a continuous production and a constant contact with other people, the risk of infection of other family members increases significantly [20, 21].

As a prophylaxis, interferon therapy has an immunomodulatory and indirect antiviral effect upon contact with COVID-19 patients, contributing to the optimal response to the penetration of the virus and the production of intracellular antiviral proteins, which seems appropriate and justified for controlling the incidence in contact persons (family, work groups, mass gatherings of people, medical institutions) [22].

In the early stages of the infection, the use of II promotes the production of anti-inflammatory cytokines and, as a result, has a positive effect on reducing the risk of developing life-threatening conditions in patients [23].

Currently, in most industrialized countries including the Russian Federation, there is a constant search for effective molecules that have a direct and indirect effect on socially significant infectious diseases in order to prevent and treat them [24].

The results of a placebo-controlled study of RADAMIN®VIRO (RNA double-stranded sodium salt) prove its effectiveness in preventing a novel coronavirus infection. In case of COVID-19, the symptoms of the disease developed less frequently than in patients in the placebo group. A decrease in the frequency of development, duration and severity of the symptom complex characteristic of COVID-19 indicates a high efficiency of the drug. That actualizes the need to study the effect of drug based on RNA double-stranded sodium salt on the cytokine response (local and systemic) and the feasibility of its use for the purpose of immunocorrection in the context of therapy and prevention of respiratory viral infections, primarily COVID-19 [25].

THE AIM of the work was to study cytokine response characteristics in the group of persons

contacted by a novel coronavirus infection depending on the development of the disease over the next 14 days. Herewith, for the immunocorrection with a preparation based on RNA double-stranded sodium salt (RADAMIN®VIRO preparation) causing a secondary reduction in the risk of COVID-19 in the analyzed group, potential targets had been selected.

MATERIALS AND METHODS

Study design

Approval by the local ethics committee at National Research Ogarev Mordovia State University (protocol No. 5 dated May 17, 2020; approval dated April 30, 2022, protocol No. 105), a study of the local and systemic cytokine response of patients with an essential arterial hypertension (EAH) exposed to a novel coronavirus infection, was carried out. The features of the cytokine status associated with an increased risk of developing COVID-19 had been identified. The study was conducted from May 2020 to May 2022 based on the outpatient department of Republican Clinical Hospital No. 3, Saransk, Russia.

In the second part of the study (from June 1, 2022 to November 20, 2022), two groups were formed to investigate the immune effector of RNA double-stranded sodium salt. The 1st (placebo group) comprised 15 people aged 45 to 55 years, with EAH, who were in "close family contact" with COVID-19 patients. The 2nd (Test drug group) comprised 14 people aged 45 to 55 years with EAH who were in "close family contact" with COVID-19 patients. They were included in the study investigating the effects of the drug as a part of an additional study to the Clinical Study Protocol RAD-012022. The principle of a double-blind placebo-controlled study was the following: at the time of the administration and until the completion of the second stage of the study, neither the doctor nor the patient knew what therapy the subject was receiving, but the patient knew that he could receive RNA double-stranded sodium salt).

For dividing contact patients, two additional criteria were also introduced: a waist circumference (less than 80/94 cm (20 people) or more than 80/94 cm (15 people), women/men), and a retrospective criterion depending on the development of COVID-19 during 14 days of observation (sick and not sick).

The diagnosis of COVID-19 was established in accordance with the current Interim Guidelines for the Prevention, Diagnosis and Treatment of a Novel Coronavirus Infection.

Contact persons (in the placebo group) were administered a saline sodium chloride solution, 0.9%–5 ml once at the dose of 5 mg on the first day of treatment of an infected family member, which corresponded to 2 days of a direct contact. In group 1, material sampling (blood, saliva) was carried out three times: on the 1st–2nd days of contact with an infected patient, then on the 3rd and 7th days.

In the Radamin group, the participants were administered a drug based on RNA double-stranded sodium salt once at the dose of 5 mg on the 1st day of treatment of an infected family member, which corresponded to 2 days of a direct contact when compared with the main group. Before the administration of the drug based on RNA double-stranded sodium salt, on the 2nd day of contact with an infected patient, 1st day after the administration (corresponding to 3 days of contact with an infected patient), 3rd days after the administration (corresponding to 6–7 days of contact with an infected patient), blood and saliva were sampled.

The sampling of blood and unstimulated saliva for the both groups was carried out in the morning on an empty stomach (12 h without food and water). The blood was centrifuged (ARMED LC-04B centrifuge, Russia), followed by a serum separation and storage in labeled test tubes at -30°C for no more than 45 days. Before the saliva sampling, the participants rinsed their mouths with distilled water for 2 min immediately before sampling, and then saliva was collected for 15 minutes by passive salivation into graduated tubes. The levels of interferon alfa (IFNα) and interferon beta (INFβ), interleukin-1β and -10 (IL1β and IL-10, respectively) were determined by enzyme immunoassay (ELISA) in the laboratory of the Department of Immunology, Microbiology, Virology, National Research Ogarev Mordovia State University, on the enzyme immunoassay analyzer "Personal Lab TM" (Adaltis, Italy). The choice of the cytokines had been justified by the data of the previously conducted study and on the role of type I interferons. The ratio of pro- and anti-inflammatory cytokines in the antiviral immunity, as well as the results of a pilot study of the immune status of contact patients. The above studies were carried out before the start of this study and included 32 cytokines, the results were not published by the authors.

This study was conducted in accordance with the rules of Good Clinical Practice of the International Conference on Harmonization (ICH GCP), the ethical principles set forth in the Declaration of Helsinki of the World Medical Association (Fortaleza, 2013) and the requirements of the Russian legislation. A biological material (blood) was obtained for research taking into account the provisions of the WMA Declaration of Helsinki (2013) and the protocol of the Council of Europe Convention on Human Rights and Biomedicine (1999), taking into account the additional protocol to the Convention on Human Rights and Biomedicine in the field of biomedical research (2005).

Randomization of study subjects into groups

The subjects were randomized using an interactive online randomization system (Interactive Web

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Randomization System, IWRS) embedded in an electronic individual registration card (eIRC). Before the start of the study, each investigator-physician who was delegated the responsibility of transferring the data to the eIRC was provided with an access code (a combination of a username and password) to the eIRC, as well as detailed written instructions for working with the eIRC, including the randomization procedure.

The randomization was carried out according to the following algorithm. Each subject that met all of the inclusion criteria and did not meet any of the exclusion criteria was assigned a three-digit randomization number by the IWRS system. The subject randomization number and other relevant data were entered by the investigator into the Subject Screening/Randomization Journal.

If a subject terminated the study participation prematurely, their randomization number was not reused and the subject was subsequently unable to participate further on.

Inclusion criteria

The inclusion criteria are as follows: the age from 45 to 55 years, the history of vaccination to prevent COVID-19 (no later than 6 months before the inclusion in the study), a presentation of the first infected family member on the 1st–2nd days when clinical symptoms of acute respiratory infections appear, a confirmation of the COVID-19 diagnosis in a family member by an express diagnostic method and then PCR, a consent of the contact person to sample their material for research on the 1st day of the infected family member's presentation, a consent of the contact person to participate in the study within 14 days with signing of a voluntary informed consent. For the main group, additionally, there should be a consent to the administration of a drug based on RNA double-stranded sodium salt, for the control group – placebo (physiological saline (NaCl) 0.9% – 5 ml.).

Non-inclusion criteria

They are associated clinical conditions in history: an acute cerebrovascular accident, myocardial infarction, angina pectoris, coronary revascularization, renal failure, type 1 diabetes mellitus, autoimmune, allergic diseases, the use of glucocorticosteroids, antiviral drugs and/or immunomodulators within 30 days before the inclusion in the study, a refusal of a patient to a long-term participation in the study.

Exclusion Criteria

A decision to exclude a subject from the study was made by the investigator. The subject was withdrawn from the study immediately if any of the following situations occurred:

1. The occurrence during the study course of any diseases or conditions that worsen the prognosis of the subject, and make it impossible for the subject to continue their participation in the clinical study.

If a subject was diagnosed with COVID-19 based on the results of SARS-CoV-2 RNA analyzes by the nucleic acid amplification test (NAAT), selected both at the screening stage and after it, and there was no need for hospitalization of the subject, he was not excluded from the study, he continued observation. If a subject needed to be hospitalized during the study course, he was excluded from the study.

2. Taking drugs of prohibited therapy or the need to be prescribed them.

3. Pregnancy (for study participants).

4. An invalid inclusion of a subject that does not meet the inclusion criteria and/or meets the non-inclusion criteria.

5. Other violations of the Protocol, which, in the opinion of the investigator, are significant.

6. A subject's refusal to participate in the study.

7. Other administrative reasons.

Statistical analysis

A statistical analysis was carried out using StatTech v. 2.8.8 (LLC "Stattech", Russia) and StatSoft Statistica 13.5. Quantitative indicators were assessed for the compliance with the normal distribution using the Shapiro-Wilk test (the number of patients in the group is fewer than 50). The data are presented as median (Me) and limits of 95% confidence interval (95% CI) or lower and upper quartiles (Q1–Q3). A comparison of two groups in terms of the quantitative indicator, the distribution of which differed from the normal one, was performed using the Mann-Whitney U-test for unrelated samples and the Wilcoxon test for dependent samples. The level of statistical significance for small samples (in this case, the effect of drug therapy on the risk of developing the disease) was calculated using the Fisher test (p <0.05).

To assess the diagnostic significance of quantitative signs in predicting a certain outcome, the method of analyzing ROC curves was used. The separating value of a quantitative trait at the cut-off point (the cut-off point was determined by the highest value of the Youden index) was calculated based on the specificity and sensitivity using the following formula: J=Sensitivity+Specificity–1 (where: J is the Youden index). The higher the specificity and sensitivity were, the higher the values of the Youden index were.

RESULTS

The persons who had been in the family contact with COVID-19 patients but did not subsequently become ill, had higher levels of INFα in the blood serum (more than 2.5 pg/ml) and saliva (more than 28 pg/ml); IFNβ in the blood serum (more than 3 pg/ml) and saliva (more than 12 pg/ml) at the time of the 1st–2nd days of contact. According to the obtained data, the threshold value of the INFα level in saliva on the 1st–2nd days of contact,

which corresponded to the highest value of the Youden index (0.79), was 14.5 pg/ml, indicating an increased risk of developing COVID-19 with a lower content of cytokine in saliva (the area under the ROC-curve was 0.917±0.055; 95% CI=0.810–1.000). The resulting model was statistically significant (p=0.004) and the level of INFα in saliva in the first days of contact had predictive information about the risk of developing COVID-19 in the exposed persons. The prediction of IFNα levels in the blood of the exposed persons on days 1–2 with respect to the changes in the risk of developing COVID-19 in the exposed persons was not detected (the resulting model was not statistically significant, $p > 0.05$).

For INFβ, the predictive value in relation to reducing the risk of developing COVID-19 in the exposed persons was its blood level of more than 2.7 pg/ml on days 1–2 (the area under the ROC-curve was 0.89±0.23; 95%, CI=0.76–0.93 The resulting model was statistically significant (p=0.0034) and in saliva, there was 13.5 pg/ml (the area under the ROC curve was 0.80±0.09; 95% CI=0.62–0.98 The resulting model was statistically significant (p=0.038).

When comparing the dynamic characteristics of the interferons contents (in the range between the 1st–2nd days and the 3rd one of contact with an infected patient) in the persons who had not fallen ill during 14 days of contact, an increase in INFα in saliva by 60–70% on day 3 was determined; it was significantly higher (p <0.001) than in the group of patients subsequently (10–23%).

The dynamics of the INFα contents in the same time period (between the 1st–2nd days and the 3rd one of contact with an infected patient) in the blood was not detected (p >0.05). The threshold value of the percentage increase in the level of INFα in saliva on the 3rd day of contact at the cut-off point which corresponded to the highest value of the Youden index (0.8), was 42%, which determined a decrease in the incidence of an acute disease when the percentage was above 42. The area under the ROC curve was 0.95 (95% CI: 0.87–1.000). The resulting model was statistically significant (p=0.002).

The data of the dynamic growth of the INFβ level in saliva and blood demonstrate significant differences in the groups: thus, the exposed persons (without COVID-19 in the next 14 days of the observation) were characterized by a threefold increase in the content of INFβ (p <0.001) by the 2nd day of contact which remained in saliva, and by 6–7 days of contact, but had leveled in the blood by days 6–7 (Tables 1, 2). On day 3, the threshold value of the percentage increase in the level of INFβ in saliva of the exposed persons at the cut-off point, which corresponded to the highest value of the Youden index (0.77), was 61%, which determined the decrease in the incidence of the acute disease when the percentage reached more than 61. The area under the ROC – curve was 0.96 (95% CI was 0.82–1.97). The resulting model was statistically significant (p=0.004).

It is important to note that on days 1–2 of contact, the lowest values (p <0.001) of the INFα levels were observed in the group of the individuals with a waist circumference of more than 80/94 cm (women/men) (in the blood 1.43 pg/ml, 95% CI [1.27–2.39] pg/ml, in saliva 11.7 pg/ml 95% CI [8.99–12.4] pg/ml), with a waist circumference of less than 80/94 cm (women / men): in the blood 2.57 pg/ml, 95% CI [1.48–2.97] pg/ml, in saliva 18.2 pg/ml 95% CI [15.1–41.7] pg/mL, and by day 3 of contact, the percentage of the increase was less than 21% [6–21]%. In this group of the exposed persons, the incidence was 85% (16 out of 20 people). The incidence among the persons with a waist circumference of less than 80/94 cm (women/men) was 30% (3 out of 15 people).

