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RESEARCH ARTICLE

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PHARMACY & PHARMACOLOGY

PHENOLIC COMPOUNDS OF LAURUS NOBILIS (REVIEW)

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One of the most famous plants of the laurel family (Lauraceae) is Laurus nobilis L.
The aim of the study was to review scientific information on the study of phenolic compounds of wild-growing and cultivated Laurus nobilis L.
Materials and methods. The study was performed using information retrieval (PubMed, ScholarGoogle) and library databases (eLibrary, Cyberleninca), as well as ResearchGate application for semantic search. The research methods are analysis and synthesis of the scientific literature data for the period from 2000 up to the present.
Results. The data presented in the review show that leaves, fruits, and shoots of Laurus nobilis L. are valuable sources of phenolic compounds, such as phenolic acids, flavonoids, and proanthocyanidins. The quantitative content of these groups of substances varies depending on the collecting ground, the source of raw materials (cultivated or wild plants), the time (phase) of their harvesting, the method of drying, extraction from raw materials, etc. Phenolic compounds exhibit a pronounced antioxidant and antiradical activity, have an inhibitory effect on NO production, sodium-potassium adenosine triphosphatase, on tumour cell lines (HeLa, MCF7, NCI-H460 and HCT15), and are characterised by an antibacterial action against gram-positive and gram-negative bacteria.
Conclusion. The analysis of the available scientific information showed that the phenolic compounds of Laurus nobilis L. are one of the main groups of the active compounds of this plant. The use of this information is essential for the development of **new effective medicines based on the raw materials of Laurus nobilis L..**
Keywords: Laurus nobilis L., phenolic compounds, quantification , antioxidant, anticancer activity

ФЕНОЛЬНЫЕ СОЕДИНЕНИЯ ЛАВРА БЛАГОРОДНОГО (ОБЗОР)

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Одним из самых известных растений семейства лавровые (Lauraceae) является лавр благородный (Laurus nobilis L.).
Целью исследования являлся обзор научной информации по изучению фенольных соединений дикорастущего и культивируемого лавра благородного.

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ОРИГИНАЛЬНАЯ СТАТЬЯ
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Материалы и методы. Исследование проводилось с использованием информационно-поисковых (PubMed, ScholarGoogle) и библиотечных баз данных (eLibrary, Cyberleninca), а также приложения ResearchGate для семантического поиска. Методы исследования – анализ и обобщение научной литературы за период с 2000 года по настоящее время.

Результаты. Представленные в обзоре данные показывают, что листья, плоды и побеги лавра благородного являются ценными источниками фенольных соединений, таких как фенольные кислоты, флавоноиды, проантоцианидины. Количественное содержание этих групп веществ варьирует в зависимости от места сбора, источника сырья (культивируемые или дикорастущие растения), времени (фазы) его заготовки, способа сушки, извлечения из сырья и т.д. Фенольные соединения проявляют выраженную антиоксидантную и антирадикальную активность, оказывают ингибирующее влияние на продукцию оксида азота, натрий-калиевую аденозинтрифосфатазу, на линии опухолевых клеток (HeLa, MCF7, NCI-H460 и HCT15), характеризуются антибактериальным действием в отношении грамположительных и грамотрицательных бактерий.

Заключение. Анализ доступной научной информации показал, что фенольные соединения лавра благородного являются одной из основных групп действующих соединений этого растения. Использование этих данных важно для разработки новых эффективных лекарственных средств на основе сырья лавра благородного.

Ключевые слова: лавр благородный, Laurus nobilis, фенольные соединения, количественное определение, антиоксидантная, противораковая активность

INTRODUCTION

The Laurel family (*Lauraceae*) includes more than 2500 plant species that grow in the subtropics and tropics of East Asia, South and North America. One of the most famous and most commonly used plants from this family is *Laurus nobilis* L. The name of the plant is dedicated to Apollo, the ancient Greek sun god, and is a symbol of peace and victory. Laurel wreaths covered the heads of emperors, generals and poets.

The natural habitats of this evergreen plant are the territories of the Mediterranean countries with high annual rainfall [1]. It is grown as a decorative species in Europe, Russia, the USA and other countries, cultivated in Turkey, Algeria, Morocco, Portugal, Spain, Italy, France, Russia and Mexico [2].

Laurus nobilis L. leaves are widely used in traditional dishes of peoples of not only the Mediterranean but also many other countries [5]. The leaves and fruits of the plant are used in traditional medicine of peoples of different countries to reduce high blood glucose levels, in the treatment of diseases caused by fungal and bacterial infections. Extracts from laurel leaves exhibit anti-inflammatory, soothing, antiepileptic properties [6–10]. Infusion of dry leaves is used for various gastrointestinal diseases, as well as for flatulence as a carminative [11]. *Laurus nobilis* L. fruits were included in the sixth edition of the Russian Pharmacopoeia and the State Pharmacopoeia of the USSR of the first edition. Laurel leaves are official raw materials (*Lauri Folium*) in Iran [12].

It has been experimentally established that the biologically active compounds in the essential oil and leaf extracts, promote healing of small wounds [13], have anti-inflammatory, analgetic [14], immunostimulating [15], neuroprotective [13, 16], anticholinergic, antioxidant, antiulcer, anticonvulsant, antimutagenic, insecticidal, antibacterial, antiviral, antifungal [13] and larvicidal [17] effects. Some publications are devoted to characterizing the anticancer potential of the essential oil [18], methanol [19], ethanol and water extracts [20] from laurel leaves and its fruits. The scientific literature describes antibacterial properties of the essential oil [21–22] and a few kinds of extracts: water [23], ethanol [24] and methanol [25]. According to some researchers, the antibacterial activity of the extracts is associated with the presence of terpene and phenolic substances [26–27]. *Laurus nobilis* L. leaves are also included in the herbal tea [28] and drugs for the treatment of diabetes [29–30], and their extracts - in the composition of biologically active food additives [31]. *Laurus nobilis* L. essential oil is used in cosmetology and in the production of perfumes and soaps.

The chemical composition of the leaves was studied quite widely in different countries where this plant grows in natural habitats or is cultivated. In previous studies, different groups of chemical compounds in *Laurus nobilis* L. leaves and fruits were found. According to the results of a lot of studies [32–33], 1,8-Cineol is the main component of *Laurus nobilis* L. leaf essential oil (up to 70%). *Laurus nobilis* L. fruits contain fatty and essential oils. It is this mixture that was previously known as “laurel oil” and included laurostearin, a lauric acid ester as one of its components. The composition of fruits fatty acids was studied by B. Ozcan et al. [7]. The roots and leaves of *Laurus nobilis L.* are a source of sesquiterpene lactones [34]. Two distinct chemical types containing laurenobiolide and costunolide, as the main substances, were identified in them [35–37]. Sesquiterpene lactones found in *Laurus nobilis L.* leaves, have different pharmacological properties: inhibition of NO production [36] and ethanol absorption [38], an increased activity of hepatic glutathione S-transferase [3]. In the last decade, the cytotoxic activity of these compounds against various tumour cell lines, has been actively studied [39–40]. Quite often, the antioxidative activity of various extracts from the leaves of wild-growing [10, 41–42] and cultivated *Laurus nobilis L.* [6, 11] was investigated. In recent years, several review papers devoted to the biologically active compounds of *Laurus nobilis L.,* have appeared [13, 43]. However, in these articles, the information on the accumulation of phenolic compounds in the plant is extremely limited. Phenolic compounds of leaves and fruits of wild-growing and cultivated *Laurus nobilis L.* have been studied in different habitats. The

growing interest in this group of natural compounds of *Laurus nobilis L.* is associated not only with the variety of identified structures but also with the relevant types of pharmacological activity (antioxidant and anticancer) that are associated with it.

THE AIM of the study was to review scientific information on the study of phenolic compounds of wild-growing and cultivated *Laurus nobilis L.*

MATERIALS AND METHODS

The study was performed using information retrieval (PubMed, ScholarGoogle,) and library databases (eLibrary, Cyberleninca), as well as ResearchGate application for the semantic search. The research methods are: analysis and synthesis of the scientific literature data for the period from 2000 up to the present.

RESULTS

In the study by H.W. Kang et al. [44], ethanol extract from laurel leaves was cytotoxic to *Staphylococcus aureus 209p* and had the highest alkylperoxy radical-trapping (ROO·) activity among 120 species of the plants studied. After processing this extract with chloroform, ethyl acetate, n-butanol and water, the ethyl acetate fraction showed the highest activity. The authors isolated the main flavonol from the leaves of the plant, which was identified as isoquercitrin by spectral characteristics **(1)** (see Table 1). The further study of the antioxidant activity of this compound established its comparability to the effect of the known antioxidants such as epigallocatechin, resveratrol and higher than that of butyl hydroxyanisole, butylhydroxytoluene and ascorbic acid.

Several compounds including phenolic glucoside (**2**) and flavonoids (**3** и **4**), were isolated by S. de Marino et al [45] from methanol extract of fresh *Laurus nobilis L.* leaves. The study of these substances' effect on nitric oxide production in the rats' macrophages activated by lipopolysaccharide (J774), showed that kaempferol-3-O-α-L-(3", 4"-di-E-p-coumaroyl) rhamnoside is the most active of them (**3**).

Methanol extracts of five types of the plant materials purchased at Droga (Portoroz, Slovenia), including *Laurus nobilis L.* leaves, were investigated by a group of authors [46]. The total amount of phenolic compounds was determined by the colourimetric method using the Folin – Ciocalteu reagent. In the extract of *Laurus nobilis L.* leaves, their content was 99.7 g/kg (in gallic acid equivalents). In acid hydrolysed extracts, proanthocyanidins were studied spectrophotometrically at the wavelength of 540 nm (29.9 g/kg in total). Free flavones (apigenin and luteolin) and flavonols (kaempferol, myricetin and quercetin) in hydrolysed extracts were determined by HPLC. The detection was carried out at 367 nm using standard samples of apigenin, luteolin, quercetin, myricetin and kaempferol as external standards. The authors identified quercetin and kaempferol. The flavonoid content was 80.1 mg/kg (in total).

The composition of the anthocyanins isolated from peeled *Laurus nobilis L.* seeds was first determined by L. Longo and G. Vasapollo [47]. The compounds were isolated with a 0.1% aqueous methanol solution acidified with hydrochloric acid, followed by purification of the extract in a C-18 solid-phase cartridge; then they were identified by HPLC-MS analysis. The content of anthocyanins in the fruits was 217 mg/g. The main anthocyanins are cyanidin 3-O-glucoside (5.90 mg/g, i.e. 41% of the total amount) and cyanidin 3-O-rutinoside (6.116 mg/g – 53%). Besides, two minor anthocyanins were identified as 3-O-glucoside (7) and 3-O-rutinoside peonidin (8) (10.6 mg/g, i.e. 5% of the total amount) (see Table 1).

Laurus nobilis L. leaves collected by V. Papageorgiou et al. in the Patra region (Greece) in the first half of February, May, August and November 2007, were divided into two parts. The first part of the samples was subjected to the air-shadow drying at ambient temperature, and the other one was freeze-dried for 6 hours at –60°C [48]. The total amount of phenolic compounds was determined by the colourimetric method using the Folin – Ciocalteu reagent [49] and gallic acid as a standard. The absorption spectrum of relatively distilled water was measured at 765 nm, and the calibration curve was plotted using the data of gallic acid. Colourimetry was also used to determine the total amount of flavonoids, with epicatechin as the reference substance. The spectrum of the mixture was measured at 510 nm. The results showed a significant difference in the content of the total amount of phenolic compounds depending on the phase of the plant development. The amount of flavonoids in the extract from *Laurus nobilis L.* leaves collected in May (at the beginning of fruiting) amounted to 2.90 mg/g in terms of epicatechin and dry raw materials. The total amount of phenolic compounds reached a maximum level during the fruiting initiation stage (80.30 mg, calculated in gallic acid equivalents / g of dry raw materials), while the lowest content was noted at the end of the fruiting period (22.90 mg/g, calculated in gallic acid equivalents and dry raw materials). These results were significantly different from those established during the period of full bloom (51.30 mg/g in gallic acid equivalents and dry raw materials) and in the budding stage (42.60 mg gallic acid equivalents/g and dry raw materials). The *Laurus nobilis L.* extracts obtained from freeze-dried raw materials, showed a similar seasonal variation. The main phenolic components in all the extracts studied, were flavonoids. The concentration of luteolin **(9)** was relatively high (ranging from 0.20 to 4.50 mg/g of dry weight). Phenolic acids – 3,4-dihydroxybenzoic **(10)**, gallic **(11)**, vanillic **(12)** and rosmarinic **(13)** ones - were found in low concentrations (see Table 1). Freeze-drying caused a significant decrease (by almost 50%) in the total amount of phenolic compounds and flavonoids in almost all the studied samples of laurel leaves. To a great extent, this was due to a decrease in the content of luteolin and most phenolic acids (mainly hydroxycinnamic acids). This study result is consistent with the data obtained by other authors, according to which freeze-drying caused a loss of 87% of the total amount of flavonols in the extracts of *Posidonia oceanica* L. [50].

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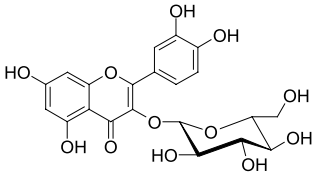

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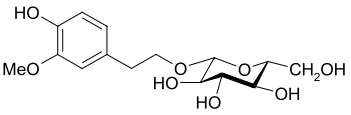

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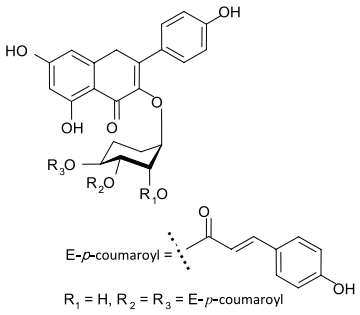

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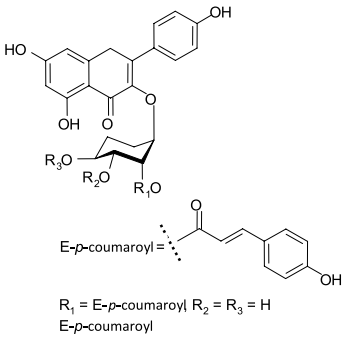

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Table 1 – Phenolic compounds of Laurus nobilis L.

Order numbers	Groups of substances / names of compounds	Morphological parts of the plant	Content, %	Pharmacological activity	Reference
		Flavonoids			
1.	Isoquercitrin (**1**)*	Leaves	+**	Antioxidant activity	[44]
2.	Kaempferol-3-O-α-L- (3", 4"-di-E-p-coumaroyl) rhamnoside (**3**)	Leaves	0.00027	Inhibitor of nitric oxide production in LPS-activated murine macrophages (J774)	[45]
3.	Kaempferol-3-O-α-L-(2"-E-p-coumaroyl) rhamnoside (**4**)	Leaves	0.00022	–***	[45]
4.	Luteolin (**9**)	Leaves	up to 0.45 ± 0.05 (dry weight)	–	[48]
5.	Kaempferol-3-O-glucopyranoside (**16**)	Leaves	0.0092	Antioxidant activity	[11]
6.	Kaempferol-3-O-rhamnopyranoside (**17**)	Leaves	0.00112	Antioxidant activity	[11]
7.	Kaempferol-3-O-(2",4"-di-E-p-coumaroyl)-rhamnoside (**18**)	Leaves	0.00916	Antioxidant activity	[11]
8.	Kaempferol-3-O-arabinopyranoside (**19**)	Leaves	0.0064	Antioxidant activity	[11]
9.	Kaempferol-3-O-[6-O-(rhamnopyranosyl) glucopyranoside] (**20**)	Leaves	0.00112	Antioxidant activity	[11]
10.	Quercetin-3-O-glucopyranoside (**21**)	Leaves	0.0152	Antioxidant activity	[11]
11.	Quercetin-3-O-rhamnopyranoside (**22**)	Leaves	0.0084	Antioxidant activity	[11]
12.	Quercetin-3-O-[6-O-(rhamnopyranosyl) glucopyranoside] (**23**)	Leaves	0.0062	Antioxidant activity	[11]
13.	3'-Methoxyquercetin-3-O-[6-O-(rhamnopyranosyl) glucopyranoside] (**24**)	Leaves	0.00488	Antioxidant activity	[11]
14.	3'-Methoxyquercetin-3-O-glucopyranoside (**25**)	Leaves	0.008	Antioxidant activity	[11]
15.	Izovitexin-2"-rhamnoside (**27**)	Leaves	0.00536	Antioxidant activity	[11]
16.	Rutin (**29**)	Leaves	0.0929 ± 0.19 (dry weight)	Antioxidant activity	[54]

Continuation of table 1

Order numbers	Groups of substances / names of compounds	Morphological parts of the plant	Content, %	Pharmacological activity	Reference
17.	Kaempferol-3-O-α-L-(3″-Z, 4″-di-E-p-coumaroyl)-rhamnopyranoside (**30**)	Leaves	0.000627	Sodium-potassium adenosine triphosphatase inhibitor. Antibacterial activity against St. aureus, B. subtilis, M. luteus, S. typhimurium, Pr. vulgaris	[55]
18.	Kaempferol-3-*O*-α-L-(3″, 4″-di-Z-p-coumaroyl)-rhamnopyranoside (**31**)	Leaves	0.000307	Sodium-potassium adenosine triphosphatase inhibitor. Antibacterial activity against St. aureus, B. subtilis, M. luteus, S. typhimurium, Pr. vulgaris	[55]
19.	Kaempferol-3-*O*-α-L-(3″, 4″-di-E-p-coumaroyl)-rhamnopyranoside (**32**)	Leaves	0.00243	Sodium-potassium adenosine triphosphatase inhibitor. Antibacterial activity against St. aureus, B. subtilis, M. luteus, S. typhimurium, Pr. vulgaris	[55]
20.	Kaempferol-3-*O*-α-L-(2″-*E*, 4″-*Z*-di-p-coumaroyl)-rhamnopyranoside (**33**)	Leaves	0.00275	Sodium-potassium adenosine triphosphatase inhibitor. Antibacterial activity against St. aureus, B. subtilis, M. luteus, S. typhimurium, Pr. vulgaris	[55]
21.	Kaempferol-3-*O*-α-L-(2″, 4″-di-E-p-coumaroyl)-rhamnopyranoside (**34**)	Leaves	0.0105	Sodium-potassium adenosine triphosphatase inhibitor. Antibacterial activity against St. aureus, B. subtilis, M. luteus, S. typhimurium, Pr. vulgaris	[55]
22.	Kaempferol-3-*O*-α-L-(2″-*Z*, 4″-E-di-p-coumaroyl)-rhamnopyranoside (**35**)	Leaves	0.00349	Sodium-potassium adenosine triphosphatase inhibitor. Antibacterial activity against St. aureus, B. subtilis, M. luteus, S. typhimurium, Pr. vulgaris	[55]
23.	2′,β-Dihydroxy-α,β-dihydrochalcon-α-O-hexoside (**37**)	Leaves	+	–	[56]
24.	2′-Dihydroxy-α,β-dihydrochalcon-α-O-hexoside (**40**)	Leaves	+	–	[56]
25.	Apigenin-6,8-di-C-hexoside (**41**)	Leaves	+	–	[56]
26.	Apigenin-6-C-(2″-O-deoxyhexosyl)-hexoside (**42**)	Leaves	+	–	[56]
27.	Apigenin-8-C-hexoside (**43**)	Leaves	+	–	[56]
28.	Quercetin-3-O-(6″-O-deoxyhexosyl)-hexoside (**44**)	Leaves	+	–	[56]
29.	Tetramethoxydihydroquercetin-3-O-pentoside (**45**)	Leaves	+	–	[56]
30.	Kaempferol-3-O-(6″-O-deoxyhexosyl)-hexoside (**46**)	Leaves	+	–	[56]
31.	Quercetin-3-O-hexoside (isomer 1 and 2) (**47**)	Leaves	+	–	[56]
32.	Isorhamnetin-3-O-(6″-O-deoxyhexosyl)- hexoside (**48**)	Leaves	+	–	[56]
33.	Quercetin-3-O-pentoside (**49**)	Leaves	+	–	[56]
34.	Kaempferol-3-O-hexoside (**50**)	Leaves	+	–	[56]
35.	Quercetin-3-O-deoxyhexoside (**51**)	Leaves	+	–	[56]
36.	Isorhamnetin-3-O-hexoside (**52**)	Leaves	+	–	[56]
37.	Kaempferol-3-O-pentoside (**53**)	Leaves	+	–	[56]
38.	Kaempferol-3-O-deoxyhexoside (**54**)	Leaves	+	–	[56]
39.	Luteolin-6-C-glucoside (**59**)	Leaves	+	–	[57]
40.	Apigenin-8-C-glucoside (**60**)	Leaves	+	–	[57]
41.	Apigenin-6-C-glucoside (**61**)	Leaves	+	–	[57]

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End of table 1

Order numbers	Groups of substances / names of compounds	Morphological parts of the plant	Content, %	Pharmacological activity	Reference
42.	Quercetin-3-O-glucoside (**62**)	Leaves	+	–	[57]
43.	Kaempferol-3-O-rutinoside (**63**)	Leaves	+	–	[57]
44.	Kaempferol-3-O-glucoside (**64**)	Leaves	+	–	[57]
	Phenolic acids				
45.	3,4-Dihydroxybenzoic acid (**10**)	Leaves	до 5.0 ± 0.4 (dry weight)	–	[48]
46.	Gallic acid (**11**)	Leaves	до 1.40 ± 0.15 (dry weight)	Antioxidant activity	[48]
		Fruits	0.02	–	[61]
47.	Vanillic acid (**12**)	Leaves	до 1.40 ± 0.15 (dry weight)	Antioxidant activity	[48, 52]
48.	Rosmarinic acid (**13**)	Leaves	до 0.02 (dry weight)	Antioxidant activity	[48]
49.	Caffeic acid (**14**)	Leaves	+	Antioxidant activity	[52]
50.	Ferulic acid (**15**)	Leaves	+	Antioxidant activity	[52]
51.	3,4-Dihydroxybenzoic acid hexoside (**36**)	Leaves	+	–	[56]
52.	Coumaric acid hexoside (**39**)	Leaves	+	–	[56]
53.	Coumaric acid (**65**)	Leaves	+	–	[59]
54.	2-Hydroxycinnamic acid (**66**)	Leaves	+	–	[59]
	Anthocyanins				
55.	Cyanidin-3-O-glucoside (**5**)	Fruits	0.56	–	[47]
56.	Cyanidin-3-O-rutinoside (**6**)	Fruits	0.73	–	[47]
57.	Peonidine-3-O-glucoside (**7**)	Fruits	0.0063 в сумме	–	[47]
58.	Peonidine-3-O-rutinoside (**8**)	Fruits	+	-	[47]
	Phenolic glycosides				
59.	2-(4-Hydroxy-3-methoxy-phenyl)-ethyl-O-β-D-glucopyrano-side (**2**)	Leaves	0.00032	–	[45]
60.	1-(2′-Hydroxyphenyl)-1-hydroxy-phenylpropane-α-O-hexoside (**38**)	Leaves	+	–	[56]
	Flavan-3-ols				
61.	Catechin (**26**)	Leaves	0.00916	Antioxidant activity	[11]
			1.06	–	[61]
62.	Epicatechin hexoside (**55**)	Leaves	+	–	[57]
63.	(+)-Gallocatechin (**56**)	Leaves	+	–	[57]
64.	(+)-Catechin (**57**)	Leaves	+	–	[57]
65.	(-)-Epicatechin (**58**)	Leaves	+	–	[57]
66.	Epicatechin (**67**)	Leaves	1.29	–	[61]
		Fruits	0.65		
67.	Epigallocatechin (**68**)	Leaves	0.40	–	[61]
		Fruits	0.51		
68.	Epicatechin gallate (**69**)	Fruits	0.16	–	[61]
69.	Cinnamtannin B1 (**28**)	Leaves	0.00092	Antioxidant activity	[11]

** – the number of the compound in the text of the article;*
*** – the compound was found out but its quantitative content was not established;*
**** – the activity of this compound was not determined in this study*

Similar changes, i.e., the destruction of hydroxycinnamic acids and flavonoids and an increase in the content of gallic acid, were known before, but no reasons had been established [51]. According to the authors, it is quite possible, that the stage of thawing of plant material after freeze-drying could lead to a loss in the content of hydroxycinnamic acids and flavonoids.