An increase in the IFNα and INFβ contents in the saliva and blood of the persons who had been in contact with a novel coronavirus infection in the absence of a disease in the next 14 days, was not accompanied by an increase in pro-inflammatory IL-1β. That significantly differed from the group of patients with a developed disease within 14 days of contact (p <0.001), but occurred against the background of an increase in anti-inflammatory IL-10 in saliva by days 6–7 of contact (Tables 1, 2).

The administration of the drug based on RNA double-stranded sodium salt intramuscularly at the dose of 5 mg once on days 1-2 to the contact patients with clinical manifestations of COVID-19, determined a decrease in the incidence in the next 14 days when compared with the placebo group. In the Test drug group, no sick persons were identified out of 14 patients, in the placebo group, 5 out of 15 contact persons which accounted for 30%, fell ill (Table 3).

Based on the data obtained, when analyzing the effect of the drug on the risk of developing the disease, statistically significant differences were revealed (p=0.042).

Taking into account the objective of the study, aimed at identifying the potential for correcting the interferon response with the administration of the drug RNA double-stranded sodium salt, the dynamics of changes in the content of IFNα and INFβ, IL-1β and IL-10 in saliva and blood serum against the background of the use of the drug was analyzed. At the time of the administration of RNA double-stranded sodium salt or placebo, the patients were characterized by comparable levels of IFNα and INFβ in the blood serum and saliva (p >0.05; Tables 4, 5). According to the previously reported data, they corresponded to an equal number of patients with IFNα and INFβ levels in saliva, to increase the risk of developing COVID-19 through contact with a SARS-CoV-2 infected person. 1 day after the administration of the drug, in serum and saliva of IFNα and INFβ in the form of the most pronounced increase in INFβ (Tables 4, 5), a statistically significant difference was noted between the Radamin and placebo groups.

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Table 1 – Analysis of cytokine levels (pg/ml) in exposed persons blood on the novel coronavirus infection, depending on the development of the disease during the observation period

Indicators	Groups	Disease risk			*p*
		Me	Q_1–Q_3	*n*, persons	
INFα days 1–2 of contact	Not sick	2.61	2.23–3.01	32	0.030*
	Sick	2.15	1.67–2.29	35	
INFα day 3 of contact	Not sick	2.74	2.38–2.96	32	0.015*
	Sick	2.17	1.33–2.24	35	
INFα day 7 of contact	Not sick	2.74	2.33–3.62	32	0.001*
	Sick	2.08	1.64–2.35	35	
INFβ days 1–2 of contact	Not sick	3.51	3.12–4.92	32	0.019*
	Sick	1.82	1.08–2.84	35	
INFβ day 3 of contact	Not sick	13.3	6.17–15.8	32	<0.001*
	Sick	1.92	1.32–2.23	35	
INFβ day 7 of contact	Not sick	5.42	4.93–6.27	32	<0.001*
	Sick	2.35	1.28–3.17	35	
IL1β days 1–2 of contact	Not sick	4.51	4.26–5.39	32	0.204
	Sick	8.87	4.44–9.23	35	
IL1 β day 3 of contact	Not sick	5.09	4.51–7.10	35	0.015*
	Sick	21.5	14.50–24.78	32	
IL1 β day 7 of contact	Not sick	4.73	4.22–5.28	35	0.011*
	Sick	19.5	16.70–22.50	32	
IL-10 days 1–2 of contact	Not sick	9,72	9.05–10.39	35	0.06
	Sick	11.8	8.99–14.72	32	
IL-10 day 3 of contact	Not sick	10.6	8.96–11.30	35	0.07
	Sick	12.2	11.20–13.70	32	
IL-10 day 7 of contact	Not sick	10.1	9.43–11.27	35	0.394
	Sick	11.1	9.95–12.20	32	

Note: * – significant when comparing groups (“Sick” and “Not sick”) by the Mann-Whitney U-test.

Table 2 – Analysis of cytokines levels (pg/ml) in exposed persons’ saliva on the novel coronavirus infection, depending on the development of the disease during the observation period

Indicators	Groups	Cytokine level			*p*
		Me	Q_1–Q_3	*n*, persons	
INFα days 1–2 of contact	Not sick	38 I	28.7–42.1	32	0.003*
	Sick	12.7	9.13–14.5	35	
INFα day 3 of contact	Not sick	51.2	44.3–58.3	32	<0.001*
	Sick	14.5	8.23–18.7	35	
INFα day 7 of contact	Not sick	38.5	35.3–45.1	32	<0.001*
	Sick	15.6	7.91–19.5	35	
INFβ days 1–2 of contact	Not sick	17.23	9.54–22.7	32	0.035*
	Sick	4.34	3.48–4.92	35	
INFβ day 3 of contact	Not sick	51.8	47.5–60.4	32	<0.001*
	Sick	4.98	4.11–5.68	35	
INFβ day 7 of contact	Not sick	51.7	50.8–60.7	32	<0.001*
	Sick	18.7	17.7–24.1	35	
IL1 β days 1–2 of contact	Not sick	20.3	16.8–22.3	32	0.057
	Sick	29.50	26.50–31.6	35	
IL1 β day 3 of contact	Not sick	25.3	21.4–26.1	32	0.005*
	Sick	43.1	42.7–51.4	35	
IL1 β day 7 of contact	Not sick	19.5	18.4–20.1	32	<0.001*
	Sick	75.4	71.6–77.3	35	
IL-10 days 1–2 of contact	Not sick	26.8	23.4–33.5	32	0.63
	Sick	27.20	25.1–28.3	35	
IL-10 day 3 of contact	Not sick	27.12	21.7–32.5	32	0.75
	Sick	30.1	25.8–32.3	35	
IL-10 day 7 of contact	Not sick	43.7	40.6–60.5	32	0.019*
	Sick	31.8	27.6–35.1	35	

Note: * – significant when comparing groups (“Sick” and “Not sick”) by the Mann-Whitney U-test.

Table 3 – Analysis of the effect of therapy with drug based on RNA double-stranded sodium salt (compared with the placebo group) on the incidence of COVID-19 among the persons exposed to the novel coronavirus infection

Groups	Risk of disease		Risk of disease
	Not sick	Sick	
Radamin	14 (58.3)	0 (0.0)	0.042*
Placebo	10 (41.7)	5 (100.0)	

Note: * – differences in indicators are statistically significant (p <0.05; Fisher criterion).

Table 4 – Analysis of the cytokines levels (pg/ml) in the contact persons' saliva on the novel coronavirus infection, depending on the administration of drug based on RNA double-stranded sodium salt

Indicators	Groups	Levels			*p*
		Me	Q_1–Q_3	*n*, persons	
INFα before the administration	Radamin	33.5	11.8–41.7	14	0.760
	Placebo	35.4	12.1–40.3	15	
INFα in 1 day after the administration	Radamin	58.3	42.4–62.7	14	0.005*
	Placebo	41.4	11.7–48.3	15	
INFα in 3 days the administration	Radamin	41.7	34.5–49.1	14	0.026*
	Placebo	29.6	21.3–37.9	15	
INFβ before the administration	Radamin	8.30	3.65–18.5	14	0.247
	Placebo	16.6	8.04–19.9	15	
INFβ 1 day after the administration	Radamin	59.1	54.3–61.9	14	0.003*
	Placebo	52.7	7.26–54.1	15	
INF1b 3 days after the administration	Radamin	52.5	46.3–56.7	14	0.541
	Placebo	54.3	24.4–61.3	15	
IL1 β level before the administration)	Radamin	20 l	17.9–24.5	14	0.600
	Placebo	19.5	17.1–23.4	15	
IL1 β in 1 day after the administration	Radamin	24.6	22.4–28.8	14	0.965
	Placebo	23.7	22.2–35.4	15	
IL1 β in 3 days after the administration	Radamin	19.5	18.6–19.7	14	0.175
	Placebo	19.7	19.3–58.1	15	
IL-10 level before the administration	Radamin	27.6	24.5–33.3	14	0.585
	Placebo	27.2	24.7–32.6	15	
IL-10 in 1 day after the administration	Radamin	22.8	20.5–26.7	14	<0.001*
	Placebo	32.7	29.8–33.7	15	
IL-10 in 3 days after the administration	Radamin	41.2	39.1–43.5	14	0.064
	Placebo	62.4	38.3–68.1	15	
	Placebo	21.1	6.53–28.4	15	

Note: * – significant when comparing groups ("Sick" and "Not sick") by the Mann-Whitney U-test.

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Table 5 – Analysis of the cytokines levels (pg/ml) in the blood of contact persons for a new coronavirus infection, depending on the administration of drug RNA double-stranded sodium salt

Indicators	Groups	Levels			*p*
		Me	Q_1–Q_3	*n*, persons	
INFα before the administration	Radamin	2.31	2,09–2,49	14	0,810
	Placebo	2.21	2,12–2,57	15	
INFα in 1 day after the administration	Radamin	2.71	2,47–3,08	14	0,043*
	Placebo	2.31	2,00–2,63	15	
INFα in 3 days after the administration	Radamin	2.98	2,45–3,91	14	0,041*
	Placebo	2.45	2,13–2,68	15	
INFβ before the administration	Radamin	2.95	1,61–4,66	14	0,371
	Placebo	4.15	2,51–4,97	15	
INFβ 1 day after the administration	Radamin	14.6	13,81–15,53	14	<0,001*
	Placebo	4.20	3,16–5,24	15	
INF1b 3 days after the administration	Radamin	5.55	4,84–6,27	14	0,071
	Placebo	4.44	3,41–5,47	15	
IL1 β level before the administration	Radamin	4.51	4,30–7,49	14	0,965
	Placebo	5.19	4,23–7,39	15	
IL1 β in 1 day after the administration	Radamin	5.97	4,78–9,23	14	0,485
	Placebo	4.75	4,13–12,9	15	
IL1 β in 3 days after the administration	Radamin	4.95	4,59–5,55	14	0,913
	Placebo	4.77	4,27–11,8	15	
IL-10 level before administration	Radamin	9,99	9,51–11,1	9	0,619
	Placebo	9,67	9,23–10,7	12	
IL-10 in 1 day after the administration	Radamin	10.8	10,4–11,4	12	0,488
	Placebo	9,51	9,03–11,6	12	
IL-10 in 3 days after the administration	Radamin	10.3	9,9–11,5	12	0,382
	Placebo	9,84	9,21–11,6	14	

Note: * – significant when comparing groups (“Sick” and “Not sick”) by the Mann-Whitney U-test.

According to the previously described data related to the group with an extremely high risk of developing the disease, when comparing individual indicators of changes in the content of interferons in the blood serum and saliva of contact persons, it was found out that in the patients (6 out of 14) with IFNα levels in the blood of less than 2.5 pg/ml and INFβ less than 3 pg/ml, and saliva less than 28 pg/ml, with the of the drug RNA double-stranded sodium salt, an increase in the concentration of these interferons by 2.5–3 times per day after the administration was noted. At the same time, it was also noted that these patients reached the indicators of individuals with a low risk of developing an acute disease. The analyzed 6 patients of this group were also characterized by a waist circumference of more than 80/94 cm (women/men). This trend in the form of a significant increase in the contents of IFNα and INFβ in individuals with comparable concentrations of interferons and waist circumferences above the norm was not registered with the administration of placebo.

DISCUSSION

In most cases, an immune response against human coronaviruses (HCoV), including a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [26, 27], is characterized by low levels of types I and III interferons (IFNs). This factor leads to an increase in the viral load followed by a hyperimmune response. The significance of the local and systemic interferon response is a significant factor both in the initiation of COVID-19 in contact persons and in the formation of clinical course features [28, 29].

In the course of the present study, it was demonstrated that the content of INFα in saliva less than 28 pg/ml and IFNβ less than 12 pg/ml on the 1st–2nd days of contact predicts an increased risk of developing COVID-19 in the persons who have been in contact with a novel coronavirus infection. However, in the interval of 2–3 days of contact, the degree of increase in these immunoregulatory peptides is also important, since an increase in IFNα and IFNβ by 250% or more makes it possible to level the negative prognosis in this category of patients.

It should be noted that the study was conducted during the period of dominance in the circulation of the Omicron virus variant, which, according to researchers, changes the synthesis of interferons to a lesser extent [28]. However, individually low IFNα and INFβ concentrations in saliva and blood, being a pathophysiological feature of patients, are primarily, with a waist circumference of more than 80/94 cm (women/men) and acting as a risk factor for developing COVID-19 in this category of patients upon the contact with a SARS-CoV-2 infected person, regardless of the virus associated with the variant, are able to suppress the data synthesis of immunoregulatory peptides.

Due to the high contagiousness of the novel variants of Omicron (Centaur, Cerberus, Kraken, Arcturus), the medical community attaches great importance to the search for the drugs that can correct the insufficient

(slow) interferon response in the patient at the time of the infection. The administration of the drug based on RNA double-stranded sodium salt (RADAMIN®VIRO) once intramuscularly at the dose of 5 mg determined the modification of both local and systemic interferon responses without a hyperactivation of the pro-inflammatory cytokine IL-1β. The most significant result of the use of the drug is an increase in the content of IFNα and INFβ in individuals with initially low levels, both in the blood and saliva, which, according to their own data, refers these patients to an increased risk of developing COVID-19 when they have a close family contact with an infected patient. In more cases, these patients were characterized by an increase in the waist circumference of more than 80/94 cm (women/men), which allows us to put forward a hypothesis about the additional significance of correcting the local and systemic interferon response in overweight and obese individuals who are in contact with a novel coronavirus infection. The use of a drug based on sodium ribonucleinate, reduces the risk of initiation and a complicated course of this infection.

The data of the present study indicate that the administration of the drug based on RNA double-stranded sodium salt did not reveal an average increase in the levels of anti-inflammatory cytokine (IL-10) in contact persons in the blood serum and saliva, which may depend on the multimodal effects of the drug, as well as on the characteristics of the individual immune response. A patient's status is realized through a protective and blocking effect on the reproduction of the virus in the incubation period, thereby leveling the pathophysiological need for the growth of IL-10.

CONCLUSION

The differences in the interferon regulation of the immune response upon a contact with SARS-CoV-2 in the form of lower INF α and β levels, as well as a slightly pronounced growth dynamics in the interval of the first 3 days, affected the increased risk of developing COVID-19, which had a pathogenetic justification. A clinically significant hypothesis put forward in the work is the association of a waist circumference of more than 80/94 cm with baseline low levels of type 1 interferons in the blood and saliva, which indicates a greater importance of correcting interferon synthesis in this category of patients. Undoubtedly, given the cascade type of work of the cytokine system, the revealed differences in the concentrations of pro-inflammatory IL-1β and anti-inflammatory IL-10 in the individuals with the development of an infectious process are of the fundamental importance, but these differences are secondary and reflect the onset of the disease in one of the groups. The levels of IL-1β and IL-10 at the time of the infection did not show a predictor role.

It is important to note that the IFN-mediated antiviral response depends on the viral load, which is the sum of the infecting dose and the degree of the immune control of replication. Interferonogen preparations have the potential to correct interferon synthesis, which is pathogenetically significant in the initiation and development of SARS-CoV-2 infection.

Despite the inhibition of endogenous IFN production, the use of an interferon inducer like RNA double-stranded sodium salt may be the factor determining the control of SARS-CoV-2 replication, especially in the early stages of the disease. It is important to note the safety of the drug, because the endogenous control mechanisms of the interferons contents in the body are preserved not higher than protective concentrations.

Thus, drug based on RNA double-stranded sodium salt can be recommended as a means of post-exposure COVID-19 prophylaxis, both for medical institutions (outpatient clinics and hospitals), and for those caring for and/or in contact with COVID-19 patients (including labor collectives and places of people's mass gathering). The use of RADAMIN®VIRO 5 mg (1 vial) intramuscularly once for the purpose of COVID-19 post-exposure prophylaxis significantly reduces the risk of contracting this disease, and has also a significant impact on reducing the burden of coronavirus infection in the socio-economic aspect of the fight against the pandemic.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Olga A. Radaeva – research conducting, text writing and editing; Larisa A. Balykova – study design implementation, study data processing; Kira Ya. Zaslavskaya – research design development, text editing; Yuliya A. Kostina – study design implementation, study data processing; Nikolai A. Pyataev – research data processing; Natalya M. Selezneva – study design implementation, study data processing; Alena V. Klimova – study design implementation, study results processing; Irina Y. Chegodaeva – study design implementation study design implementation, study data processing; Ksenia N. Koryanova – study data processing; Alexey V. Taganov – text editing, research data processing Petr A. Bely – study design implementation, study data processing.

All authors confirm that their authorship complies with the international ICMJE criteria (all authors have made a significant contribution to the development of the concept, research and preparation of the article, read and approved the final version before the publication).

ОРИГИНАЛЬНАЯ СТАТЬЯ
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ОБЗОРЫ

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“Off-label” drugs: legal problems and socio-economic aspects of application practice

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The aim of the work was to analyze Russian and foreign experience in the regulation and application practice of “off-label” drugs in order to develop recommendations on the optimization of their application in clinical practice.

Material and methods. The analysis of scientific articles and legal documents of the Russian Federation and foreign countries published from 2011 to 2022 on the websites Consultant Plus, FDA, EMA, NCBI, e-library, as well as a qualitative sociological study conducted in May-August 2022 – 11 in-depth interviews with experts in the field of the healthcare system of the Russian Federation.

Results. The social and economic aspects have been considered and the list of legal problems in the application practice of “off-label” drugs has been disclosed. A state analysis of the regulatory and legal framework on the drugs application practice by healthcare professionals in the absence of registered indications for “off-label” drugs use has been presented. The use of an unregistered medicinal product in the territory of the Russian Federation in everyday medical practice has been considered. The analysis of the Russian and foreign experience in regulating the use of drugs in the absence of their registration in the country, as well as the absence of registration of some indications for their prescription in the instructions for the medical use of such drugs has been also carried out. The authors have formulated the key problems of the use of “off-label” drugs in clinical practice. Based on the results of the in-depth interviews, the recommendations of the expert community on the ways to optimize the use of “off-label” drugs have been identified and concretized.

Conclusion. The results of this study made it possible to formulate recommendations for expanding the ability of specialists to prescribe “off-label” drugs treatment while maintaining a proper degree of the state control over this process: a legislative consolidation of the regional health authorities’ obligations and responsibilities on the drug provision; creating an open and transparent system for the “off-label” drugs use by patients and their legal representatives, the mandatory full information of the patient about the fact of using the “off-label” drug, as well as the risk and nature of the development of possible adverse reactions. When prescribing these drugs, the patient safety should be the top priority.

Keywords: drugs; misuse; “off-label” prescriptions; regulation and practice of drug use; patient safety

Abbreviations: RLA – regulatory legal act; AR – adverse reaction; EMA – European Medicines Agency; GOLUP – Good Off-Label Use Practices; US FDA – United States Food and Drug Administration; VED – Vital and Essential Drugs; CTs – clinical trials; CMI – compulsory medical insurance; HTMC – high-tech medical care.

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Лекарственные препараты «off-label»: правовые проблемы и социально-экономические аспекты практики применения

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Цель. Проанализировать российский и зарубежный опыт регулирования и практики применения лекарственных препаратов (ЛП) «off-label» для выработки рекомендаций по способам оптимизации их использования в клинической практике.

Материал и методы. Анализ научных статей и нормативно-правовых документов Российской Федерации и зарубежных стран, опубликованных с 2011 по 2022 года на сайтах КонсультантПлюс, Food and Drug Administration, European Medicines Agency, National Center for Biotechnology Information, e-library, а также качественное социологическое исследование, проведённое в мае-августе 2022 года, а именно 11 глубинных интервью с экспертами в области системы здравоохранения Российской Федерации.

Результаты. Рассмотрены социально-экономические аспекты и раскрыт перечень правовых проблем практики применения ЛП «off-label» Представлен анализ состояния нормативно-правовой базы по вопросам использования ЛП специалистами здравоохранения в условиях отсутствия зарегистрированных показаний по применению лекарственного средства; применения незарегистрированного лекарственного средства на территории Российской Федерации в повседневной врачебной практике; анализ российского и зарубежного опыта регулирования применения ЛП в условиях отсутствия их регистрации в стране, а также отсутствия регистрации некоторых показаний к их назначению в инструкции по медицинскому применению лекарственного средства. Авторами были сформулированы ключевые проблемы применения ЛП «off-label» в клинической практике. По результатам глубинных интервью были выявлены и конкретизированы рекомендации экспертного сообщества по способам оптимизации применения ЛП «off-label».

Заключение. Результаты настоящего исследования стали основой для формулирования рекомендаций для расширения возможностей специалистов назначать лечение ЛП «off-label» при одновременном сохранении должной степени государственного контроля за данным процессом: законодательное закрепление обязательств и ответственности региональных органов управления здравоохранением по вопросам лекарственного обеспечения; создание открытой и прозрачной системы применения ЛП «off-label» для пациентов и/или их законных представителей, обязательное полноценное информирование пациента о факте применения ЛП «off-label», а также риске и характере развития возможных нежелательных реакций. Основным приоритетом при назначении таких препаратов должна выступать безопасность пациента.

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Ключевые слова: лекарственные препараты; неправильное применение; назначения «off-label»; регулирование и практика применения лекарственных препаратов; безопасность пациентов

Список сокращений: ЛП – лекарственные препараты; НПА – нормативный правовой акт; НР – нежелательная реакция; EMA – Европейское агентство лекарственных средств; GOLUP – Надлежащая практика использования лекарств не по прямому назначению; US FDA – Управление по санитарному надзору за качеством пищевых продуктов и медикаментов США; ЖНВЛП – жизненно необходимые и важнейшие лекарственные препараты; КИ – клинические исследования; ОМС – обязательное медицинское страхование; ВМП – высокотехнологичная медицинская помощь.

INTRODUCTION

The use of drugs for indications not specified in the instructions, including prescriptions for other diagnoses, changing the administration methods and dosages, has been an inevitable practice for quite a long period of time [1–4]. Prescribing and dispensing drugs outside the registered indications (informally "off-label" ones) still remains an urgent legal problem and a problem in the clinical practice, caused primarily by unmet clinical needs [5–8]. This is due to a number of reasons: the need, in some cases, to provide medical care to a patient in the absence of registered drugs [9, 10]; the reluctance of pharmaceutical companies to invest in clinical trials and registration of additional indications [11]; the doctor's lack of temporary (high workload), organizational and technical possibilities to clarify the indications for the use of a particular drug specified in the registration dossier [12], as well as a number of individual characteristics of the medical specialist and the patient (insufficient qualifications, ignorance of the responsibility for prescribing the "off-label", false beliefs) [13, 14]. In some cases, it is not one, but a combination of the above factors that leads to the incorrect use of drugs [15].

Although the effectiveness of the "off-label" drugs use has not been officially confirmed, their use in clinical practice can serve as a means of saving patients' lives with rare diseases, infancy or senile patients, pregnant and lactating women, as well as the patients in case of detection of new infectious diseases, for which the etiotropic therapy has not been developed yet (for example, COVID-19) [16].

One of the important problems of the "off-label" drugs use outside the registered indications is their "confusion" in terminology. According to the definition given by the Food and Drug Administration of the United States of America (Food and Drug Administration, US FDA), an "off-label" prescription is the use of a medical device or drug for the indications not approved in the instructions, in a different dosage form or regimen, as well as in the populations not specified in the instructions [17]. At the same time, in regulatory legal acts and publications regarding the use of "off-label" drugs, there is such a formulation of this phenomenon as the prescription of the drug for "the indications that are different from the indications for the use contained in the instructions", i.e., "the incorrect use", which significantly narrows the concept [18].

The cases in which the use of drugs occurs "off-label", include the following ones: the use of the drugs not registered in the country; prescribing a drug contraindicated in this pathology; prescribing a drug for unregistered indications; prescribing a drug to the populations not specified in the instructions (pregnant women) or age groups (children, the elderly); a simultaneous prescribtion of adverse drugs combinations; the use of drugs in violation of the methods of application (multiplicity, dosing, route of administration, duration of treatment) [17–19].

Any practicing physician, regardless of their specialization, repeatedly resorts to "off-label" prescriptions [20]. The main groups of patients, among which "off-label" drugs are most widespread, are children, pregnant women, elderly and senile patients, patients with oncological and orphan diseases, patients receiving palliative care, psychiatric patients [18, 21–23].

In these groups, conducting clinical trials (CTs) necessary for the inclusion of the relevant indications in the instructions for the use of medicinal products is usually difficult, and in some cases – completely impossible for ethical reasons. These are the main reasons for the impossibility of including a number of drugs in the list of "Vital and Essential Drugs" (VED), clinical guidelines and standards of medical care. As a result, the use of "off-label" drugs cannot be paid for by the compulsory medical insurance (CMI) system or budgetary funds as parts of a high-tech medical care (HTMC) provision.

THE AIM of the work was to analyze Russian and foreign experience in the regulation and application practice of "off-label" drugs in order to develop recommendations on the optimization of their application in clinical practice.

Research objectives:

1. Assess the prevalence of "off-label" drug use in the international clinical practice.
2. Analyze the regulatory and legal framework on the drugs application practice in the Russian Federation and abroad.
3. Find out the experts' opinion on the use of "off-label" drugs.

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MATERIALS AND METHODS

The main methods used in this work are the analysis of domestic and foreign scientific articles on the regulation of circulation and practical application of the "off-label" drugs, published from 2011 to 2022 on the websites of Consultant Plus, FDA, EMA, NCBI, e-library, and regulatory legal documents of the Russian Federation and foreign countries; qualitative sociological research using the methodology of in-depth interviews.

In-depth interviews[1] with experts in the field of the healthcare system of the Russian Federation citizens were conducted in May-August 2022. The in-depth non-formalized interview questionnaire was a list of topics to be clarified. The topics were formulated in both narrative and interrogative forms. These grammatical forms were interchangeable, with the aim of choosing any of them, or a combination of them, by researchers to their liking. Depending on the type of a semantic connection, the topics of the questionnaire were divided into narratives, descriptions and reasoning. Compared to other qualitative research methods, this method provided in-depth information due to the fact that the entire time duration of the topic under study discussion is focused on the interviews with one participant. It should be noted that the absence of other participants' influence makes it possible to uniquely identify the author of the answer and compare the results obtained with the respondent's characteristics.