Using high-performance liquid chromatography (HPLC), M. Muchuweti et al. [52] established the presence of caffeic **(14)**, ferulic **(15)** and vanillic **(12)** acids in the laurel leaf extracts (see Table 1).

The antioxidant activity and the total amount of the phenolic compounds of some spices (*Mentha piperita* L., *Rhus coriaria* L., *Thymbra spicata*, *Salvia officinalis*, *Rosmarinus officinalis* L., *Capparis ovata* L., *Origanum vulgare* L., *Laurus nobilis* L. and *Capsicum annum* L.) were determined by A. Ünver et al. [53]. The highest values of the antioxidant activity in TEAC units (Trolox Equivalent Antioxidant Capacity) were obtained for sage (1.783) and rosemary (1.241). For the extraction from *Laurus nobilis* L. leaves, it amounted to 1.001 ± 0.020 mmol TE/g of the extract. The values of the antiradical activity were IC_{50}, mg/ml) – 1.901 ± 0.034 mg/ml. The total amount of phenolic compounds (colourimetry with the Folin – Ciocalteu reagent) was 288.15 ± 1.34 mg/g of the extract, in terms of the equivalent amount of gallic acid.

Phytochemical studies of the infusion of the *Laurus nobilis* L. leaves collected in November 2003 in S. Basilio (Cagliari, Sardinia, Italy) were carried out by a group of authors [11] using semi-preparative HPLC with a diode matrix as a detector and tandem mass spectrometry. The following substances were found in the aqueous leaf infusion: kaempferol-3-O-glucopyranoside (16); kaempferol-3-O-rhamnopyranoside **(17)**; kaempferol-3-O-(2”, 4”-di-E-p-coumaroyl)-rhamnoside **(18)**; kaempferol-3-O-arabinopyranoside **(19)**; kaempferol-3-O-[6-O-(rhamnopyranosyl) glucopyranoside] **(20)**; quercetin-3-O-glucopyranoside **(21)**; quercetin-3-O-rhamnopyranoside**(22)**;quercetin-3-O-[6-O-(rhamnopyranosyl) glucopyranoside] **(23)**; 3’-methoxyquercetin-3-O-[6-O-(rhamnopyranosyl) glucopyranoside] **(24)**; 3’-methoxyquercetin-3-O-glucopyranoside **(25)**; catechin **(26)**; 2”-rhamnosylisovitexin **(27)**; cinnamtannin B1 **(28)** (see Table. 1).

The content of kaempferol and quercetin derivatives in the infusion was 0.31 ± 0.01 mg/100 ml and 2.11 ± 0.01 mg/100 ml, respectively. Thus, by the researchers’ data, per 200 ml of infusion, the quantitative content of flavonoids was approximately 5.0 mg.

M. Lu et al. [54] found out the presence of flavonoids and phenolic acids in ethanol extracts of *Laurus nobilis L.* leaves. The content of phenolic acids established by the method of ultra-performance liquid chromatography, was 474.1 ± 12.7 (mg/g dry weight), rutin **(29)** –929.4 ± 19.3 (μg/g dry weight) and unidentified flavonoids – 2138.2 ± 42.7 (mg/g dry weight). The total amount of phenolic compounds (colorimetry according to Folin – Ciocalteu) in gallic acid equivalents and dry raw materials was 46.79 ± 3.22 mg/g.

B. Kaurinovic et al. studied the leaves of cultivated laurel collected in June 2008 in the vicinity of Ulcinj (Montenegro) [42]. The amount of flavonoids in the dried leaves was determined by the colourimetric method based on the property of flavonoids and flavone glycosides to form complexes with aluminium ions. The absorption of the investigated solutions was measured at λ = 430 nm.

The determination results are shown in Table 2.

The maximum amount of flavonoids was found in the ethyl acetate fraction and the smallest in water. The results of the study of the antiradical activity of these extracts against free radicals (DPPH, NO, $O_2^{\bullet-}$) are presented in Table 3. Ethyl acetate extract showed the strongest inhibitory effect since the IC_{50} value was reached at the lowest concentration.

The results obtained, characterise the pronounced inhibitory effect of flavonoids from ***Laurus nobilis L.*** leaves against DPPH radicals.

Solid amorphous substances have been isolated from the ***Laurus nobilis L.*** leaves purchased in Turkey (Orege Forest Agricultural and Food Products Foreign Trade Ltd.) in August 2007, as a result of extraction (CH_2Cl_2, MeOH), subsequent fractionation and separation, using normal phase vacuum flash chromatography on silica gel and semi-preparative HPLC [55]. Metabolites have been identified as kaempferol-3-O-α-L-(3”-Z, 4”-E-di-p-coumaroyl) -rhamnopyranoside **(30)**, kaemperol-3-O-α-L-(3”, 4”-di-Z-p-coumaroyl)-rhamnopyranoside **(31)**, kaempferol-3-O-α-L-(3”, 4”di-E-p-coumaroyl)-rhamnopyranoside **(32)**, kaempferol-3-O-α-L-(2”-E, 4”-Z-di-p-coumaroyl)-rhamnopyranoside **(33)**, kaempferol-3-O-α-L-(2”,4”-di-E-p-cumaroyl)-rhamnopyranoside **(34)** and kaempferol-3-O-α-L-(2”-Z, 4”-E-di-p-coumaroyl)-rhamnopyranoside **(35)** (see Table 1).

All the compounds have been tested in vitro for their ability to inhibit sodium-potassium adenosine triphosphatase isolated from pig cerebral cortex.

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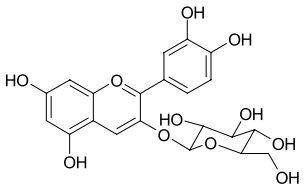

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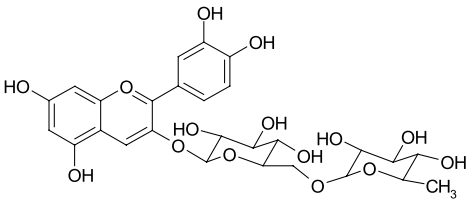

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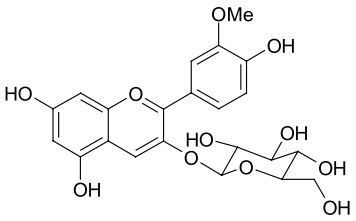

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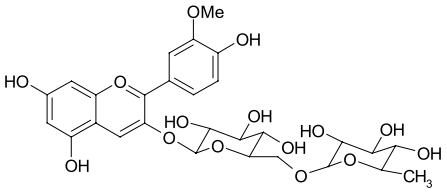

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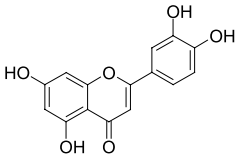

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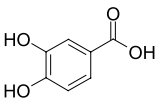

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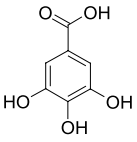

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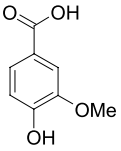

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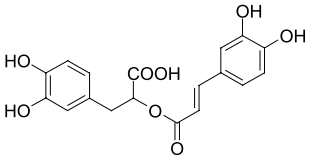

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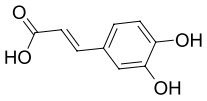

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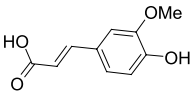

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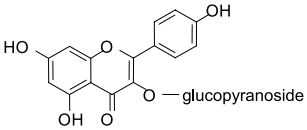

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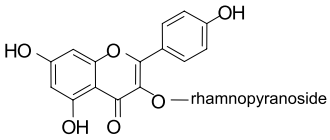

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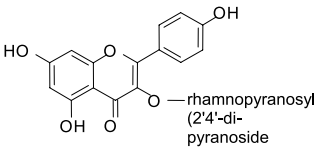

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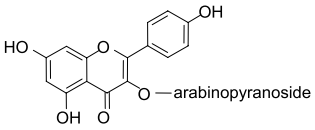

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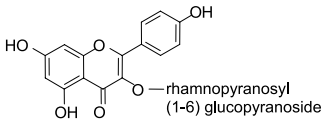

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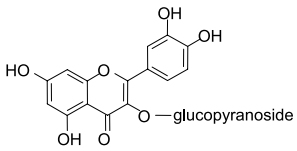

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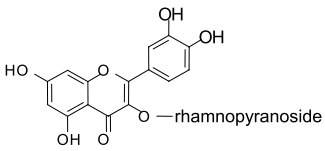

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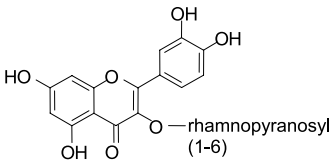

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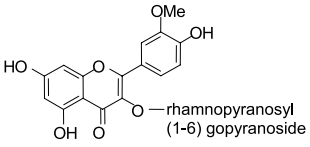

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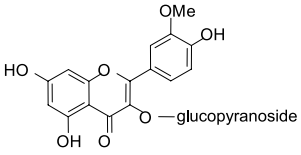

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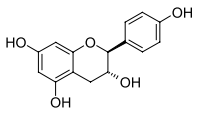

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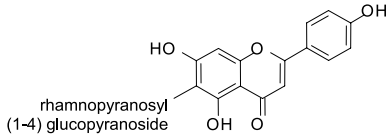

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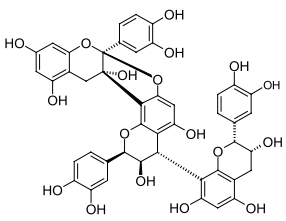

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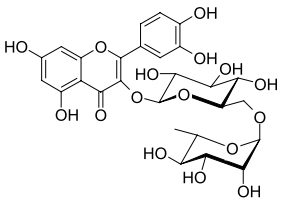

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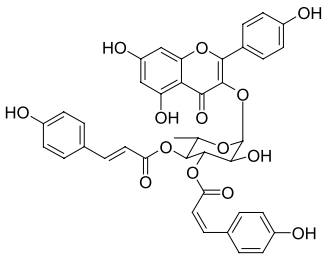

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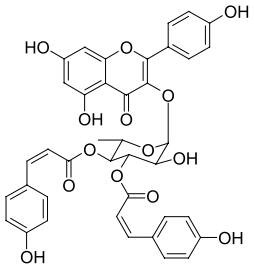

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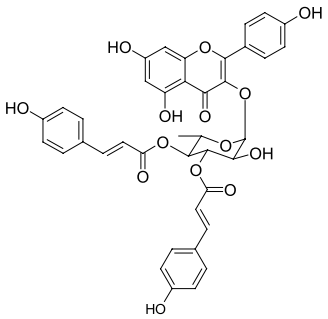

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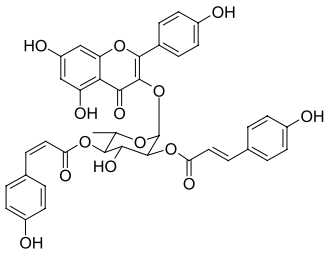

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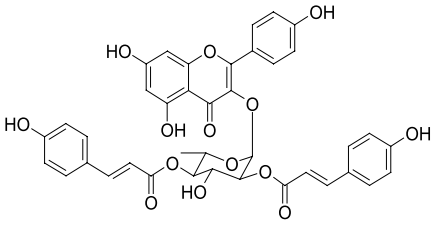

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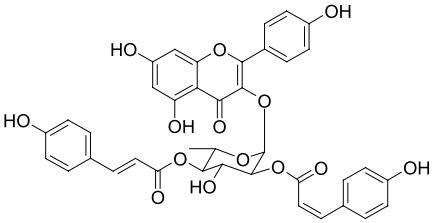

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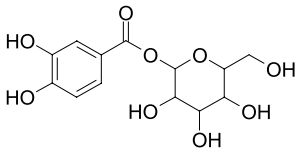