11 specialists of various profiles took part in this study as experts: a representative of the State Duma of the Federal Assembly of the Russian Federation (1 expert); the Regional Children's Clinical Hospital of the National Medical Research Center for Radiology (1 expert); Institute of Management and Translational Medicine of Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology (2 experts); Hematological Research Center (2 people); Institute of Hematology, Immunology and Cell Technologies of Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology (2 experts); Blokhin National Medical Research Center of Oncology (3 experts); pharmaceutical concern Bayer JSC (1 expert); vice president of the Society of Evidence-Based Medicine Specialists (osdm.org) (1 expert). The average age of the respondents was 45±5 years. For all the respondents, the same conditions had been created for the place and time of the interview. The interviewees were also informed and, in turn, agreed to publish the results of the survey.

[1] An in-depth interview is unstructured, direct, personal interview, single respondent probed by highly skilled interviewer to uncover underlying motivations, beliefs, attitudes, and feelings on a topic.

RESULTS AND DISCUSSION

Use of "off-label" drugs in international clinical practice

The phenomenon of using "off-label" drugs has been studied by a number of domestic and foreign researchers [24–27]. According to Kuznetsova E.Yu. et al. (2020), more than 62% of doctors noted the need to prescribe "off-label" drugs in their clinical practice [27]. At the same time, experts indicate the lack of alternative treatment options and the need to provide palliative care to seriously ill patients as the main reasons for the use of drugs in violation of the instructions [27, 28].

Most often, this practice is found in oncology, hematology, and pediatrics [29–31]. A systematic review published in 2017 summarized the results of 23 studies published between 1975 and 2016. The frequency of "off-label" drugs use in oncology was 6–82%, depending on the type of tumor, stage, drug, and age of the patient. It has been shown that "off-label" drugs are equally often prescribed for both outpatient and inpatient treatment. The main reasons for the use of "off-label" drugs were the absence of indications for the use of drugs in a certain type of tumor (9–46%), as well as a change in the regimen of the drugs use (10–40%). "Off-label" drugs in oncology are used most often as a part of palliative care (34–76%), less often for curative (10–41%) or adjuvant therapy (8.5–49%) [32].

Another clinical specialty in which "off-label" drug prescriptions are widespread is pediatrics [33-35]. The frequency of the "off-label" drugs use outside the registered indications in this area reaches 87.7% [36].

According to Pryadkina E.A. et al. (2018), when analyzing the frequency and structure of "off-label" drugs prescriptions indicated in the medical records of the Department of Pediatric Day Hospital for Hematology and Oncology, 69% of case histories contained 93 "off-label" drugs prescriptions, among which 39 prescriptions did not match instructions on the recommended age of the patient, 14 – by the dose of the drug, 39 – according to the registered use indications, 1 – according to the drug dosage form. The main groups of "off-label" drugs were immunosuppressants (29%); interferons (15%); complexing agents (12%). In 68% of cases, "off-label" drugs were prescribed for the treatment of the underlying disease, in 32% – for the concomitant pathology [37].

Such a high prevalence of "off-label" prescriptions is due to the laboriousness and cost of conducting large clinical trials in a relatively small population of patients, and the lack of economic incentives for such studies. As a result, in the instructions for the use of drugs, the aspects related to the possibilities and features of their use in children are the least developed. Among the registered

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drugs, only 33% are approved for use in children: 23% – in an early childhood (1–3 years old), 9% – in infancy (up to 1 year old). At the same time, it is known about an increased risk of developing toxic effects on the drugs in children compared with the adult population, as well as the presence of specific adverse reactions (ARs) [18, 38, 39]. The frequency of complications in the use of "off-label" drugs in pediatrics ranges from 23 to 60%, and the relative risk of their occurrence is 4.43 times higher than in cases of their use according to indications [33, 36].

The study by Matveev A.V. et al. (2018) showed that 47.7% of cases of the ARs development in the children of the Republic of Crimea in the period from 2011 to 2016 were associated with the prescription of "off-label" drugs. While 36.1% of the identified episodes were classified as a drug use for unregistered indications, 31% occurred with a frequency violation of the administration and the administration timing; in 20.7%, contraindications for use were not taken into account; 19.4% were characterized by a dosing violation; 18.7% – by an unspecified route of administration; in 2.1%, there was ignoring of an allergy history. The majority (52.7%) of the "off-label" drugs prescriptions were for the patients aged 29 days to 1 year [40].

The uncontrolled use of "off-label" drugs can cause negative consequences for both doctors and patients who have turned to them [27, 41]. It should be noted that once a drug meets the safety and efficacy standards required to obtain the FDA approval, it might be used for new indications and/or in the populations other than those for whom it had been approved. An example of such a use of "off-label" drugs is a therapy of a new coronavirus infection specified in the clinical recommendations of various countries. At the beginning of the pandemic, for the treatment of COVID-19, medical specialists used various drugs registered for the treatment of a similar infectious pathology [42, 43]. At present, 16 versions of interim guidelines for the treatment of coronavirus infection have already been formed. However, during the pandemic, an active search was made for effective treatment regimens using "off-label" drugs, since in the initial period of the pandemic there were no drugs which indications for use would include the infection caused by SARS-CoV-2 strains [27, 42].

The use of "off-label" drugs has also a negative experience. Thus, one example of the development of severe ARs with an uncontrolled prescription of drugs is the case of the blindness development caused by using the antitumor agent bevacizumab in Romania. The doctor who had prescribed the drug was accused of negligence [44]. At the same time, the relevant ministry made a statement that in cases of using an "off-label" drug, the healthcare professionals involved in the process of prescribing and using it, bear a full responsibility[2].

In Russia, there were also several high-profile stories associated with harm to the life and health of patients as a result of the use of "off-label" drugs [45]. One of them is associated with the prescription of misoprostol in the Republic of Dagestan in 2016. According to the official instructions, this drug is a treatment for gastric and duodenal ulcers, but it was prescribed to a patient for the purpose of terminating a pregnancy, which led to the woman's death. The relatives of the deceased patient filed a lawsuit for damages. During the proceedings, it was established that the cause of death was the use of this "off-label" drug, the claim of the plaintiffs was satisfied, and the medical organization was brought to a civil liability[3].

In 2014, for the use of "off-label" ceftriaxone when diluted with an incorrect concentration of a lidocaine solution (10% instead of 1%) and the patient's death associated with this, an Ulan-Ude paramedic was sentenced to imprisonment under Part 2 of Article 109 "Causing death by negligence due to improper performance by a person of his professional duties" of the Criminal Code of the Russian Federation"[4].

The described cases indicate the need to develop and introduce additional measures of detailed legal regulation into wide practice, and control over the use of "off-label" drugs.

Legal regulation of "off-label" medicinal products use in the Russian Federation and abroad

A legal regulation of prescribing and use of "off-label" drugs in the Russian Federation today is very ambiguous. The possibility of using a drug in this variant is rather superficially indicated in paragraph 15 of Art. 37 "Organization of delivery of health care" of Federal Law No. 323-FZ "On health protection of citizens in the Russian Federation". According to this law, "the prescription and use of medicines, medical devices and specialized medical food products that are not included in the relevant standard of medical care or not provided for by the relevant clinical recommendation, are allowed if there are medical indications (individual intolerance, according to vital indications) by decision of the medical commission." Herewith, it is emphasized that "the effect of this requirement may be changed in relation to

[2] Press Release of ANMDM Regarding the Off-Label Use of AVASTIN [Comunicat de Presa al ANMDM Referitor la Utilizarea off_Label a AVASTIN (bevacizumab)]. Available from: https://www.anm.ro/anunt-important-11-03-2011/. Romanian

[3] The decision on the case on compensation for moral damage in connection with the infliction of harm to life and health. Available from: https://sudact.ru/regular/doc/6vIcquF4dy1e/. Russian

[4] The decision on the case of causing death by negligence due to improper performance by a person of his professional duties. Available from: http://oktiabrsky.bur.sudrf.ru/modules.php?name=press_dep&op=1&did=135. Russian

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medical organizations of the private healthcare system – participants in the experimental legal regime in the field of digital innovation in accordance with the program of the experimental legal regime in the field of digital innovations"[5].

At the same time, according to the order of the Ministry of Health of Russia dated December 2, 2013 No. 886n, the composition and procedure for the work of the medical commission is determined by the head of the medical organization. The medical commission should include a chairperson, one or two deputy chairpersons, a secretary and members of the commission (heads of the departments, medical specialists)[6].

The procedure for prescribing a drug is indirectly mentioned in a number of regulatory legal acts, but it has been clearly stated nowhere about the permission or prohibition of the use of an "off-label" drug. One of these documents that previously regulated the use of drugs was the Procedure for prescribing drugs (Appendix No. 1 to the order of the Ministry of Health of the Russian Federation dated December 20, 2012, No. 1175n)[7]. In paragraph 6.1 of this document, it was said that medical specialists were prohibited from prescribing drugs in the absence of medical indications, but it was not indicated that these should be precisely the indications prescribed in the instructions for the drug. At the same time, in 2017, there was a public discussion of the Draft Order of the Ministry of Health, amending the specified procedure, according to which, by decision of the medical commission, in certain cases it was proposed to allow the use of the drug "for health reasons other than the indications for the use contained in the instruction." The document caused a wide public outcry, but these changes to the Procedure were not made. The current document Appendix N 1 to the Order of the Ministry of Health of the Russian Federation dated November 24, 2021 No. 1094n "On Approval of the Procedure for Prescribing Medicines" in Art. 1, Paragraph 7 states that "Medical specialists are prohibited from issuing prescriptions if a patient has no medical indications, and from unregistered medicinal products[8]. However, in the same regulatory legal act (RLA) in Art. 1, paragraph 5 refers to the possibility of prescribing a drug that is not included in the standards of medical care through a medical commission with reference to Art. 37, paragraph 15 of the Federal Law No. 323-FZ "On health protection of citizens in the Russian Federation". The decision of the medical commission of a medical organization is recorded in the patient's medical records and in the journal of the medical commission.

On June 29, 2022, amendments to Federal Law No. 323-FZ "On health protection of citizens in the Russian Federation" came into force. They allow the use of medicines outside the instructions ("off-label") for the treatment of the minors with certain diseases or conditions. Their list was approved by the Decree of the Government of the Russian Federation No. 1180n-r dated May 16, 2022 ("Check-list of diseases in which the use of "off-label" medicines is possible)"[9]. This list includes neoplasms, diseases of blood, respiratory organs, endocrine and nervous systems, mental disorders, etc. The codes of the International Classification of Diseases (the 10th revision, ICD-1), conditions and groups of conditions for pregnancy, childbirth, a postpartum period have also been inserted.

An important aspect in prescribing "off-label" drugs is informing a patient (a minor, one of their parents (a legal representative)) by the attending physician about the use of the "off-label" drug. According to Art. 20 of the Federal Law No. 323-FZ "On health protection of citizens in the Russian Federation", the patient must be informed about the safety of the drug, the expected result, the degree of risk and the actions in case of unforeseen effects.

In accordance with Art. 37, paragraph 14.1 of Federal Law No. 323-FZ "On health protection of citizens in the Russian Federation", in the amendment dated July 13, 2022, there is also a permission to include an "off-label" drug in the clinical recommendations and standards of medical care for children. In the instructions (in the section of dosages and methods of application), this category of the population is absent. It is important to note that these amendments apply to the medicinal products registered in the Russian Federation, to the diseases and conditions included in the "Check-list of

[5] Federal Law of November 21, 2011 No. 323-FZ (as amended on March 26, 2022) "On the fundamentals of protecting the health of citizens in the Russian Federation" (as amended and supplemented, effective from April 10, 2022) Available from: https://www.consultant.ru/document/cons_doc_LAW_121895/f7964563436c4bb0aed73d388df1a95d9103b632/. Russian

[6] Order of the Ministry of Health of the Russian Federation of December 2, 2013 No. 886n "On Amendments to the Procedure for the Creation and Activities of the Medical Commission of a Medical Organization, approved by Order of the Ministry of Health and Social Development of the Russian Federation of May 5, 2012 No. 502n, and to the Procedure for Appointment and Discharge medicinal products, approved by the order of the Ministry of Health of the Russian Federation dated December 20, 2012 No. 1175n" (as amended). Available from: https://base.garant.ru/70551698/. Russian

[7] The procedure for prescribing and prescribing drugs. Appendix No. 1 to the order of the Ministry of Health of the Russian Federation of December 20, 2012 No. 1175n. Available from: http://base.garant.ru/70404898/53f89421bbdaf741eb2d1ecc4ddb4c33/#ixzz5lCKu1Xnz. Russian

[8] Order of the Ministry of Health of Russia dated November 24, 2021 No. 1094n "On approval of the Procedure for prescribing medicines, forms of prescription forms for medicines, the Procedure for issuing these forms, their accounting and storage, forms of prescription forms containing the prescription of narcotic drugs or psychotropic substances, the Procedure for their manufacture, distribution, registration, accounting and storage, as well as the Rules for issuing prescription forms, including in the form of electronic documents" Available from: http://www.consultant.ru/document/cons_doc_LAW_401865/. Russian

[9] Ibid.

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diseases in which the use of "off-label" medicines is possible)".

In Paragraphs 1 and 2, Art. 67 of Federal Law No. 61-FZ "On the Circulation of Medicines" dated April 12, 2010 with the amendment dated July 14, 2022, it has been pointed out that information about a drug may be contained in specialized publications, instructions for the use of the drug, in scientific publications, etc., but it is has not been regulated how a doctor can use the information obtained from various sources[10]. Separately, it has been emphasized that promotional materials about an over-the-counter drug must comply with the instructions for the use of the drug [46].