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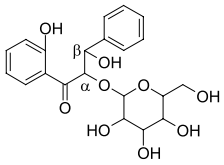

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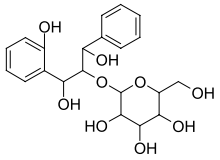

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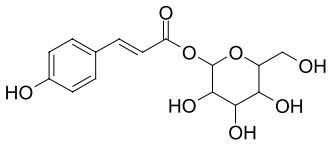

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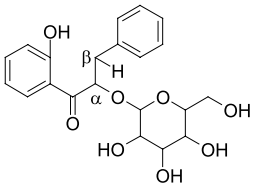

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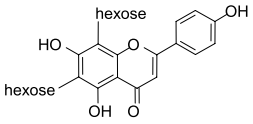

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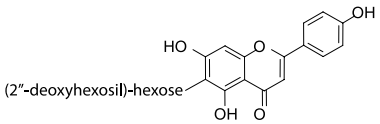

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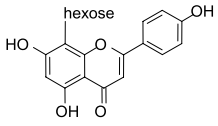

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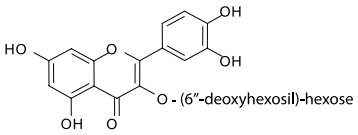

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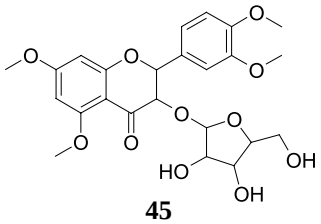

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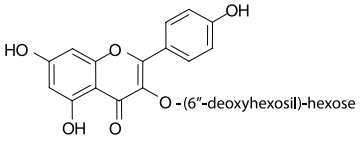

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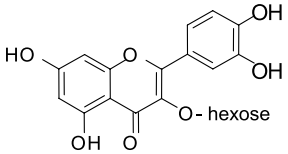

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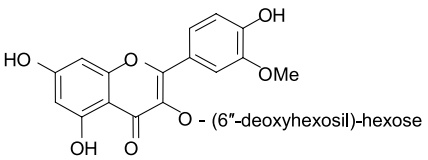

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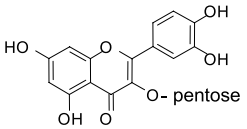

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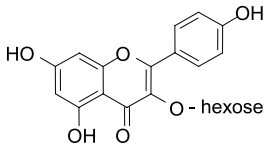

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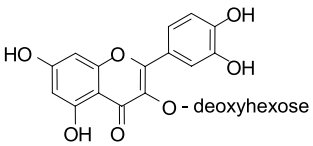

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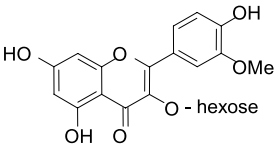

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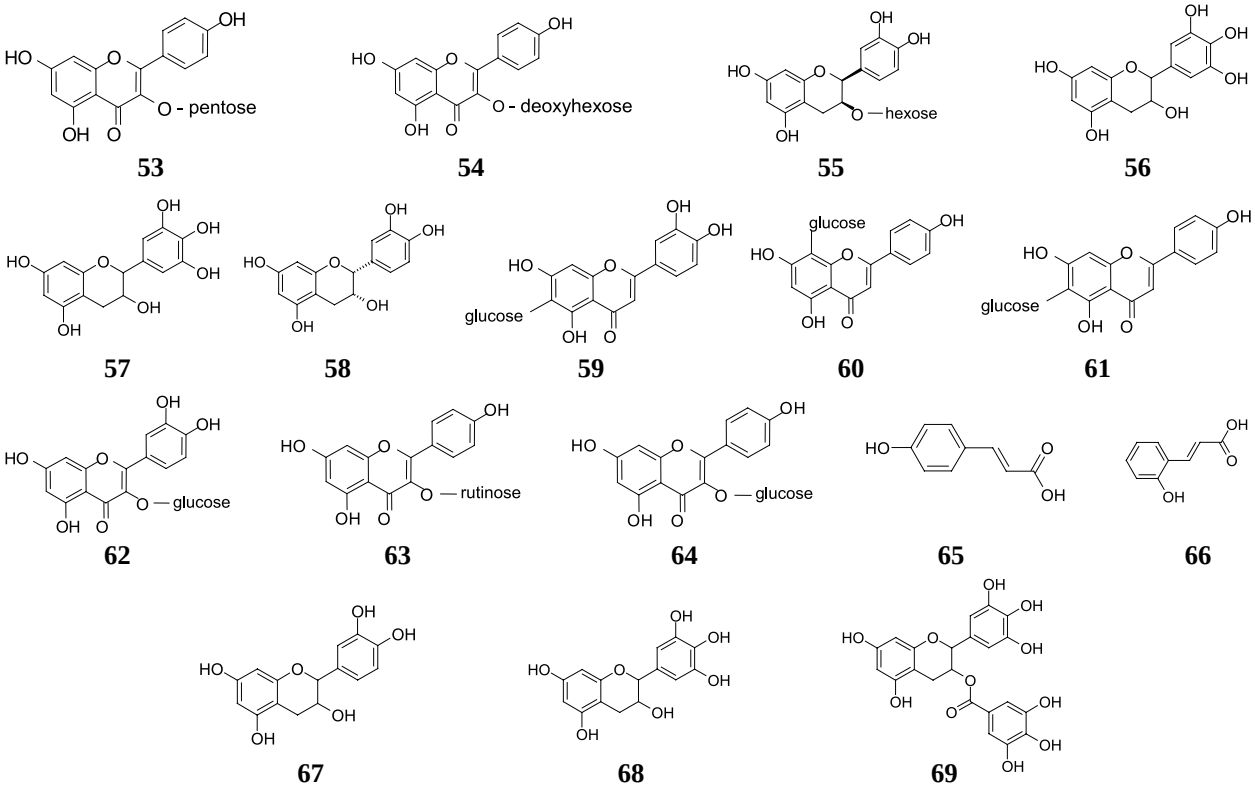

Table 2 – The content of the total amount of flavonoids in the extracts from *Laurus nobilis L.* leaves according to Kaurinovic et al. [42]

The total amount of flavonoids (mg/g) in the extracts				
Ethanolic	Chloroformic	Ethyl acetate	Butanol	Aqueous
0.76	1.02	1.56	1.07	0.68

Table 3 – Antiradical activity of extracts from *Laurus nobilis L.* leaves according to Kaurinovic et al [42]

Radicals	IC_{50} (μg/cm³)				
	Extracts				
	Ethanol	Chloroformic	Ethyl acetate	Butanol	Aqueous
DPPH*	127.38	139.42	83.24	181.35	161.83
$O_2^{\bullet-}$	327.60	429.43	163.57	288.64	486.32
NO	168.77	322.84	158.63	386.80	618.42

*Note: * – DPPH – 1,1-diphenyl-2-picrylhydrazyl*

Table 4 – The inhibitory activity of kaempferol glycosides from the *Laurus nobilis L.* leaves in regards to sodium-potassium adenosine triphosphatase according to Lee et al. [55]

Order numbers	Compounds	IC_{50} (μM)
1 (**30**)*.	Kaempferol-3-O-α-L-(3"-Z, 4"-E-di-p-coumaroyl)-rhamnopyranoside	6.4±0.3
2 (**31**).	Kaempferol-3-O-α-L-(3", 4"-di-Z-p-coumaroyl)-rhamnopyranoside	10.4±0.6
3 (**32**).	Kaempferol-3-O-α-L-(3",4"-di-E-p-coumaroyl)-rhamnopyranoside	5.0±0.1
4 (**33**).	Kaempferol-3-O-α-L-(2"-E,4"-Z-di-p-coumaroyl)-rhamnopyranoside	4.0±0.1
5 (**34**).	Kaempferol-3-O-α-L-(2",4"-di-E-p-coumaroyl)-rhamnopyranoside	5.2±0.2
6 (**35**).	Kaempferol-3-O-α-L-(2"-Z,4"-E-di-p-coumaroyl)-rhamnopyranoside	5.1±0.1
7.	Kaempferol**	>669.3
8.	Afzelin**	>463.0
9.	*p*-Coumaric acid**	>1218.0
10	Ouabain**	4.6±0.1

*Note: * – The number indicated in brackets, corresponds to the connection number in the text.*
*** – The compounds used as reference samples.*

Table 5 – Groups of phenolic compounds identified in *Laurus nobilis* L. leaves and extracts from them (mg/g, n = 18) [58]

Sample of raw materials/extract	The quantitative content of phenolic compounds			
	Flavan-3-ols	Flavones	Flavonoids	Phenolic compounds
Cultivated	*56 ± 8	4.4 ± 0.2	26 ± 2	86 ± 11
Wild	60 ± 4	2.6 ± 0.4	7 ± 2	71 ± 6
Methanolic extract	63.6 ± 0.4	4 ± 1	19 ± 10	86 ± 11
Aqueous extract	52 ± 5	3 ± 1	15 ± 9	70 ± 5

*Note: * – average value.*

Table 6 – Results of the quantitative determination of phenolic compounds in the leaves and shoots of *Laurus nobilis* L., according to Musienko and Kyslychenko [60]

Order numbers	Quantitative content (x ± Δx),% in terms of dry raw materials (n = 5)		
	Oxidizable phenols	Hydroxycinnamic acids	Flavonoids
Shoots			
1	4.80 ± 0.12	1.35 ± 0.08	0.85 ± 0.03
2	4.54 ± 0.17	1.29 ± 0.07	0.81 ± 0.07
Leaves			
1	5.25 ± 0.16	1.73 ± 0.05	0.95 ± 0.06
2	5.04 ± 0.11	1.71 ± 0.05	0.91 ± 0.05

Note. 1 – a sample from the vicinity of Alushta, 2 – a sample from the vicinity of Rybachye

Table 7 – The quantitative content of phenolic compounds and flavonoids in Laurus nobilis L. leaves, according to Vinha et al. [62]

Extracts	Phenolic compounds, mg/g, in terms of gallic acid	Flavonoids, mg/g, in terms of epicatechin
Aqueous	14.37±0.79	14.12±0.93
Hydroalcoholic (water-ethanol 1: 1)	43.03±0.35	30.15±0.25
Alcoholic	31.09±0.31	20.88±0.88

Table 8. Composition of monomeric (catechin and epicatechin) and oligomeric flavan-3-ols (A-type proanthocyanidins) in Laurus nobilis L. leaves, according to Vinha et al. [62]

Flavan-3-ols	Extracts		
	Aqueous	Hydroalcoholic (water-ethanol 1: 1)	Alcoholic
(+)-Catechin	0.41*	0.58	0.04
(-)-Epicatechin	0.99	3.44	0.67
Amount of the monomers	1.40	4.02	0.71
Dimeric proanthocyanidins	1.49	16.97	5.25
Trimeric proanthocyanidins 1	0.48	1.24	0.32
Trimeric proanthocyanidins 2	1.73	5.05	2.46
Tetrameric proanthocyanidins	1.02	1.16	0.32
Amount of flavan-3-ols	6.12	28.44	9.06

** – the results are given in mg/g in terms of epicatechin and dry weight.*

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The comparison of the relationship between the structure and activity of acylated kaempferol glycosides shows that substance 3**(32)**, which has an E-p-coumaroyl group in position C-3" in the rhamnopyranoside ring, was more active than substances 1(**30**) and 2**(31)** with Z-p-coumaroyl groups in position C-3". Substances 5**(34)** and 6**(35)** had almost the same IC_{50} values in regards to Na / K adenosine triphosphatase. Of all the tested substances, substance 4**(33)** showed the most powerful inhibitory potential. According to the authors' data, the presence of E-p-coumaroyl in position C-2"and Z-p-coumaroyl in position C-4" in the rhamnopyranoside ring, determined a high inhibitory activity of the studied compounds. Sodium-potassium adenosine triphosphatase inhibitors are known to have a significant therapeutic potential for some heart diseases, such as heart failure and cardiac arrhythmias. Therefore, the obtained results allow us to further continue the research in this direction. The antibacterial activity of acylated kaempferol glycosides has also been studied in regards to several gram-positive and gram-negative bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Salmonella typhimurium*, *Proteus vulgaris*, *Escherichia coli*. Substances 1–6 (Table 4) showed an inhibitory activity in regards to all the studied bacteria, with the exception of E. coli. Substances 4**(33)** and 6**(35)** showed a minimum inhibitory concentration in the range of 0.65–2.08 μg/ml. The activity of the studied compounds was slightly inferior to the effect of the ampicillin comparison drug.

S. Pacifico et al., isolated and identified more than 20 phenolic compounds [56] from the polar fractions of the methanol extract of the *Laurus nobilis* L**.** leaves collected in Caserta (Italy) in May 2011. They are: hexoside 3,4-dihydroxybenzoic acid **(36)**; 2',β-dihydroxy-α, β-dihydrochalcon-α-O-hexoside **(37)**; 1-(2'-hydroxyphenyl)-1-hydroxyphenylpropan-α-O-hexoside **(38)**; coumaric acid hexoside **(39)**; 2'-hydroxy-α, β-dihydrochalcon-α-O-hexoside **(40)**; apigenin-6,8-di-C-hexoside **(41)**; apigenin-6-C- (2"-O-deoxyhexosyl) hexoside **(42)**; 8-C-hexosyl apigenin **(43)**; quercetin-3-O-(6"-O-deoxyhexosyl) hexoside **(44)**; tetramethoxydihydroquercetin-3-O-pentoside **(45)**; kaempferol-3-O-(6"-O-deoxyhexosyl) hexoside **(46)**; quercetin-3-O-hexoside (isomers 1 and 2) **(47)**; isorhamnetin-3-O-(6"-O-deoxyhexosyl) hexoside **(48)**; quercetin-3-O-pentoside **(49)**; cinnamtannin B1 **(28)**; kaempferol-3-O-hexoside **(50)**; quercetin-3-O-deoxyhexoside **(51)**; isorhamnetin-3-O-hexoside **(52)**; kaempferol-3-O-pentoside **(53)**; kaempferol-3-O-deoxyhexoside **(54)** (see Table 1).

The fractions which these compounds had been isolated from, showed their high antioxidant activity. The authors of the study have arrived at the conclusion that the extracts, rich in phenolic compounds from Laurus nobilis L. leaves, are of interest from the point of view of searching effective herbal remedies in the prevention and treatment of Alzheimer's and other age-related degenerative diseases.

A study by M. Dias et al. was aimed at a comparative study of cultivated and wild Laurus nobilis L. leaf samples by their nutritional value, some groups of natural compounds, including phenolic ones [57]. For that, a sample of raw materials (air-dried leaves) from cultivated plants was purchased from Ervital in Castro Daire, Portugal. According to the manufacturer, the leaves were collected in 2012. Wild raw materials (fresh leaves) were harvested in the autumn of that year in Bragança, Portugal, and subsequently dried. The both samples were lyophilised to preserve, as far as possible, their native chemical composition for the analysis. Phenolic compounds were determined by HPLC and identified by their UV and mass spectra, retention times and comparison with the standard samples. The phenolic profile of the studied samples was characterised by the presence of flavan-3-ols, flavonols and flavones. The compounds of these groups included: epicatechin hexoside **(55)**, (+)-gallocatechin **(56)**, procyanidin tetramer, (+)-catechin **(57)**, procyanidin dimer, (-)-epicatechin **(58)**, procyanidin tetramer (A- and B-type bonds), procyanidin trimer, luteolin 6-C-glucoside **(59)**, apigenin 8-C-glucoside **(60)**, 2"-O-rhamnosyl-C-hexosyl-apigenin, quercetin 3-O-rutinoside (29), apigenin 6-C-glucoside **(61)**, quercetin 3-O-glucoside **(62)**, quercetin O-hexoside, kaempferol 3-O-rutinoside **(63)**, quercetin O-pentoside, kaempferol 3-O-glucoside **(64)** (see Table 1), isorhamnetin O-rutinoside, quercetin O-rhamnoside, isorhamnetin O-hexoside, kaempferol O-pentoside, isorhamnetin O-pentoside, kaempferol O-hexoside, isorhamnetin O-rhamnoside.

The cultivated raw materials contained phenolic substances in higher concentrations, especially derivatives of flavones and flavonols. However, the flavan-3-ols content was similar in the both samples. It was this group of phenolic compounds that was predominant in the cultivated and wild-growing Laurus nobilis L. Methanol extract and the infusion obtained from the leaf sample of cultivated plants, in addition, showed their higher antioxidant activity.

The studies carried out by these authors later [58], revealed the (*in vitro*) activity of phenolic extracts against human tumour cell lines, as well as bacterial and fungal cells. It was established that the extracts from the samples of wild Laurus nobilis L. leaves, inhibited tumour cell lines stronger (HeLa, MCF7, NCI-H460, and HCT15). Methanol extracts had a higher antibacterial activity. According to the authors, the differences in their biological activity could be associated with different contents of phenolic compounds.

According to the results presented in Table 5, the cultivated samples showed higher concentrations of flavonoids and flavones. On the other hand, methanol extracts were characterised by a high content of flavan-3-ols.

The dried *Laurus nobilis* L. leaves purchased on the market in Saltillo, Coahuila, Mexico, in November 2010,

were investigated for the content of phenolic compounds and the influence of several experimental factors on the processes of their extraction from the raw materials. Among the studied factors, special attention was paid to the ratio of the solid liquid and the solvent concentration [59]. The best results were obtained by ultrasonic extraction of 1 g of a plant sample with 12 ml of 35% ethanol for 40 minutes. The yield of phenolic substances was 17.32 ± 1.52 mg/g. HPLC analysis revealed the presence of two phenolic acids in the extracts - coumaric **(65)** and 2-hydroxycinnamic **(66)**.

A study of the chemical composition of the two leaf and shoot samples of Laurus nobilis L collected in November 2013 in the Crimea in the vicinity of Alushta (1) and the village of Rybachye (2) showed, that they contain carbohydrates, fatty acids, amino acids, and phenolic substances [60]. The authors of the study determined the content of the main groups of biologically active substances, including phenolic compounds, in different samples of shoots and leaves of *Laurus nobilis* L. See Table 6.

As follows from the data presented in Table 6, the content of oxidizable phenols, hydroxycinnamic acids and flavonoids in the leaf samples did not differ significantly. Hereby, the total amount of oxidizable phenols was at least 4.5%, the amount of hydroxycinnamic acids was at least 1.3%, and the amount of flavonoids was at least 0.8%. The data obtained by the authors show, that the content of the studied groups of phenolic compounds is slightly higher in the samples of Laurus nobilis L. leaves in comparison with the shoots. Sample No. 1 of the leaves and shoots of Laurus nobilis L. showed the highest content of phenolic compounds.

The raw materials of the *Laurus nobilis* L. (leaves and fruits), harvested in the Crimea in 2013, was studied by HPLC-UV method [61]. Three compounds of the flavan nature were found out in Laurus nobilis L. leaves. The dominant component was epicatechin **(67)** with its content of 1.29%. In addition, catechin (1.06%) and epigallocatechin (0.40%) **(68)** were found out. In the fruits, 3 compounds of the flavan nature were also established, the dominant components being epicatechin (0.65%) and epigallocatechin (0.51%). The chemical difference between the studied raw materials samples was the presence of catechin in the leaves and gallic acid (0.02%) and epicatechin gallate (69) (0.16%) in the fruits.

The phenolic profile and the antioxidant activity of *Laurus nobilis* L. leaves collected in northern Portugal, the Azores, and Madeira, were analysed by A. Vinha et al. [62]. The dried leaves were used to obtain aqueous, alcoholic and hydroalcoholic (water-ethanol 1: 1) extracts. The phenolic profile of the extracts was determined using HPLC with a diode array detector combined with a mass spectrometer. The results of the study are presented in Tables 7 and 8.

In the study with 2,2-diphenyl-1-picrylhydrazyl, the highest antioxidant activity was detected in alcoholic extract and the lowest one – in aqueous.

CONCLUSION

The data presented in the review, characterize the leaves, fruits, and shoots of the *Laurus nobilis* L. as valuable raw materials for phenolic compounds, such as phenolic acids, flavonoids, proanthocyanidins, etc. Their total amount in the leaves can reach 99.7 g/kg (in terms of gallic acid). In the fruits of *Laurus nobilis* L., anthocyanins are usually accumulated in the quantity up to 217 mg in terms of cyanidin-3-glucoside/g of seed-free raw materials. The quantitative content of these groups of substances varies depending on the place of collection, the source of the raw materials (cultivated or wild plants), the time (phase) of their harvest, the method of drying and extraction from raw materials, etc. According to the results of the studies, the phenolic compounds of *Laurus nobilis* L. exhibit pronounced antioxidant and antiradical activity, have an inhibitory effect on the production of NO, sodium-potassium adenosine triphosphatase, on the tumour cells line (HeLa, MCF7, NCI-H460 and HCT15). They are characterised by an antibacterial action against gram-positive and gram-negative bacteria.

A further, more rigorous research of the Laurus nobilis L. cultivated in Russia, is relevant, and it will make it possible to assess the quality of its raw materials by the content of phenolic compounds and to develop methods for its standardization for this group of active substances. In addition, the development of regulatory documentation for raw materials of *Laurus nobilis* L. will significantly expand the introduction of new medicines based on it, into medical practice.

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AUTHOR CONTRIBUTIONS

All authors have equally contributed to the research work.

CONFLICTS OF INTEREST

The authors of this paper report no conflicts of interest.

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COLLAGENASES IN MEDICAL PRACTICE: MODERN COLLAGENASE-BASED PREPARATIONS AND PROSPECTS FOR THEIR IMPROVEMENT

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The aim of this study was to assess the coverage of studies of collagenolytic enzymes (collagenases) in the sphere of their use in wounds and scars treatment, the resource of their production and the range of collagenase products to identify the areas for their improvement.

Materials and methods. The information from retrieval and library databases (eLIBRARY, PubMed, Scopus, ScholarGoogle, ResearchGate), patent information databases (freepatent.ru, ntpo.com, fips.ru), the State register of medicines (GRLS) and the State register of medical devices, as well as technical information provided by manufacturers of medicines and medical devices, was used in the article.

Results. The analysis of the information database has shown that collagenolytic enzymes are effective proteolytic complexes because of their ability to provide the breakdown of collagen, the main component of wounds and scars.

Hepatopancreas crustaceans is currently one of the available raw resources of collagenases in Russia. It is noted that proteolytic enzymes from the Paralithodes camtschatica hepatopancreas are characterized by a broad specificity: they are able to hydrolyze both native collagen and other protein substrates. There are data confirming the capability of collagenases to accelerate the process of reparation in addition to wound cleansing from a necrosis. The results of clinical studies of collagenases anti-scar properties, indicate the effectiveness of their use for the skin scar correction. The content analysis has shown that there is a small amount of collagenase-based products in the Russian pharmaceutical market: lyophilized powder for preparation of the injection solution “Collalysin”, recommended for scars treatment; a medical dressing “Digestol” with collagenase, recommended for wounds and necrotic lesions treatment; “Fermencol” (gel and powder), the “Karipain plus” gel for scars treatment. Drugs are represented by only powder lyophilisate “Collalysin”.

Conclusion. The development of gel compositions (Aerosil-based oleogels) and atraumatic dressings with collagenase from Paralithodes camtschatica hepatopancreas as the most affordable raw materials can be considered problem number one of practical pharmacy at present. This provides for the creation of the dosage forms, improved in terms of stability and efficiency, as well as ease of use.

Keywords: collagenolytic enzymes, Paralithodes camtschatica collagenase, scars, wounds, preparations, gels

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КОЛЛАГЕНАЗЫ В МЕДИЦИНСКОЙ ПРАКТИКЕ: СОВРЕМЕННЫЕ СРЕДСТВА НА ОСНОВЕ КОЛЛАГЕНАЗЫ И ПЕРСПЕКТИВЫ ИХ СОВЕРШЕНСТВОВАНИЯ

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Цель. Оценка состояния изученности применения коллагенолитических ферментов (коллагеназ) в терапии ран и рубцов, источников их получения, ассортимента средств с коллагеназой для выявления направлений их совершенствования.