According to the Order of the Ministry of Health of the Russian Federation No. 203N "On Approving Quality Criteria for Evaluating Medical Care" dated May 10, 2017, the prescription of the drugs for medical use should be carried out taking into account the instructions for the use of the medicinal product[11]. However, there is no information on the rigidity of the instruction to be taken into account. The same Order establishes the mandatory entry into the outpatient card when prescribing drugs for a medical use and using medical devices by decision of the medical commission of the medical organization.

At the same time, one should not forget about situations of a criminal liability when prescribing a drug. In case of a serious injury to the health or death of the patient, a doctor's actions may fall under the provisions of Art. 109 "Causing death by negligence due to improper performance by a person of his professional duties", Art. 118 "Causing serious harm to health through negligence due to improper performance by a person of his professional duties", or Art. 238 "Provision of services that do not meet safety requirements" of the Criminal Code of the Russian Federation. In this case, the exceptions are adverse outcomes in conditions of an extreme necessity and/or a reasonable risk. It should be understood that, according to Art. 39 of the Criminal Code of the Russian Federation, a situation of an extreme necessity is the presence of a danger "directly threatening the personality and rights of this or other persons, the legally protected interests of the society or the state, if this danger could be not eliminated by other means and, at the same time, not exceeding the limits of the extreme necessity[12]. At the same time, according to Art. 41 of the Criminal Code, a risk is justified in a situation where the set goal cannot be achieved by other actions or inaction that are not related to the risk, and the subject that allowed the risk has taken sufficient measures to prevent the development of undesirable side effects.

The use of an "off-label" drug may also entail disciplinary (in case of non-compliance with official duties or a violation of the requirements of the Labor Code of the Russian Federation) or a civil (for a medical organization) liability [47].

In the article by Gabay P.G. and Bagmet N.A. (2017), indicate the possibility of exempting a medical organization from compensating for harm to a patient's health in the event that an "off-label" drug was prescribed to eliminate the danger to life or health in the absence of other possible methods and when signing an informed voluntary consent before prescribing the drug. However, it is emphasized that this possibility may not be always realized [48].

The problem of regulating the use of "off-label" drugs in the United States and European countries has a longer history. In the United States, an "off-label" prescription is considered acceptable after the drug has been registered with the FDA at the discretion of the attending physician in case of the patient's informed consent [21]. In Germany in 2007, the following conditions were developed for the use of drugs outside the indications: a patient has a life-threatening pathology or disease that significantly reduces his quality of life; lack of clinically proven specific drugs for the treatment of this disease; availability of clinical trials demonstrating the effectiveness of "off-label" drugs in similar clinical situations; making a decision on prescribing a drug by an expert commission [18, 49].

Working at the principles of regulating the use of medicines outside the instructions took place at the pan-European level, resulting in a proposal for good practice for the use of drugs outside the instructions (Good Off-Label Use Practices, GOLUP), formulated in the EMA Declaration (European Medicines Agency, European Medicines Agency), according to which the use of the "off-label" medicinal product should take place only if all the following conditions are met:

1. The presence of a serious illness that endangers vital functions, or life-threatening.
2. The absence of a drug approved for use or a recurring adverse outcome of the use of the existing drugs.
3. The lack of alternative treatments prescribed for a particular condition.
4. The availability of the data confirming the effectiveness of the use of this "off-label" drug.
5. The availability of an informed voluntary consent of the patient to the use of an "off-label" drugs.

[10] Federal Law "On the Circulation of Medicines" dated April 12, 2010 No. 61-FZ, as amended. dated July 14, 2022. Available from: http://www.consultant.ru/document/cons_doc_LAW_99350/7072622692d160a804b7ba320189a2338752ee81/. Russian

[11] Order of the Ministry of Health of the Russian Federation of May 10, 2017 No. 203N "On approval of quality criteria for assessing medical care". Available from: https://normativ.kontur.ru/document?moduleId=1&documentId=293575. Russian

[12] Criminal Code of the Russian Federation of June 13, 1996 No. 63-FZ (as amended on March 25, 2022). Available from: http://www.consultant.ru/document/cons_doc_LAW_10699/.

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6. The availability of the established methods for reporting on the adverse reactions associated with the use not provided for by the instructions [46, 50].

It should be noted that this Declaration in the EU does not have the status of a regulatory legal act; the issue of introducing documents regulating the use of "off-label" medicinal products is currently being resolved. At the same time, in European countries, there are separate regulatory documents, such as temporary recommendations on the use of drugs, the measures to regulate reimbursement [19].

Opinions and recommendations of experts on the practice of using "off-label" drugs based on the results of in-depth interviews

The majority of experts (8 out of 11 respondents) assessed the fact that specialists of subdivisions prescribed "off-label" drugs as a routine practice. At the same time, unregistered drugs in Russia are not used by doctors. However, if necessary, their use is possible only by submitting an application to the Ministry of Health under expanded access programs.

Experts noted oncological and hematological diseases as the indications requiring the most frequent use of "off-label" drugs. At the same time, in ARVI, "off-label" drugs are prescribed to adults a little, and to almost every one of the children.

Most experts (9 out of 11 respondents) specified that, as a rule, "off-label" drugs are used for underlying medical conditions, and not for the treatment of their complications or a concomitant pathology. According to experts, the main goal of such prescriptions is to increase the possibility of recovery of a patient suffering from a potentially fatal disease. Usually, "off-label" prescriptions are made in the situations where there are no available registered drugs for the treatment of a particular group of patients, or the possibilities of therapy according to the instructions have been exhausted.

The minority of experts (2 out of 11 respondents) noted that there are cases of non-payment for an "off-label" drug prescription or fines from insurance companies, indicating that there are fines, and the frequency of their imposition is not known. The main reason for such sanctions is the quality of the consultations design (for example, if an alternative regimen for taking the drug has not been indicated).

It is important to mention that the experts mention the main organizational shortcomings of the "off-label" prescription system:

- "repeated consultations are required; for example, to reduce the doses of drugs, the consultation is paid again. There are frequent refusals to implement the recommendations of the federal center at the place of residence";
- "there is a need to create conditions in Russia under which the registration of a Russian drug in the EU and the USA would be as close as possible in terms of the time of registration in our country and the countries of the Eurasian Economic Union (EAEU)";
- "difficulties in registering new indications, the lack of activity of the manufacturer to correct indications in a registered drug that has been used for a long time";
- "a low activity of pharmacological companies in relation to informing about the efficacy and safety of the "off-label" drug use in the treatment of narrow groups of patients, including children. To increase the activity of drug manufacturers, incentives are necessary, for example, tax benefits";
- "an unequal territorial distribution of reference centers (obtaining a second opinion – technical capabilities and competencies) and interregional centers for pediatric oncology and hematology. There are large centers only in Moscow and St. Petersburg, as a result, they are overloaded with patients";
- "imposition of fines by medical insurance organizations for the use of "off-label" drugs where it is justified; "off-label" drugs are not accepted for payment by insurance companies; there is a low level of funding for clinical and statistical groups".

The proposals of experts on the ways of optimizing the use of "off-label" drugs in clinical practice are of serious interest. They include:

- the development of ethical standards for prescribing "off-label" drugs;
- the development of directly effective laws that do not limit the doctors opportunities to prescribe drugs outside the indications specified in the instructions;
- the entry of the regions' obligations on the issues of the drug provision into the National Project "Healthcare"[13];
- the simplification of the system for entering new indications for prescribing drugs into the existing instructions for medical use;
- recommendations on the creation of a list of medicines used "off-label", having a clinical evidence base in order to ensure an unhindered use and payment in the CHI and HTMC systems. Let the doctor justify the prescription of the drug. But it is necessary to systematically analyze the work of doctors for the validity of the "off-label" drug use for a particular disease.

Thus, according to the results of the in-depth

[13] National projects "Healthcare" and "Demography". – [Electronic resource]. - Access mode: https://minzdrav.gov.ru/poleznye-resursy/natsproektzdravoohranenie

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interviews, the experts confirmed the fact that doctors regularly prescribe “off-label” drugs, and there is a need to improve the existing rules regarding their prescription. Practicing experts noted that there are still some opportunities to justify the prescription of such drugs to a patient in the current legislation – the prescription of “off-label” drugs for health reasons through a medical commission, through clinical recommendations and standards of medical care approved by the Ministry of Health. The purpose of prescribing drugs “off-label” is to save the patient’s life.

According to the opinions of the interviewed experts, in resolving conflicts in legislation and medical practice in matters of “off-label” prescription of drugs, an organizational solution can be a simplification of the system for introducing new indications for drugs into the existing instructions for medical use.

CONCLUSION

Based on the analysis of literary sources, regulatory legal documents, as well as a qualitative study conducted by the authors, it can be concluded that the use of “off-label” drugs is inevitable in almost any medical practice, and all participants in the treatment process should be aware of it.

The main priority in the use of “off-label” drugs should be a patient safety. In this regard, the obligatory conditions for prescribing “off-label” therapy should be the following: the absence the application of the decision on the appointment of “off-label” drugs only within the framework of specialized medical institutions; obtaining voluntary informed consent of a patient; obligatory compliance with the reporting requirements for cases of development of adverse events.

Both in the Russian Federation and in other countries, the legal regulation of the prescription of “off-label” drugs is ambiguous. In order to expand the ability of specialists to prescribe “off-label” drugs while maintaining a proper degree of a state control over this process, the following organizational measures can be applied: legislative consolidation of the obligations and responsibilities of regional health authorities on drug provision; creation of an open and transparent system for the use of “off-label” drugs for patients and/or their legal representatives, the mandatory full information of the patient about the fact of the use of the “off-label” drug, as well as the risk and nature of the development of possible adverse reactions; creation of a list of “off-label” medicines that have a clinical evidence base in order to ensure an unhindered use and payment in the CHI and HTMC systems; a simplification of the procedure for making changes to the registration certificate for “off-label” drugs, the effectiveness of which has been demonstrated in high-quality clinical trials.

Despite the current lack of regulations allowing or prohibiting the use of “off-label” drugs in the Russian Federation, under certain circumstances, medical specialists and/or organizations may be subject to disciplinary, administrative or criminal liability for prescribing “off-label” drugs. This fact translates the issue considered in this study from the medical plane into a wide area of related disciplines.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

Sergey V. Russkikh – research design development, data collection, analysis and interpretation, article editing; Elena A. Tarasenko – scientific advice, analysis and data interpretation, article writing and editing; Lyudmila I. Moskvicheva – data collection, article writing and editing; Sergey A. Orlov – scientific consulting, article editing; Aleksey A. Tryakin – scientific advice; Anna V. Vorobieva – data collection, analysis and interpretation, article writing; Olga A. Beneslavskaya, Veronika I. Makarova, Ekaterina A. Putilina, Sergey A. Utkin – data collection, article editing. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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ОБЗОРЫ

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New role of extemporaneous manufacturing in regulating drug products access onto the market

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The aim of the work was to study the legal aspects of the legislative regulation for manufacturing medicines in a pharmacy organization.

Materials and methods. Databases of ConsultantPlus, Cyberleninka, Food and Drug Administration (FDA), European Medicines Agency (EMA), National Center for Biotechnology Information (NCBI), PubMed, e-library, WIPO Lex were used as search sources. The search was based on the following keywords and phrases: intellectual property, pharmacies+invention, patent, drugs, extemporal+production, orphan+diseases, as well as their Russian counterparts. 133 sources of information, including scientific articles and regulations, were found out; 50 have been included in this review. The analysis of information sources published from 2013 to 2023, was determined by the peculiarities of legislation changes in this area.

Results. The article provided an overview of modern, including regulatory practice, pharmaceutical manufacturing in the Russian Federation, and also analyzed the benefits of this activity for the medical community, patients and the state. At the same time, the individualization of drug treatment has made it possible to work out systemic solutions for developing drug therapy methods for special groups of patients for whom the economic feasibility of a pharmaceutical registration and launching such drugs onto the market has been brought into challenge. In addition, pharmacy manufacturing is an accessible tool in the study of the drugs prescribed by a doctor not in accordance with the instructions for medical use (off-label) or in the dosage forms/dosages that are not on the market. Extemporaneous manufacturing can be also a part of the process of "repositioning" drugs on the market, subject to compliance with the requirements for pharmacy manufacturing and control of the prescribed drugs safety. The possibility of pharmaceutic drug manufacturing also makes it possible to partially resolve issues related to intellectual property. As a result of the carried out analysis, the following hypothesis was confirmed: the legislative changes have a similar legal assessment both in Russia and abroad and correspond to the legal practice in resolving intellectual property issues in relation to pharmacy organizations.

Conclusion. The renewal of a pharmacy production will improve the availability of the drug care to the population, taking into account individual dosages and dosage forms in various therapeutic areas, and can also become a tool for repositioning drugs or clinical testing of new molecules for rare incurable diseases.

Keywords: drugs; pharmacy organizations; extemporaneous production; orphan diseases; patent; invention; intellectual property

Abbreviations: DF - dosage form; SRMRs – State Register of Medicinal Remedies; CCRF – Civil Code of the Russian Federation; EPC – Eurasian Patent Convention; WIPO – World Intellectual Property Organization; DPA – Dutch Patent Act.

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Новая роль экстемпорального изготовления в регулировании доступа лекарственных препаратов на рынок

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Цель. Изучение юридических аспектов законодательного регулирования по изготовлению лекарственных препаратов в аптечной организации.