Материалы и методы. В работе использованы информационно-поисковые и библиотечные базы данных (eLIBRARY, PubMed, Scopus, ScholarGoogle, ResearchGate), патентно-информационные базы (freepatent.ru, ntpo.com, fips.ru), Государственный реестр лекарственных средств и Государственный реестр медицинских изделий, а также техническая информация, представленная производителями лекарственных средств и медицинских изделий.

Результаты. Анализ информационных источников показал, что коллагенолитические ферменты являются эффективными протеолитическими комплексами, т.к. обладают способностью обеспечивать расщепление коллагена, являющегося главным компонентом ран и рубцов. Одним из доступных сырьевых источников коллагеназ в России в настоящее является гепатопанкреас ракоообразных. Отмечается, что для протеолитических ферментов из гепатопанкреаса камчатского краба характерна широкая специфичность: они способны гидролизовать как нативный коллаген, так и другие белковые субстраты. Имеются данные исследований, подтверждающие, что коллагеназы наряду с очисткой раны от некроза способны ускорять процесс ее репарации. Результаты клинических исследований противорубцовых свойств коллагеназ свидетельствуют об эффективности их использования для коррекции рубцовых изменений кожи. Контент-анализ показал, что на российском фармацевтическом рынке присутствует незначительное количество средств на основе коллагеназ – лиофилизированный порошок для приготовления раствора для инъекций («Коллализин»), рекомендуемый для лечения рубцов; медицинская повязка с коллагеназой, рекомендуемые для лечения ран и некротических поражений «Дигестол»; гель и порошок «Ферменкол», гель «Карипаин плюс» для лечения рубцов. Лекарственные препараты представлены только порошком-лиофилизатом «Коллализин».

Заключение. Разработка состава гелей (олеогелей на основе аэросила) и атравматических салфеток с коллагеназой из гепатопанкреаса краба как наиболее доступного сырья можно считать актуальной задачей практической фармации. Это предусматривает создание лекарственных форм, улучшенных с позиций стабильности и эффективности, а также удобства использования.

Ключевые слова: коллагенолитические ферменты, коллагенза камчатского краба, рубцы, раны, препараты, гели

INTRODUCTION

The problem of effective and rapid healing of wounds resulting from injuries of different origins, remains one of the most relevant in modern medical practice. Currently, the effectiveness of wound healing is determined not only by the term, but also by the aesthetic result achieved. In addition, special attention is paid to the treatment convenience to combine the optimal therapeutic effect and a patient's life quality [1].

In medical practice, preparations of proteolytic enzymes are used to accelerate the granulation process and reduce the time of wound healing by the ability to cleanse wounds from necrotic tissues and exudate. Preparations of proteolytic enzymes (collagenase, hyaluronidase) are also used for scars medical correction [2, 3].

The use of enzyme preparations in the composition of external therapy, has some peculiarities caused mainly by their instability, which makes it relevant to improve the existing dosage forms both in terms of stability and efficiency, and ease of use. Collagenases are one of the most effective proteolytic enzymes due to their ability to provide collagen, the main component of wounds and scars. Thus, studuing the problems related

to collagenolytic enzymes raw resources, mechanisms of their action, clinical efficacy of collagenases in wounds and scars, as well as the range and features of the use of collagenase-based products in medical practice, are relevant.

THE AIM of the review is to assess the research of collagenolytic enzymes (collagenases) use in the treatment of wounds and scars, the sources of their production, the range of collagenase products to identify the areas for their improvement.

MATERIALS AND METHODS

The information from retrieval and library databases (eLIBRARY, PubMed, Scopus, ScholarGoogle, ResearchGate for the time interval from 2000 to 2019), patent information databases (freepatent.ru, ntpo.com, fips.ru), reference literature, the State Register of Medicinal Remedies (SRMR) and the State Register of Medical Products, (SRMP) as well as technical information provided by manufacturers of medicines and medical products, the sites dedicated to cosmetic products "Fermencol" and "Karipain", is used in the article. The depth of the patent search was 30 years. The keywords in the search process are: collagenase, collagenolytic enzymes, enzyme immobilization, wounds, treatment, scars.

RESULTS AND DISCUSSION

The role of enzyme preparations in the correction of pathological wound healing

Wound healing is a complex biological process that consists of overlapping phases: inflammation, proliferation, and remodeling. Wound healing is a regulated process in which several cell types (keratinocytes, fibroblasts, endothelial cells, macrophages and platelets) and a network of signaling molecules (cytokines, chemokines and growth factors) are in effect. In its course, the disorders which can lead to hard-to-heal chronic wounds or scars are possible. The most common factors impeding normal healing are diabetes, venous disease, old age, peripheral neuropathy, impaired microflora and malnutrition. A wound is considered chronic when it does not show a tendency to repair for more than 4 weeks. So, according to the data, full and complete wound closure, is achieved in only 25-50% of cases of chronic or hard-to-heal wounds after 20 weeks' treatment, especially in the case of venous and diabetic ulcers. Chronic hard-to-heal wounds are often characterized by a number of microbiological (increased microbial contamination, including the presence of biofilms), biochemical and cellular pathologies that prevent or slow down the process of healing. Unlike acute wounds, chronic wounds are not completed by the process of remodeling, but are considered as a process stopped at the stage of inflammation or proliferation [1–4].

The presence of cellular detritus, necrotic tissues and bacterial toxins leads to the prolongation of inflammation and increased production of cytokines by macrophages and neutrophils, resulting in the activation of macrophages and fibroblasts. An excessive activity of tissue proteases is observed [3].

The so-called "wound bed preparation" technology is used to remove the necrotic component to transfer the wound from a chronic state to an acute one. Traditionally, to remove necrotic, damaged or infected tissues, different methods are used: surgical, autolytic, enzymatic, chemical and physical. Wound cleansing helps to reduce the presence of inflammatory cytokines and metalloproteinases, which are produced during chronic wounds inflammation [4].

One of the problems of pathological wound healing is the scars (keloid and hypertrophic) formation, which occurs when the regulation by fibroblasts and keratinocytes balance of collagen synthesis-breakdown process disrupts, and the disturbance of the collagen remodeling process takes place. A stimulating effect on fibroblasts is provided by a chronic inflammation of the scar tissue, a long-term wound healing, secondary infections and epithelialization disruption [1].

Enzymatic wound cleansing is an effective and selective method, often used in combination with other methods of treatment, e.g., in combination with moisturizing dressings. In chronic wounds, it is necessary not only for cleansing (removal of necrotic tissue), but also for the migration of cells involved in epithelialization, as well as the elimination of inflammation. Unlike acid preparations used for treatment of wounds with a high content of necrotic tissues, e.g., the ointment containing salicylic acid 40%, proteases have no effect on intact tissues [2, 3].

Collagen is the most stabile protein of tissue detritus. In this case, the wounds containing collagen fibers, hardly give way to enzymatic cleansing by means of other proteolytic enzymes: trypsin, chymopsin, papain, terrilitin, streptokinase, etc. [2]. Collagenase preparations are successfully used to treat wounds with massive purulonecrotic discharge, trophic ulcers, frostbite, burns, scars. Proteolytic enzymes with collagenolytic activity are the most effective for the wounds' treatment and elimination of scarring [5–11].

Characteristics of collagenases used in medical practice: sources of production, mechanism of action, data of pharmacological and clinical studies

Collagenase is a specific proteolytic enzyme that breaks down peptide bonds in natural collagen, the main structural element of a connective tissue. According to the active ingredient resource, collagenase preparations are biological agents, because their industrial raw materials are microorganisms' cultures or animals' digestive glands.

The analysis of the commercial drugs, veterinary and medical products, cosmetics composition with collagenases showed, that modern products contain enzyme

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complexes, the source of which are the Clostridium family bacteria or digestive tract glands (hepatopancreas) of the *Paralithodes camtschaticus* [12–15].

Collagenases derived from Clostridium histolyticum, the most commonly used drug-derived microbial proteases, are single-stranded proteins with masses ranging from 68 kDa to 130 kDa. Thus, the "Santil" ointment contains two collagenases (collagenase G, ~114 kDa, and collagenase H, ~110 kDa), nonspecific neutral metalloproteinase (~35 kDa), a small amount of cysteine proteases (clostripain ~58 kDa). The disadvantages of microbial collagenase preparations are: a relatively low activity, difficulties in cultivating producers, as well as potential allergenicity, which may be caused by the usage of pathogenic microorganisms-producers [4, 11, 12].

In Russia, hepatopancreas *Paralithodes* of crustaceans is widely used as a source of collagenase, which is considered as an affordable, cheap and non-toxic raw material, being a waste of commercial crab processing. The research of this group of proteases is devoted to both the production of stable and highly active enzyme complexes and the development of effective drugs [13–19].

Hepatopancreas crustaceans produces a number of digestive enzymes that hydrolyze different classes of biopolymers: collagenolytic (serine, trypsin-like) proteases, collagenases, phosphotases, phosphodiesterases, elastases, RNAses, DNAses, etc. It has been proved that proteolytic enzymes of *Paralithodes camtschaticus,* possess a broad specificity. They disintegrate both native collagen and other protein substrates – casein, gelatin, fibrinogen and serum albumin, which, in many respects, leads to the high efficiency of these complexes [14, 16, 20]. The enzyme complexes obtained from the *Paralithodes camtschaticus* hepatopancreas, vary according to the degree of purity, activity and composition. Thus, the sum of collagenolytic proteases of crab hepatopancreas, which is a mixture of nine proteins with a molecular weight of 23–36 kDa, was isolated. In the preparations "Collalitin" and "Collagenase KK", collagenolytic proteases of *Paralithodes camtschaticus* hepatopancreas are represented in the form of three isoenzymes, the molecular weight of which is in the range from 18 to 27 kDa [12, 15–20].

The mechanism of a therapeutic action of collagenases is based on the ability to convert native insoluble collagen into a soluble form by hydrolysis of peptide bonds. True collagenases, particularly of microbial and animal origin (the metalloproteases class), cleave the triple helix of collagen at one specific point, forming large soluble fragments, further destruction of which is relatively slow. It has been established that purified clostridial collagenase, as well as the complex of enzymes present in the "Santil" preparation, hydrolyze native and denatured collagen, as well as collagen-associated proteins of the intercellular matrix to peptides. Serine proteases (collagenolytic proteases) obtained from the gastrointestinal tract of fishes and invertebrates, break down the three polypeptide chains of tropocollagen, and the resulting peptides are further hydrolyzed to amino acids [2, 11, 12, 20–26].

It is noted that in the wound, proteolytic enzymes contribute to the exudate colliquation, facilitating the access for antiseptic and antibiotic drugs to the bacterial cell, enhancing the effect of antibacterial therapy. On the model of an infected rats' burn it was established that the collagenase tretment of the wound formation from day 5 from the beginning of the process reduced a bacterial load from 108 to 105 or fewer bacteria per gram of tissue. The level of bacterial load had a beneficial effect on normal wound healing, which contributed to the reparation acceleration. The authors suggest that collagenases can be safely used without concomitant local antimicrobials in chronically infected wounds due to their antimicrobial properties and effectiveness of wound healing [25, 26].

In the studies it has been established, that in addition to cleansing the wound of necrotic tissues, collagenases directly affect the reparation process. Thus, collagenase isolated from *C. histolyticum,* was found to enhance migration and proliferation of keratinocytes, endothelial cells and fibroblasts. The study of the effect of bacterial collagenase preparations on the model of full-layer wound in Yucatan pigs showed, that the daily treatment with the enzyme from the first day of the wound formation, made it possible to achieve purification, exudation, as well as effective angiogenesis and epithelization and, as a result, effective wound healing in a shorter time [28].

The reparative effect of proteolytic complexes obtained from crustaceans, has also been revealed. The study of moricrasa (a lipophilic base ointment with *Paralithodes camtschaticus* collagenase) reparative properties on the spontaneous purulent-ulcer injuries in rats, showed a complete wound healing after 6–10 days, when applied daily. It has also been noted that the further application of the ointment, contributed to the resumption of the wool cover at the wound location [29].

The study of reparative properties of oleogel with *Paralithodes camtschaticus* collagenase on the rats' burn skin model showed, that the test ointment, applied every day beginning from the 3rd day after the infection and formation of spontaneous purulent-necrotic wounds, activated the processes of epithelization and proliferation in the damaged tissues, which significantly reduced the healing time – there was complete epithelization of the burn surface, desquamation of the scab on the 10th day, in comparison with the control group, where incomplete epithelization was observed [30].

In the experiments on the animals (rats) it was demonstrated, that the wounds treatment with chitosan-modified textile with immobilized collagenase of *Paralithodes camtschaticus,* significantly reduced the necrotic tissue to 3 days (14 days in the control) and the

purulent wounds healing time reduced to 12 days (27 days in the control) [18].

Attempts to research the mechanism of microbial collagenases influence on the wound healing effectiveness were carried out. *In vitro* research has established, that the intercellular matrix enzymolysis with bacterial collagenase and the "Santil" ointment, containing collagenases, releases peptides that activate cellular migration, proliferative and angiogenic processes in trauma and promote wound healing. *In vitro* research has also revealed that collagen hydrolysis fragments and collagen-associated peptides obtained as a result of endothelial collagenase hydrolysis of dermal capillaries and human fibroblasts, increase cell proliferation and promote angiogenesis. On the model of full-layer long-term non-healing wounds of the mice, it was demonstrated that collagenase of "Santil" ointment, as well as peptides obtained from extracellular matrix, increase wound reepithelization by 60–100% compared to the control (saline solution) during the daily treatment, starting from the first day of damage [10].

The effect of the "Santil" ointment on the resolution of inflammation in long-term unhealed wounding was investigated. The research was carried out on macrophages, isolated from wounds treated with ointment or petrolatum (a comparison drug), and implanted in mice. A significant increase in pro-reparative and a decrease in pro-inflammatory macrophages polarization in both acute inflammatory process and chronic diabetic wound, have been revealed. Wound macrophages in the ointment-treated group showed an increased production of anti-inflammatory cytokines IL-10 and TGF-β, as well as a reduced production of pro-inflammatory cytokines TNF-α and IL-1β. Wound treatment with clostridial collagenase attenuated the transactivation of factor NF-KB and significantly reduced STAT6-phosphorylation. These results, make it possible to consider collagenase as a potential anti-inflammatory agent that can be effective in chronic wound inflammation, including diabetic wounds [31]. There are numerous data on the effectiveness of the "Santil" ointment, used as a wound healing agent in clinical practice. The data confirm that the ointment based on collagenase, is safe and effective for the treatment of skin ulcers and burn wounds, helping to reduce the healing time and the severity of pain symptoms, reduce the risk of infection. The effectiveness of the "Santil" ointment for surgical treatment of diabetic foot ulcers, bedsores and trophic varicose ulcers, as well as burns, has been demonstrated in a number of clinical trials in various institutions (for example, inpatient, outpatient and long-term kinds of care) [32–34].

The data of clinical studies of the "Moricrol" ointment containing *Paralithodes camtschaticus* collagenase, were registed. The use of the ointment in patients with hard-to-heal skin wounds and the wounds after skin transplantation, contributed to the engraftment of transplants in patients and the absence of rough deforming scars. A reduction of the healing time of extensive wounds in the mucosa of the oral cavity when using the "Moricrol" ointment, was revealed. It has been noted that the treatment with "Moricrol" in purulent wounds, contributed to a faster wound surface cleansing [35].

In clinical research it was found out, that the combined application of wound coating "Multiferm" (the collagenase *Paralithodes camtschaticus* and chitosan complex), photodynamic and NO-therapy, accelerated the defect reparation in the case of trophic ulcers. It was manifested in accelerate purifying of purulent necrotic content от гнойно-некротического содержимого and the granulation formation 2.4 times faster. The average healing time also decreased by an average of 9.2 days [36].

Clinical efficacy of various collagenase-based anti-scar agents has been studied. The effectiveness of collagenases in the scar therapy is associated with their ability to hydrolyze collagen excess [37–41]. For example, the possibility of hypertrophic scars correction by means of electrophoresis in a solution of weak electrolytes with the "Polycollagenase-K" preparation has been investigated. During the treatment, the analysis of the scars state was carried out by EHF-dielectrometry method. The tendency of the moisture content increase in the scar tissue has been revealed. It was associated with an increase in the fraction of intracellular structured water. It has been detected that the hydration of the scar tissue under the influence of "Polycollagenase-K", approached the values peculiar to those of healthy skin at the similar localization. According to the authors' data, the increase in the moisture content in the scar tissues, was due to the water release during the collagen hydrolysis. Alongside with the destruction of the excess collagen, the normalization of microcirculation was noted [8, 39].

The anti-scar activity of the collagenolytic complex from sea stars has been proved: the ability to influence the reduction of collagen gel, the activity of matrix metalloproteinases (MMP), the release of hydroxyproline and the regulation of the activity of fibroblast genes. It was found out, that the complex significantly inhibited the reduction of collagen gel after 2 days of incubation. The expressed activity of MMP-2 and MMP-9 was revealed, which was manifested in the form of a large amount of hydroxyproline release. The fibroblast cell culture treatment significantly reduced fibrocyte proliferation in 3-day cultures. The ability to influence the expression of genes controlling the inflammatory response in fibroblasts, has been established [42].

The clinical studies of the anti-scar properties of the "Moricrol" ointment, detected a decrease in the manifestations of redness, itching and a sense of tightening of keloid scars. In 46 patients, a significant improvement in the condition of the skin, namely paling of tissues and some flattening of the scar tissue in the scar area was revealed. Some softening of the scar and a decrease in the turgor of the scar skin on palpation. was assessed How-

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ever, in some patients (18 patients), no tissue softening occurred, and in 5 patients with chronic burn scars, the expected effect was absent [35].

There are research data on gel (group 1 – applications and group 2 – phonophoresis) and a solution (group 3 – electrophoresis) for scars correction with "Fermencol" containing *Paralithodes camtschaticus* collagenase in patients with hypertrophic and keloid scars of different origins. A statistically significant slowdown in the scar growth, paresthesia and itching reducing, scar thickness reducing, the disappearance of inflammation signs was detected when using the gel and solution "Fermencol" after the treatment course of 10-15 days. It was revealed that the use of therapeutic electrophoresis and ultrasound with "Fermencol"®, made it possible to significantly increase the drug intake into the skin [41].

The influence of electro- and ultraphonophoresis "Fermencol" on clinical manifestations of pathological scars in 89 patients with hypertrophic and keloid skin scars, has been investigated. The scars treatment with "Fermencol" electro-and ultraphonophoresis, contributed to a more significant dynamics of clinical signs, compared with "Contractubex" and "Lidase" electro – and ultraphonophoreses. The maximum reduction in clinical manifestations – type, consistency, color and sensitivity of the scar – was observed under the "Fermencol" ultraphonophoresis. The defibrosing effect was most evident with the combination of the drug and the ultrasound exposure [43].

A new method of treatment and prevention of hypertrophic and keloid scars, which implies lidase injection into the thickness of the scar tissue, followed by wet coating containing chitosan and collagenase application to the scars surface in the course of 10-15 procedures, has been developed, patented and clinically tested.

This method's clinical approbation showed a significant improvement in both – the clinical picture and the patient's life quality – in relation to the comparison course (collagenase was administered transdermally in therapeutic doses, and lidase was administered intradermally by the course of 10-15 daily procedures) [44].

The method of skin scars after the acne correction by means of a course treatment with the "Fermencol" electrophoresis. In group 1, applications of the «Fermencol» gel were used twice a day, in the 2nd group, electrophoresis of the fermencol solution was carried out. A positive result in 86.4% of patients of group 1 and 94.1% of patients of group 2 was revealed: slowing down of the active growth, regression of the scar, disappearance of unpleasant subjective sensations, alignment of the color of the scar and surrounding tissues [45].

In clinical studies, the evaluation of the treatment of hypertrophic scars on patients after surgical interventions on the thyroid gland. The treatment was carried out by collagenase in the form of the dry "Collalysin" powder (collagenase Clostridium histolyticum), mixed up with petrolatum. The positive effect, was observed in a month from the beginning of the treatment by this drug. Collagenase treatment has shown the results similar to the administration of triamcinolone, a hormonal anti-inflammatory drug, into the scar [46]. An emulsion-based composition of "Collalysin" has also been developed for the prevention and treatment of hypertrophic and keloid scars resulting from burns, dermabrasion and plastic surgery [47].

Modern collagenase-based products: assortment in the Russian pharmaceutical market, opportunities for improvement

In the literature data, there is information about the developed and patented medicines based on collagenase. So, the "Collagenase KK" preparation was produced in the form of the lyophilized powder for the application to damaged tissues or aqueous solutions prepared ex tempore and used for wetting wipes or tampons [6, 17, 48]. The disadvantage of this form is the following: the activity of proteolytic enzymes, when directly introduced into the wound, lasts 15-30 minutes, making the therapy ineffective because of inactivating the enzymes. The process is as also characterized by a the relative complexity [49].

Some hydrophilic gels have been developed, e.g., a polyethylene oxide gel with collase [50, 51]. To include *Paralithodes camtschaticus* collagenase, a composition consisting of a mixture of vinylglutarate, vinyl acetate and vinyl alcohol, which turns into a gel-like state when interacting with the wound contents, was used by I. Yu. Sakharov et al. [24]. The ointments on lipophilic bases were offered. So, the ointment with collagenase from Clostridium histolyticum was based on petrolatum. The moricrase "Moricrol" ointment on the lipophilic basis of "Eikonal" (a mixture of fatty acids and vitamins A, E, D and F) is known [19]. Currently, these drugs are not available on the pharmaceutical market [6, 52].

The analysis of the literatyre data showed that currently, collagenase-based products are available for the preparation of solutions for injection and electrophoresis forms, ointments, creams and medical dressings, in the form of powders [4–8, 52–55].

The following commercial drugs are produced abroad: the "Iruksol" and "Santil" ointments produced by "Smith&Nephew". They include collagenase C. histolyticum as a proteolytic complex. It should be noted that these drugs are not registered in the Russian pharmaceutical market [10, 52].

General Residual Life of the System (GRLS) – Encyclopedia of drugs – presents information about the "Collalysin" drug (the INN "Collagenase"), LSR-005615/09 is a proteolytic agent, an enzyme preparation obtained from the culture of C. histolyticum. "Collalysin" has a keloidolytics effect**.** It is available in the form of a powder-lyophilizate dosage form and strength from 100 КЕ to 1000 КЕ for a solution for injections and electrophoresis preparation and is recommended for treatment of burns, correction of scars, etc. [6, 52].

"Fermencol" (Russia) is a cosmetic product based on the enzymes of collagenase hydrobionts, designed for the correction of scars. It is available in the form of gel and a set for preparing an electrophoresis solution [54, 55].

A self-absorbable biological dressing "Digestol", Russia) is a wound set (RCF 2008/02946). It contains the sum of collagenolytic trypsin-like proteases (collagenases) of *Paralithodes camtschaticus*. It is recommended for the use in purulent and infected wounds, bedsores, trophic ulcers, burns, a diabetic foot syndrome. Dissolving in the wound contents, the wound coating releases the enzyme in the active form. A necrolytic effect of the wound dressing is combined with an anti-inflammatory and regeneration activating effect due to the presence of collagen [8].

Collagenase is also included in the cosmetic product – a dry "Karipain Plus" balm, containing a complex of enzymes: papain, bromelain and collagenase, intended for the treatment of scars [56].

Thus, the Russian pharmaceutical market currently has a small number of collagenase-based products: a lyophilized powder for the preparation of an injection solution ("Collalysin"), recommended for the scars treatment; a medical dressing with collagenase, recommended for the treatment of wounds and necrotic tissues "Digestol"); cosmetic gel and powder "Fermencol", a dry balm "Karipain Plus", recommended for the scars treatment. The drugs are represented by only powder-lyophilizate for the preparation of injection solutions and electrophoresis.

A pharmaceutical development of external drugs with proteolytic enzymes, in addition to justifying the optimal dosage form, makes provision for an ointment base or carrier, that ensure the enzyme stability. The analysis of the scientific and technical literature database as well as patents showed the following methods used: enzyme stabilization, the use of lipophilic base, immobilization on a polymer carrier [16, 24, 57, 58].