Материалы и методы. В качестве поисковых источников использовали базы данных КонсультантПлюс, Киберленинка, Food and Drug Administration (FDA), European Medicines Agency (EMA), National Center for Biotechnology Information (NCBI), PubMed, e-library, WIPO Lex. Поиск проводился по следующим ключевым словам и словосочетаниям: intellectual property, pharmacies+invention, patent, medicinal+preparations, extemporal+production, orphan+diseases, а также по их русскоязычным аналогам. Были найдены 133 источника информации, включавшие научные статьи и нормативно-правовые акты; 50 включены в настоящий обзор. Анализ источников информации, опубликованных с 2013 по 2023 гг., определяли особенностями изменения законодательства в указанной сфере.

Результаты. В статье был приведён обзор современной, в т.ч. нормативной практики, аптечного изготовления в Российской Федерации, а также проанализированы преимущества указанной деятельности для медицинского сообщества, пациентов и государства. Одновременно индивидуализация медикаментозного лечения позволила выработать системные решения для отработки методик лекарственной терапии для особых групп пациентов, для которых экономическая целесообразность регистрации и вывода лекарственных препаратов на рынок поставлена под сомнение. Кроме того, аптечное изготовление является доступным инструментом в изучении лекарственных препаратов, назначенных врачом не в соответствии с инструкцией по медицинскому применению («off-label») или в лекарственных формах/дозировках, отсутствующих на рынке. Экстемпоральное изготовление также может быть частью процесса «репозиционирования» лекарственных препаратов на рынке при условии соблюдения требований к аптечному изготовлению и контроля безопасности назначаемых средств. Также возможность аптечного изготовления лекарственных препаратов позволит частично разрешить вопросы, связанные с интеллектуальной собственностью. В результате поведённого анализа была подтверждена гипотеза о том, что законодательные изменения имеют схожую правовую оценку как в России, так и за рубежом и соответствуют правовой практике при разрешении вопросов интеллектуальной собственности применительно к аптечным организациям.

Заключение. Возрождение аптечного производства позволит улучшить доступность лекарственной помощи населению с учётом индивидуальных дозировок и лекарственных форм в различных терапевтических направлениях, а также может стать инструментом «репозиционирования» лекарственных препаратов или клинической апробации новых молекул для редких неизлечимых заболеваний.

Ключевые слова: лекарственные препараты; аптечные организации; экстемпоральное изготовление; орфанные заболевания; патент; изобретение; интеллектуальная собственность

Список сокращений: ЛП – лекарственный препарат; ЛФ – лекарственная форма; ГРЛС – Государственный реестр лекарственных средств; ГК РФ – Гражданский Кодекс Российской Федерации; ЕАПК – Евразийская Патентная Конвенция; ВОИС – Всемирная организация интеллектуальной собственности; DPA – Закон о патентах Нидерландов.

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INTRODUCTION

In Russia, pharmacy organizations at the legislative level are allowed to be in the manufacturing of drugs[1]. An extemporaneous production had been widespread in the country until the 1990s, but practically disappeared against the backdrop of the economic crisis and development of the pharmaceutical industry in the 2000s [1–4].

From September 2023, amendments to Art. 56 "Manufacturing and dispensing of medicinal products" of the federal law "On the circulation of medicines" come into force[2]. The changes will provide entitle pharmacies to use the drugs included in the State Registers of Medicinal Products for Medical Use, as well as Common Register of the Authorized Medicinal Products of the Eurasian Economic Union, in manufacturing medicinal products according to doctors' prescriptions, in the prescribed manner.

The authors of the article set a goal to study the legal aspects of the legislative innovation for manufacturing drugs in a pharmacy organization. The authors also reviewed the legal regulation in terms of the possibility of using industrially produced drugs in the individual manufacturing of the drug without violating patent laws [1, 5]. The issues of the legal regulation of the patent legislation in the Russian Federation, were studied within the framework of the Eurasian Patent Convention and at the international level.

THE AIM of the work was to study the legal aspects of the legislative regulation for manufacturing medicines in a pharmacy organization.

MATERIALS AND METHODS

General scientific methods were used in the research. The information search was carried out in the databases of the National Center for Biotechnology Information (NCBI) and the scientific electronic library elibrary.ru; referenced bibliographic lists were studied; the ConsultantPlus, Cyberleninka, Food and Drug Administration (FDA) databases, European Medicines Agency (EMA), PubMed, WIPO Lex were also used. The regulatory framework was formed by the legislation of the Russian Federation in the field of the drug circulation, as well as by foreign countries and patent conventions. The preference was given to the sources describing the relationship between an extemporaneous pharmacy production and its regulatory framework, including the field of the intellectual property.

The search was based on the following keywords and phrases: intellectual property, pharmacies+invention, patent, drugs, extemporal+production, orphan+ diseases, as well as their Russian counterparts. 133 sources of information including scientific articles and regulations were found; 50 have been included in this review. The analysis of information sources published from 2013 to 2023 was determined by the peculiarities of legislation changes in the in this area. A number of sources found were deleted due to the absence of the indicated relationship and repetitions.

RESULTS AND DISCUSSION

State of production pharmacies in Russia and its legal regulation

There is no Common Register of pharmacy organizations in the Russian Federation. According to AlphaRM, as of September 2021[3], there were 70 238 pharmacy organizations of various forms of ownership in the country, which had the right to manufacture medicines, while, according to Roszdravnadzor in 2017, only 460 production pharmacies actually worked. The practice of pharmaceutical manufacturing was widespread in the country; however, since 2007, there has been a trend towards a reduction in intra-pharmacy manufacturing of drugs and a decrease in the number of manufacturing pharmacies [5–7]. Simultaneously, this coincided with the growth of the pharmaceutical industry in the 2000s. Pharmacy organizations began to receive fewer prescriptions issued by doctors. This was facilitated by an increased import of finished dosage forms (DFs) in the Russian Federation, the formation of wholesale and retail links [3, 7].

A special role was played by the state policy in the field of streamlining and standardizing approaches to the provision of medical care, which in the end led to the formalization of doctors' prescriptions. At the legislative level, state guarantees for the medical care provision were fixed. They apply exclusively to the list of industrially produced drugs[4], which automatically limited the doctors' ability to use all possible options for the drug therapy. At the same time, there remains a necessity to meet the needs of health care in the drugs that are not commercially produced [8–12], the need to provide patients with individual pre-dosed drugs [13–15], to manufacture drugs and dosage forms for the geriatric [16] and pediatric populations [17–20], patients with rare diseases [21, 22].

[1] Federal Law No. 502-FZ dated December 5, 2022 "On Amendments to Art. No. 56 of the Federal Law "On the Circulation of Medicines". Effective from September 1, 2023; adopted by the State Duma on November 22, 2022: approved by the Federation Council on November 30, 2022]; Moscow, 2022. Available from: https://www.consultant.ru/document/cons_doc_LAW_433279/. Russian

[2] Ibid.

[3] Kalinovskaya E. Skol'ko proizvodstvennyh aptek ostalos' v Rossii [How many industrial pharmacies are left in Russia]. Pharmvestnik. Available from: https://pharmvestnik.ru/content/articles/Skolko-proizvodstvennyh-aptek-ostalos-v-Rossii.html. Russian

[4] On the Program of State Guarantees of Free Provision of Medical Care to Citizens for 2023 and for the Planning Period of 2024 and 2025: Decree of the Government of the Russian Federation dated December 29, 2022 No. 2497. Official website of the Government of the Russian Federation. Available from: http://static.government.ru/media/files/FQATIOfojXIUYX8cwI2X7ugkeKRrRGjb.pdf. Russian

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Today, pharmacy manufacturing is designed to solve a number of problems associated with the provision of rare drugs to the population [23–26]; they will provide doctors and patients with individual dosages in individual dosage forms in various therapeutic areas [27–29]. This creates the need to develop legal norms to regulate extemporaneous production [30-34], a drug provision of individualized therapy and social guarantees for the patients who need them [35–38].

In 2022, due to the adoption of amendments to the legislation[5], from September 1, 2023, pharmaceutical production organizations will have the opportunity, in drugs manufacturing, in addition to pharmaceutical substances (as provided for by the existing procedure), to use the drugs included in the State Register of Medicinal Remedies (SRMRs). This change in legislation opens up a lot of additional benefits for pharmacy organizations, doctors and patients [39]. In case of using factory-made finished dosage forms for drugs manufacturing for individual purposes, the main advantage is the possibility of individual packaging of finished DFs [40]. This will improve control over the drug intake for the patient, reduce the risk of errors in the use of it by patients on an outpatient basis, and also reduce the cost for the patient if the prescribed number of doses is significantly fewer than the minimum amount in the registered package [41].

In addition, the prescription of a drug according to an individual doctor's prescription, which will be repackaged by a pharmacy organization, actually changes the legal status of the indicated medicinal product. If the instruction for the use of the drug does not contain any indication, but at the same time, in medical practice, the specified active substance is indicated for the treatment of another disease, then the prescription of such a drug can be paid by the state for a privileged category of citizens[6]. This is especially important for drug researchers, who can thus study the effect of a drug for a limited cohort of patients, which can later become the basis for conducting a clinical study in order to expand the indications in the instructions. Such a process is often referred to as "repositioning" of the drug if the indication is fundamentally different from that for which primary clinical studies had been conducted [43].

In case of manufacturing medicinal products from the pharmaceutical substances approved for the release into the civil circulation, the advantage of manufacturing individual dosage forms requires a separate discussion. First, modern technology options of pharmacy organizations make it possible to convert a solid form into a liquid one, but here, it is necessary to take into account the physicochemical properties of the active substance. For example, the pharmaceutical substance digoxin is insoluble in water, which leads to the formation of a precipitate in an aqueous solution. At the same time, digoxin can be dissolved in ethyl alcohol, but many doctors in neonatological practice see serious risks in prescribing alcohol-containing drugs to newborns [44]. In this regard, it is possible to use a suspension base to obtain a uniform distribution of the substance and the desired properties. Second, the availability of pharmaceutical substances makes it possible to combine various active substances to ensure the ease of use, which opens up opportunities for researchers to form new combined forms. This is especially important in cases where it is necessary to take into account the effect of different substances on different mutations of the same genetic disease, or where it is required to combine several active substances into one dosage form for the ease of use. Based on the data collected from patients on the use of such drugs (case series), it is possible to substantiate the scheme of further drug research [42]. Third, in the presence of a pharmaceutical substance (imported or self-produced), it is possible to resume pharmacotherapy of a patient population for which the drug had been previously prescribed, but for some reason its production and/or supply was stopped. A similar situation exists with a number of orphan drugs, which are currently not supplied to the Russian Federation. Fourth, the availability of raw materials solves the issue of a quick access of drugs to the market, since they are not subject to the state registration [45]. Accordingly, the time for passing registration procedures is reduced. For example, for a rare disease hyperammonemia (in which there is a violation of the urea formation cycle), sodium benzoate is used as a part of complex therapy. This substance is present in the composition of the combined preparation of a cough mixture and is not used in any way in wide medical practice. However, the presence of a pharmaceutical substance made it possible to develop an extemporaneous form for orphan patients and provide them with this drug in the shortest possible time [13]. This aspect is important for a regulation, as it can remove a social tension in the absence of any drug on the market. Fifth, pharmacy manufacturing can bring significant benefits to the budget if the drugs manufactured according to individual prescriptions provide significant budget savings, which is especially relevant for ultra-expensive drugs used in the treatment of orphan diseases [46]. A pharmacy organization's own synthesis of pharmaceutical substances and the manufacture of drugs from these substances can provide a significant number of patients with life-saving drugs [47].

Regardless of the aim of using an extemporaneously

[5] Federal Law No. 502-FZ dated December 5, 2022 "On Amendments to Art. No. 56 of the Federal Law "On the Circulation of Medicines". Russian

[6] Federal Law No. 482-FZ of December 30, 2021 "On Amendments to the Federal Law "On the Fundamentals of Protecting the Health of Citizens in the Russian Federation"; adopted by the State Duma on December 22, 2022: approved by the Federation Council on December 24, 2022]; Moscow, 2022. Available from: https://www.consultant.ru/document/cons_doc_LAW_405472/. Russian

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manufactured drug, developers, doctors and the state must be sure that this practice does not contradict the current international and national legislation in the field of an intellectual property rights protection, since the manufacture of drugs is carried out both for scientific and therapeutic purposes, and also with commercial benefits for the pharmacy organization [48].

The existing regulatory framework prohibits pharmaceutical organizations licensed to carry out pharmaceutical activities from producing pharmaceutical substances, because Russian legislation distinguishes between pharmaceutical and manufacturing activities. Now, pharmacy organizations can only manufacture drugs from pharmaceutical substances that are included in the SRMRs. The inclusion in the SRMRs provides for the presence of an applicant, who is both a manufacturer of medicines and his legal representative.

From the point of view of the terminology used in the national legislation, including the sectoral federal law[7], a clear explanation is given of what is a drug and what is a pharmaceutical substance. Thus:

- "drugs are substances or combinations thereof that come into contact with a human or animal body, penetrate into the organs, tissues of the human or animal body, used for a prevention, diagnosis (with the exception of substances or combinations thereof that do not come into contact with the human or animal body), treatment of a disease, rehabilitation, for the preservation, prevention or termination of pregnancy and obtained from blood, blood plasma, organs, tissues of the human or animal body, plants, minerals by synthesis methods or using biological technologies. Medicinal products include pharmaceutical substances and drugs";
- "drugs are pharmaceutical products in the form of dosage forms used for the prevention, diagnosis, treatment of a disease, rehabilitation, for the maintenance, prevention or termination of pregnancy".

Thus, within the framework of the implementation of Art. 56 of the branch federal law[8], pharmacy organizations manufacture drugs, since, in accordance with the federal law[9], the doctor prescribes the drug and not pharmaceutical substances to the patient.

Intellectual property and pharmacies

The circulation and production of medicines may be further restricted by the legislation of the Russian Federation in the field of an intellectual property.

A patent for an invention related to a drug can affect both the pharmaceutical substance – a directly active substance, and extend its effect to the drug as a whole – the system of active and auxiliary components [49].

At the same time, in order to limit the ability of large pharmaceutical companies to implement the so-called "renewal strategy" [50], the legislation of different countries contains antimonopoly restrictions on the manufacture of drugs by pharmacy organizations. Two main provisions must be observed: a pharmacy production must be in the interests of protecting public health, as well as preventing situations where individual treatment is impossible.

Similar provisions have been implemented under the Russian law. In accordance with subparagraph 5 of Art. 1359 of the Civil Code of the Russian Federation[10], a one-time production of a medicinal product by a pharmacy organization on a doctor's prescription does not violate the exclusive right to an invention, a utility model or an industrial design. Conventionally, the norm of this subparagraph 5 can be presented in a schematic form, where a production in a pharmacy assumes a one-time nature according to an individual prescription from the attending physician (Fig. 1).

At the same time, the mentioned wording indicates the extension of the effect of this article to the concept of a medicinal product. That implies a broader interpretation and effect of this article not on an industry basis, but in relation to the essence of the applied subject of regulation, which is a medicinal product, and the pharmaceutical substance in it.

The Eurasian legislation does not affect the regulation of manufacturing medicines in a pharmacy organization. Thus, in the legislation of the Russian Federation, there are prerequisites and a legal basis for the formation of the practice of widely using the possibilities of industrial pharmacies.

With regard to inventions in force on the territory of the Russian Federation related to the Eurasian Patent Convention (EAPC), it can be noted that it is similar to the domestic one. According to rule 19 of the Patent Instructions to the EAPC[11], the actions related to the one-time manufacture of medicines in pharmacies according to a doctor's prescription are not recognized as infringement of a Eurasian patent.

The regulation of this provision of the countries, the members of the Eurasian Economic Union (EAEU), can be also considered similar.

[7] Federal Law "On the Circulation of Medicines" dated April 12, 2010 No. 61-FZ (last ed.); adopted by the State Duma on March 24, 2010: approved by the Federation Council on March 31, 2010]. Available from: https://www.consultant.ru/document/cons_doc_LAW_99350/. Russian

[8] Ibid.

[9] Federal Law No. 502-FZ dated December 5, 2022 "On Amendments to Art. No. 56 of the Federal Law "On the Circulation of Medicines". Russian

[10] Civil Code of the Russian Federation. Part Four: Federal Law No. 230-FZ of December 18, 2006; adopted by the State Duma on November 24, 2006: approved by the Federation Council on December 8, 2006]. Available from: https://www.consultant.ru/document/cons_doc_LAW_64629/. Russian

[11] Patent Instruction to the Eurasian Patent Convention; approved by the Administrative Council of the Eurasian Patent Organization at the 2nd (1st regular) meeting on December 1, 1995: in the current version]. Available from: https://www.eapo.org/ru/documents/norm/instr2022-p1.html. Russian

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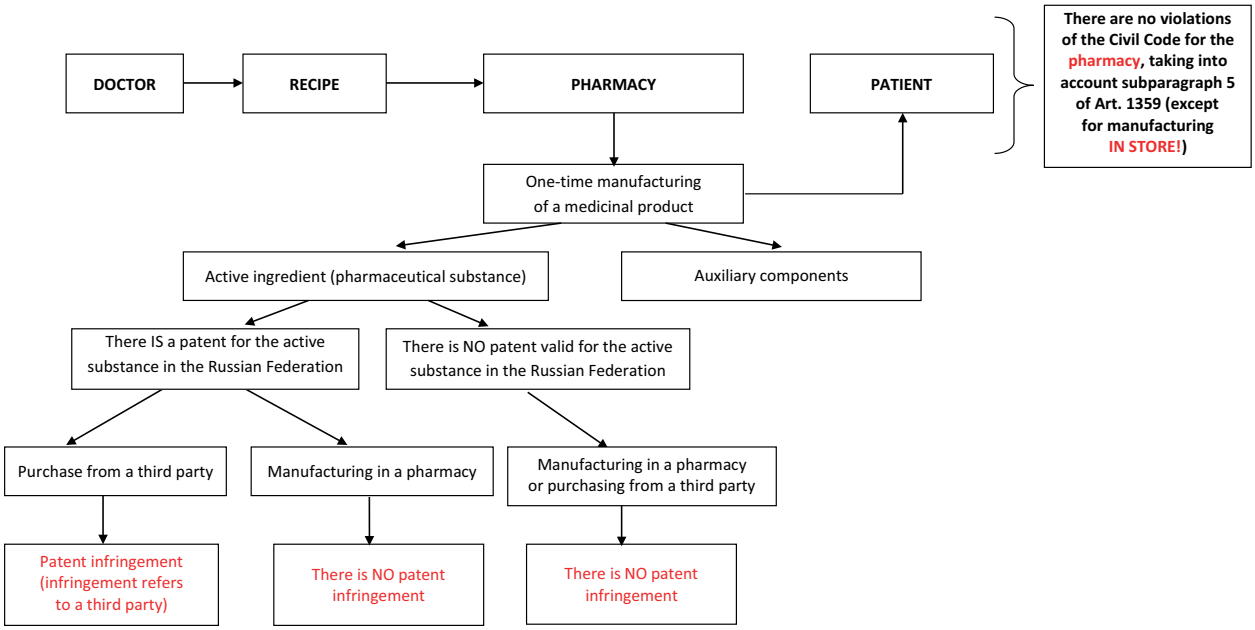

Figure 1 – One-time production of medicines by prescription in a pharmacy organization

Article 10 of the Republic of Belarus Law No. 160-3 "On Patents for Inventions, Utility Models, Industrial Designs"[12] (dated December 16, 2002) indicates that they are not recognized as a violation of the exclusive right of the patent holder: one-time manufacturing of medicines in pharmacies according to a doctor's prescription using a protected invention patent. Similar provisions are contained in Art. 12 of the Kazakhstan Patent Law (the concept "in emergency cases" is used)[13], and in Art. 17, subparagraph 1, of the Armenia Law "On Patents"[14]. At the same time, the provisions of the patent legislation of the Kyrgyz Republic do not contain such a provision.

With regard to the international position of the countries belonging to the outside of the Commonwealth of Independent States, it is possible to bring the following consolidated position, which reflects the approaches of a number of foreign states to this problem. In 2014, the World Intellectual Property Organization (WIPO) conducted a survey of the countries regarding the granting of the discussed exceptions. That resulted in the completed Questionnaire[15] and the Document of the Secretariat of the WIPO Standing Committee on Patent Law following the results of the 20th session (Geneva, January 27–31, 2014) "Limitations and exceptions to patent rights: one-time preparation of medicines" (Document)[16]. The document includes information provided by 39 states and participating patent organizations regarding legislative aspects, to restrict the use of extemporaneous DFs. Their position was presented by: Albania, Armenia, Azerbaijan, Bosnia and Herzegovina, Brazil, Bulgaria, Croatia, Cyprus, Czech Republic, Democratic People's Republic of Korea, Denmark, Finland, France, Germany, Greece, Hong Kong (China), Hungary, Italy, Japan, Latvia, Lithuania, Morocco, Norway, Philippines, Poland, Portugal, Republic of Korea, Republic of Moldova, Russian Federation, Serbia, Slovakia, Spain, Sweden, Tajikistan, Thailand, Turkey, United Kingdom, Viet Nam and Eurasian Patent Organization (EAPO) .

Given the detailed presentation of positions, the following aspects can be focused on:

1. As regards the aim of regulation, the position of Brazil provides for the need to take into account both the interests of the right holder and the opinion of users of the relevant rights, as well as issues of the social justice through the use of limited exceptions to exclusive rights that are provided by the patent law, however, only on the condition that such exceptions do not come into critical conflict with its application and do not lead to the unjustified damage to the legitimate interests of the patent owner, as well as the interests of third parties. Accordingly, it is assumed that these exceptions facilitate the access of innovative technologies, taking into account the mutual benefit of producers and users of innovations, with the aim of maintaining social and

[12] Law of the Republic of Belarus No. 160-Z of December 16, 2002, on Patents for Inventions, Utility Models, and Industrial Designs (as amended up to Law No. 275-Z of December 18, 2019). Available from: https://www.wipo.int/wipolex/ru/legislation/details/21279.

[13] Patent Law of the Republic of Kazakhstan No. 427 of July 16, 1999; amended in accordance with the Law of the Republic of Kazakhstan No. 268-VI of October 28, 2019. Available from: https://www.wipo.int/wipolex/ru/text/539217. Russian

[14] Law of the Republic of Armenia of June 10, 2008, on Inventions, Utility Models and Industrial Designs. Available from: https://www.wipo.int/wipolex/ru/legislation/details/5496.

[15] Questionnaire on Exceptions and Limitations to Patent Rights. WIPO. Available from: http://www.wipo.int/scp/en/exceptions/.

[16] Exceptions and Limitations to Patent Rights: Extemporaneous Preparation of Medicines (SCP/20/5). Available from: https://www.wipo.int/edocs/mdocs/patent_policy/en/scp_20/scp_20_5.pdf

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economic well-being while respecting the balance of rights and obligations.

The Republic of Cyprus and France adhere to the postulate that granting of exemptions is based on the principles of social justice and should be in the interests of protecting the health of citizens.

Poland's position is that exceptions are acceptable in cases where an "individual" treatment of the patient is necessary in order to prevent a situation where it is not possible.

In the Republic of Moldova, regulators insist on the need for exceptions in order to increase the availability of medicines.

Representatives of Portugal consider it important not to restrict an access to patient treatment and not to disrupt the communication between the doctor and the patient.

Spain's doctrine is to increase a patient access to extemporaneous medicines. Thus, patients can receive an individualized drug care, while not damaging the object of patent rights.

Regulators in Germany and Italy also believe that the aim of the exceptions is to allow physicians to prescribe the medicines produced in manufacturing pharmacies to patients in individual cases, regardless of the presence of patent rights, since patents should not limit the doctor's ability to treat a patient.

Sweden and the United Kingdom insist that "Pharmaceutical professionals should be able to manufacture individual drugs on prescription without the risk of patent infringement".

Hungarian representatives voice the aim of the state regulation of exceptions as an opportunity to provide patients with inexpensive and high-quality extemporaneous medicines and reduce financial costs in the public health system.

Sweden expresses a similar opinion: the aim of the government regulation is to provide a quality medical and pharmaceutical care. At the same time, the application of exceptions is used for a small number of patients and does not harm a patent holder.

Norway adheres to the fact that "manufacturing of extemporaneous DFs in pharmacies is legitimate, since they are prepared according to the doctor's prescriptions."

Japan and the Republic of Korea clearly point out to the fact that a pharmacy production is a socially significant mission of the state in order to help patients restore their health, respectively, extemporaneous production procedures cannot be considered from the point of view of the patent law.

Serbia's position is determined by ethical and medical aspects: when personalizing treatment, the presence of a patent should not prevent manufacturing of medicines in a pharmacy.

The response of the Republic of Latvia states that the main aim of the exception is to harmonize the country's patent legislation with the regulatory legal acts of the EU member states.

2. As for the legislative aspects of the regulation, the analysis of the above Document and the Questionnaire additionally illustrates how the specified rule on the pharmacy production exclusion is implemented in the legislation of a number of countries.

In France, patent legislation does not cover the "one-time or non-systematic" manufacturing of extemporaneous medicines by prescription (Article L613-5 of the French Code of Intellectual Property)[17].

Art. 23 of the Azerbaijan Law on Patents states: the "episodic" nature of manufacturing medicines in pharmacies makes it possible to avoid violating the exclusive rights of the patent owner.

Under the Thai law, patent infringement does not occur in the "manufacture of a specific medicinal product by prescription" (Section 36 of the Thai Patent Law)[18].

In Turkey, "the right of the patent holder does not extend to the manufacture of extemporaneous DFs, since they are not intended to be industrially produced, and this procedure is carried out within the framework of the execution of a specific doctor's prescription" (Article 75 (c) of the Turkish Patents Decree)[19].

According to the Italian law, "the exemption does not apply to the use of active ingredients manufactured industrially" (Article 68.1(c) of the Italian Industrial Property Code)[20].

The Serbia patent legislation declares the legitimacy of exceptions, but stipulates that the exception does not apply to the manufacture of extemporaneous DFs for the purpose of storage; only to the manufacture in pharmacy conditions for specific medical prescriptions, for a specific patient by prescription (Article 21, paragraph 3 of the Law on Serbian patents)[21].

At the same time, the categorization of the parties in whose interests the aforementioned exception is used is usually not specified in the relevant legislative acts of the Member States. It should be noted that in most states, the legal documentation does not stipulate the

[17] Intellectual Property Code (version of September 1, 2012). Available from: https://www.wipo.int/wipolex/en/legislation/details/11987.

[18] Patent Act B.E. 2522 (1979) (as amended up to Patent Act (No. 3) B.E. 2542 (1999)). Available from: https://www.wipo.int/wipolex/en/legislation/details/3807.

[19] Law No. 6769 of December 22, 2016, on Industrial Property (Türkiye). Available from: https://www.wipo.int/wipolex/en/legislation/details/16609.

[20] Industrial Property Code (Legislative Decree No. 30 of February 10, 2005, as amended up to Law No. 10829 of July 29, 2021; Italy). Available from: https://www.wipo.int/wipolex/en/legislation/details/21556.

[21] Law on Patents (Official Gazette of the Republic of Serbia No. 99/2011, 113/2017, 95/2018 and 66/2019). Available from: https://www.wipo.int/wipolex/en/legislation/details/19365.

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practice of applying exceptions, but the location where medicines can be manufactured according to a doctor's prescription (for example, pharmacies or accredited laboratories).

The bulk of the responses from Member States indicate that the exception regulates the right of the "pharmaceutical specialist" to manufacture such drugs.

The Norwegian legislation states that the exception concerns the "authorized personnel" of pharmacies, while the Swedish response refers to the "pharmacy personnel" in general.

The responses from France, Germany and Cyprus mention both pharmacists and prescribers.

Thailand's profile regulations contain information on "professional pharmaceutical and/or medical professionals".

In Italy, the right to use the exception belongs to the "pharmacists".

In Portugal, the exemption is relevant for "any person who has a legitimate right to manufacture under the conditions of a pharmacy organization" (Art. 102 of the Portuguese Industrial Property Code)[22].

Art. 43 of the Brazilian Industrial Property Law specifies that the "qualified person" is the addressee of the exception[23].

The Philippine legislation allows a "health professional" to exercise his right to use the exception, regardless of the position of the patentee.

In Thailand, the main requirement for the practice of exclusion is the professional status: "a professional pharmaceutical specialist" or "a medical specialist ".

Only general provisions are included in the legislation of individual Member States, but some Member States establish additional requirements for the persons entitled to the pharmacy manufacture and prescription.

The patent law of Hong Kong states that a medicinal product must be manufactured under the prescription of the doctor who holds the appropriate certificate (Section 75(c) of the Hong Kong Patent Ordinance, China)[24].

In Japan, pharmaceutical invention protection regulations do not prevent the pharmacy from making medicines prescribed by a doctor or dentist (Article 69(3) of the Japanese Patent Law)[25].

In the United Kingdom, relevant legislation states that only a board-certified doctor or dentist is authorized to issue a prescription (Section 60(5)(c) of the UK Patents Act)[26].

3. With regard to the volume of the extemporaneous production when using the exemption, in most states, the legislation does not directly regulate the maximum number of units of the medicinal product that can be manufactured under the exemption. However, separate responses indicate that the exception is applicable in case of "one-time" (RF and Republic of Moldova), "non-systematic" (France, Sweden and EAPO) or "singular" (France), "individual" (Finland, Hungary, Italy and Serbia) production for the manufacture of medicines in pharmacies.

Thus, all states under the Document were positive on the problem of whether the regulatory framework for the application of exceptions related to patent rights relating to pharmacies is sufficient to achieve the stated goals and objectives. An important point is that all states noted that there were no difficulties in the practical implementation of the discussed exception.

However, it should be noted that in the countries of northern Europe (Denmark, Norway and Sweden), such an exception is not an accepted practice, since pharmacy manufacturing of drugs is not a common procedure.

Taking into account the above opinions, the law enforcement practice of using this norm in the Netherlands is interesting, since the discussion has shown the difficulties that may be encountered in its practical implementation.

As of February 1, 2019, the Dutch Patent Act (DPA) has been amended to include a limitation on the exclusive right of a patent holder to a medicinal product. The operation of the exclusive right now contains an important point in Art. 53(3) of the DPA[27]: "The exclusive right ... does not apply to the preparation of drugs for an immediate use by individuals on the basis of a medical prescription in pharmacies, as well as acts relating to drugs prepared in this way". This part of Section 53(3) of the DPA allows pharmacists to manufacture patented medicines in the above circumstances for the immediate use by individuals on the basis of a medical prescription.

The public interest in addressing the problem of excessively high drug prices in the Netherlands prompted the legislature to make this provision official. According to Minister of Health and Sports, Mr. Bruins, "the elimination of liability for patent infringement in some cases of impromptu drug manufacturing could be a reasonable alternative to expensive drugs." In his Explanatory Memorandum on the Enactment of the Improvisation Provision, the Minister of Economy, Mr. Vibes, stated that "In order to prevent the abuse of extemporaneous preparation of medicines, the exception should apply only to rare cases when the

[22] Código da Propriedade Industrial (alterado pelo Lei n. 46/2011, de 24/06). Available from: https://www.wipo.int/wipolex/en/text/420570.

[23] Law No. 9.279 of May 14, 1996 (Law on Industrial Property, as amended up to Law No. 14.200 of September 2, 2021). Available from: https://www.wipo.int/wipolex/en/legislation/details/21166.

[24] Patents Ordinance (Chapter 514); Hong Kong, China. Available from: https://www.wipo.int/wipolex/en/legislation/details/2049.

[25] Patent Act (Act No. 121 of April 13, 1959, as amended up to Act No. 63 of May 13, 2011); Japan. Available from: https://www.wipo.int/wipolex/en/legislation/details/13137.

[26] Patents Act 1977 (Chapter 37, updated up to March 27, 2014); United KingdomGB324. Available from: https://www.wipo.int/wipolex/en/text/330537.

[27] Rijkswet van 15 december 1994. Available from: https://wetten.overheid.nl/BWBR0007118/2021-08-01.

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pharmacist himself prepares the medicine; the cases prescribed by a doctor; a preparation of medicines for individual patients"[28].

Scientists in the Netherlands agree that this formulation does not allow the production of patented drugs broad based. The recently adopted exception in the DPA should be interpreted restrictively. The public opinion in the Netherlands supports an immediate preparation of medicines as an alternative to expensive medicines. Obviously, such stakeholders as patent owners and the pharmaceutical industry will oppose a broad interpretation of the new law.

DISCUSSION

The article provided an overview of the modern practice of pharmaceutical manufacturing in the Russian Federation, as well as analyzed the benefits of this activity for the medical community, patients and the state. One of the most important advantages of pharmaceutical manufacturing is an individual approach to drug therapy. At the same time, the individualization of drug treatment has made it possible to work out systemic solutions for developing drug therapy methods for special groups of patients for whom the economic feasibility of a pharmaceutical registration and launching such drugs onto the market has been brought into challenge. Ensuring the availability of drug therapy, however, responds to the need to respect the rights of citizens.

In addition, pharmacy manufacturing is an available tool in the study of drugs prescribed by a doctor not in accordance with the instructions for a medical use (off-label) or in dosage forms/dosages that are not on the market.

Extemporaneous manufacturing can also be a part of the drug repositioning process on the market, subject to compliance with the requirements for pharmacy manufacturing and safety control of prescribed drugs.

A review of the foreign patent legislation, including the EAEU countries, in relation to the drugs manufactured in pharmacy organizations, shows that the legislative norm under discussion as a whole does not have an innovation characteristic exclusively for the Russian Federation. It is legally enshrined in many countries that are members of WIPO, as a rule concerning the exceptions related to patent rights in relation to the pharmacy production.

It is important to note that, in fact, this rule is conceptually formulated in approximately the same way in more than 30 WIPO Member States, including France, Spain, Germany, Italy, Sweden, the United Kingdom and many others. The norm states that it is pharmacy specialists who should be able to prepare medicines in an individual case in accordance with a doctor's prescription, without being exposed to the risk of patent infringement.

In the Russian Federation, as one of the mechanisms that allow the use of medicinal products without the permission of the right holder, правообладателя is the implementation of the provisions of subparagraph 5 of Art. 1359 of the Russian Federation Civil Code[29]. In other words, a one-time pharmacy production according to an individual prescription cannot be regarded as a patent infringement. First of all, this applies to such inventions that relate to methods for obtaining drugs or medicinal products as such, since these technical solutions use either industrial technological methods for obtaining compounds or compositions that are parts of drugs not used in pharmacies, or refer to methods of the drug use, the scope of which is focused on patients.

As for the compositions themselves, as objects of the invention, the sign of a one-time production is an individual order or an individual prescription issued to a specific individual in a certain period of time, which has other signs of a one-time nature – an individual number and date of issue, a limited shelf life, an individually selected DF, dosage, etc.

The opposition to the term "one-time" may be the term "serial". Therefore, a one-time production of a medicinal product should not have serial features in itself, i.e. have differences from other recipes (or orders) and signs of individuality.

However, the main problem in the manufacture of drugs in a pharmacy is the presence of a patent for an invention that relates directly to the compound, i.e. to the pharmaceutical substance. This is due to the fact that the sale of a pharmaceutical substance (the alienation of property rights for the purpose of obtaining commercial benefits), in respect of which exclusive intellectual property rights are distributed on the territory of the Russian Federation, is a violation of the rights of the patent owner. Herewith, in accordance with Art. 56 of the branch federal law, "in the manufacture of medicines by pharmacy organizations, veterinary pharmacy organizations, individual entrepreneurs licensed for pharmaceutical activities, pharmaceutical substances that are included in the state register of medicines for medical use and, respectively, the state register of medicines for veterinary use in the prescribed manner, are used". Accordingly, the pharmaceutical substance included by the manufacturer or supplier in the SRMRs, can be used for a pharmacy production in a licensed organization.

At the same time, a manufacturing pharmacy cannot independently produce patented pharmaceutical

[28] Radboud Ribbert, Amendment to Dutch Patents Act (Rijksoctrooiwet) Allows for Extemporaneous Preparation of Medicine in Certain Circumstances, March 14. 2019. Available from: https://www.gtlaw.com/en/insights/2019/3/amendment-to-dutch-patents-act-rijksoctrooiwet-allows-for-extemporaneous-preparation-of-medicine.

[29] Civil Code of the Russian Federation. Part Four: Federal Law No. 230-FZ of December 18, 2006; adopted by the State Duma on November 24, 2006: approved by the Federation Council on December 8, 2006].

substances. Therefore, it is necessary to clarify or introduce a similar article or supplement the current article with provisions applicable to manufacturers of pharmaceutical substances, where a one-time production of pharmaceutical substances will not be recognized as a violation of the exclusive right to an invention, a utility model or a design invention.

The combination of technological capabilities of Russian drug developers and obtaining the right to manufacture in pharmacies can open a new way to "reposition" existing drugs or become the basis for creating drugs for orphan diseases prescribed as the only possible therapy.

CONCLUSION

Thus, new regulatory changes in the field of the drug circulation, in the context of a changing political and economic landscape and sanctions against the Russian Federation, will serve to increase the availability of a drug provision and a personalized drug care to the country's population.

The renewal of the pharmacy production will improve not only the availability of the drug care to the population, but also taking into account individual dosages and dosage forms in various therapeutic areas, and can also become a tool for drug repositioning or clinical testing of new molecules for rare incurable diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Alexey V. Alekhin – goals setting, concept working out, article writing and editing, manuscript final approval; Tatyana N. Erivantseva – critical analysis of scientific literature and legal documentation, writing the manuscript text; Vasily V. Ryazhenov – scientific and methodological literature analysis, making comments of intellectual content; Nikolai B. Lyskov – material collection, critical analysis of literary sources, writing the manuscript text; Natalya A. Alekhina – material collection, critical analysis of literary sources, writing the manuscript text; Maria M. Kuznetsova – analysis of scientific and methodological literature, editing and design of the manuscript text. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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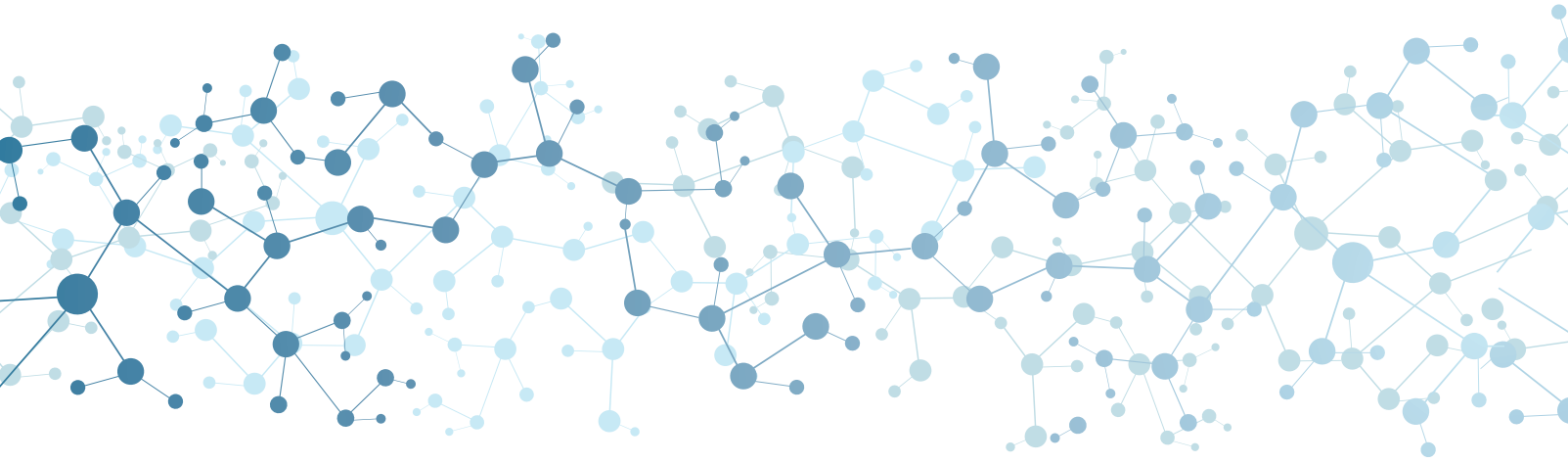


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