So, to preserve the activity of the enzyme in the drug, it is possible to use stabilizers, for example, salts. In particular, ammonium sulfate in a certain concentration reversibly inactivates proteolytic enzymes by precipitation, preventing their autolysis. Sulfate ions interact with positively charged amino acids, giving the protein molecule a more compact shape, making it less soluble. This method of enzyme stabilization is implemented in the "Fermencol" gel [59].

In the ointments with collagenase from C. histolyticum, petrolatum is used ("Iruxol", "Santil"). Based on petroleum jelly and paraffin, the veterinary ointment "Iruxovetin" containing collagenase, was previously produced. The use of these bases is due to the fact that lipophilic components do not contain water, which is a medium for the enzymes autolysis and reproduction of microorganisms-destructors, which makes it possible to preserve the activity of enzymes for a long time [3, 4, 25].

But as a base, petroleum jelly is characterized by a number of drawbacks: an occlusive effect and a low osmotic activity, which, for a favorable repair, can negatively affect the wound state requiring oxygen and an exudate outflow..

The ointments based on petrolatum, cause inconvenience, i.e., they are poorly washed off with water, because of the pronounced viscosity they are distributed over the surface of the skin with effort. In this regard, the search for the bases devoid of these shortcomings, but at the same time ensuring the preservation of the enzyme activity during the storage period, can be considered a hot topic [5, 52].

Oleogels, e.g. based on Aerosil, can be considered promising bases for preparations of proteolytic enzymes. Thanks to the gel-like structure, gels are easily applied and distributed over the skin. An additional advantage of this base. can be considered the fact, that Aerosil exhibits a high sorption capacity against the decay products of tissues, toxins, microorganisms. The oleogel containing silicones, has also been proven to be effective as a scars treatment [60, 61].

Currently, in external medicines, immobilized enzymes are used on polymer carriers. Immobilization makes it possible to limit the activity of proteolytic enzymes by the damaged area and increases their stability in the wound environment. Thus, when using the enzymes immobilized on fiber-forming carriers, the terms of purification and wound healing are significantly reduced, the consumption of drugs is also reduced in comparison with the free enzymes use [62, 63].

Preparation of fibrous materials with proteolytic enzymes by covalent immobilization, providing activation of the fiber surface, in particular by treatment with oxidants to obtain dialdehyde cellulose, can serve an example, Active aldehyde groups interacting with functional groups of the enzyme to form covalent bonds, are formed on the fiber surface resulting in its fixation [63, 64].

For example, in the "Multiferm" dressing, a copolymer on the basis of dialdehydecellulose treated with chitosan with immobilized enzyme complex of *Paralithodes camtschaticus* hepatopancreas was used. However, one of the problems of chitosan-containing textile dressings, is the "keratinization" of the dressings edges, associated with structural changes in chitosan during immobilization, sterilization and storage. Currently, studies to obtain more stable preparations of immobilized enzymes with improved functional characteristics, are being conducted in this direction [7, 65].

One of the options for improving the properties of textile dressings, is conferring atraumatic characteristics provided by water-repellent impregnation, e.g., ointments or gel coatings. E.g., a hydrophobic ointment base (the "Branolid" dressing) or wax ("Voscopran") prevent sticking to the wound and traumatization of the granulations in the process of dressing changes. Changing such a dressing is painless for the patient [66].

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Based on this, the research on the creation of atraumatic wipes with immobilized collagenase on a hydrophobic basis can be considered perspective, in our opinion.

CONCLUSION

Collagenases are among the most effective proteolytic enzymes, because of their ability to provide the cleavage of collagen, the main component of wounds and scars. In addition, there are data from preclinical and clinical studies confirming, that collagenases of various origins, alongside with their necrolytic activity, are able to accelerate the process of reparation. Biochemical distinctions of collagenase influence on the wound healing process, as well as biochemical aspects of collagenase anti-inflammatory effect, have been investigated. The results of clinical studies of anti-scar properties of collagenases of different origins confirming their effectiveness in this pathology, have been registed. One of the available raw resources of collagenolytic enzymes is currently the hepatopancreas of crustaceans, in particular, *Paralithodes camtschaticus*. Due to the composition peculiarities, the enzymes derived from *Paralithodes camtschaticus* hepatopancreas, have a wide specificity as they hydrolyze both native collagen and other protein substrates (gelatin, casein, fibrinogen and serum albumin).

On the pharmaceutical market of the Russian Federation, the range of products with collagenase is not wide, so its expansion can be relevant. The collagenase derived from *Paralithodes camtschaticus* hepatopancreas, can be used as the most affordable raw material with pronounced collagenolytic properties. As the dosage form, more convenient in application and production, gels have their set of advantages**.** Thanks to their potential sorption properties, the oleogels based on aerosil, can be considered a promising base for external remedies with collagenolytic enzymes,. The development of gels and atraumatic dressings with collagenase obtained from *Paralithodes camtschaticus* as the most affordable raw materials, can be considered significant for practical pharmacy.

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All authors had equally contributed to the research work.

CONFLICTS OF INTEREST

The authors and peer reviewers of this paper report no conflicts of interest.

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STUDY OF DISTRIBUTION OF BIOLOGICALLY ACTIVE SUBSTANCES FROM FLOWERS OF HELICHRYSUM ARENARIUM BETWEEN PHASES OF THE EXTRACTION SYSTEM

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The aim of this study is to confirm the adequacy of the proposed hypothesis, which explains and quantitatively describes the distribution of biologically active substances (BAS) within the extraction system consisting of Helichrysum arenarium flowers and the solvent using a regressive analysis for the theoretically predicted coordinates.

Materials and methods. For this research, milled officinal flowers of Helichrysum arenarium (Helichrysum arenarium L. flores) were used. The analysis of the extractions was carried out by RP HPLC method. Isosalipurposide, salipurposide, and chlorogenic acid of ≥98.0% purity were used as reference substances. The analytical wavelengths were 370, 290, and 325 nm.

Results. The obtained experimental data are well-approximated by regressive linear equations in the theoretically predicted coordinates 1/C=f(V) and ln(b/a)=f(1/T). Wherein, the coefficient of determination of regressive equations was $R^2 \geq 0.998$, which indicates functional dependence between the studied parameters and confirms the adequacy of the developed mathematical model. The experimental work identified the necessity of implementation of additional constant values into the mathematical model.

Conclusion. A new hypothesis was proposed to explain and quantitatively describe the distribution of BAS in the extraction system of Helichrysum arenarium flowers and 80% ethanol. With this working hypothesis, mathematical models were developed and their adequacy was proved using a regressive analysis in the theoretically predicted coordinates. The results obtained could not deny that a mechanism of BAS distribution between the phases is explained and described by the classic Boltzmann distribution for discrete values of molecular energy (or quantum distribution according to Fermi and Dirac).

Keywords: flowers of Helichrysum arenarium; isosalipurposide; salipurposide; chlorogenic acid; equilibrium; classic Boltzmann distribution for discrete values of molecular energy

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ИЗУЧЕНИЕ РАСПРЕДЕЛЕНИЯ БИОЛОГИЧЕСКИ АКТИВНЫХ ВЕЩЕСТВ ИЗ ЦВЕТКОВ БЕССМЕРТНИКА ПЕСЧАНОГО МЕЖДУ ФАЗАМИ ЭКСТРАКЦИОННОЙ СИСТЕМЫ

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Цель. Проверка адекватности выдвигаемой рабочей гипотезы, которая объясняет и количественно описывает распределение БАВ в экстракционной системе из цветков бессмертника песчаного и растворителя, с помощью регрессионного анализа в предсказанных теорией координатах.

Материалы и методы. Для исследований использовали измельченное фармакопейное растительное сырье «Бессмертника песчаного цветки» (*Helichrysum arenarium* L. flores). Анализ извлечений проводили с помощью ОФ ВЭЖХ метода. В качестве стандартных веществ использовали изосалипурпозид, салипурпозид, хлорогеновую кислоту ФСО ГФУ, содержание ≥98,0 %. Аналитические длины волн 370, 290 и 325 нм.

Результаты. Экспериментальные данные хорошо аппроксимируются регрессионными линейными уравнениями в предсказанных теорией координатах 1/C=f(V) и ln(b/a)=f(1/T). При этом коэффициент детерминации регрессионных уравнений, имеет значение R^2≥0,998, что говорит о функциональной зависимости между изучаемыми параметрами и подтверждает адекватность разработанных уравнений. Эксперимент выявил необходимость введения в математическую модель дополнительной константы.

Заключение. Предложена рабочая гипотеза, которая объясняет и количественно описывает распределение БАВ в экстракционной системе из цветков бессмертника песчаного и этанола 80 % об. С помощью рабочей гипотезы разработаны математические модели, адекватность которых доказана с помощью регрессионного анализа в предсказанных теорией координатах. Полученные результаты не отвергают гипотезу, что механизм распределения БАВ между фазами в экстракционной системе объясняется и описывается классическим распределением Больцмана для дискретных значений энергии молекул (или квантовым распределением Ферми-Дирака).

Ключевые слова: цветки бессмертника песчаного; *Helichrysum arenarium*, изосалипурпозид; салипурпозид; хлорогеновая кислота; равновесие; классическое распределение Больцмана для дискретных значений энергии молекул

INTRODUCTION

Flowers of Helichrysum arenarium (*Helichrysum arenarium* L. flores) are the officinal raw material within the territory of the Russian Federation, the Republic of Belarus, Ukraine, the Republic of Kazakhstan, etc. This raw material is used for the production of the drug product Flamin, which is manufactured in the form of tablets, granules and in-bulk substance. It is used in treatment of liver and gallbladder diseases. In addition, biologically active substances (BAS) from that raw material have antioxidant, antibacterial, antiviral, antihyperlipidemic, and cytotoxic effects [1–20].

In our previous research [21], the authors justified the mechanism of dielectric constant impact on equilibrium concentration of isosalipurposide in the extractions. However, the obtained model neither explains nor describes the mechanism of BAS distribution in the extraction system when the equilibrium status is reached within it. That is why the studies aimed at the development of the equilibrium status of the extraction process are relevant.

THE AIM of this study is to confirm the adequacy of the proposed hypothesis, which explains and quantitatively describes the distribution of BAS in the extraction system of *Helichrysum arenarium* flowers and the solvent using a regressive analysis in the theoretically predicted coordinates.

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MATERIALS AND METHODS

Raw materials and chemical reagents

For this study, milled plant raw material of *Helichrysum arenarium* was used, and it was bought at pharmacy Medical herbs Ltd., Kharkiv, Ukraine, lot number 530617, expiry date 07.2020 [22].

An aqueous solution of ethanol 80%±1%, was used as an extracting solution. Qualitative and quantitative analyses were carried out using RP HPLC with reference substances.

Isosalipurposide, salipurposide, and chlorogenic acid of ≥98.0% purity were used as reference substances. Analytical wavelengths were 370, 290, and 325 nm.

The main validation parameters of the analytical method and suitability of RP HPLC system for the assay of isosalipurposide, salipurposide, and chlorogenic acid, are shown in Table 1.

Methods of obtaining extracts

A precisely weighed amount of the raw material (1 g) was put into a hermetic flask, the required volume of the solvent was added, which additionally was weighted and put into a refrigerator/thermostat at the temperature of 4, 20, 40 and 60±1°C. The proportions of the raw material and the solvent at each temperature, were 1:5 (1:10), 1:15, 1:20, 1:40 w/v. The extraction mixture was decocted for 24 hours. After that, the extract was removed and its assay was performed using RP HPLC method. The mean value and the standard error of mean were calculated at the repeat count n=3 and the significance level P=0.95.

Analysis methods via RP HPLC

The analysis of the extractions was carried out using the chromatographic equipment of Agilent Technologies, "Agilent 1200 Infinity" series, manufactured in the USA. The detailed description is available in the following articles [21, 23].

Theoretical part

In order to explain the mechanism and a quantitative description of BAS distribution between a solid phase of the medicinal plant raw material and a liquid phase of the solvent, the authors proposed the following hypothesis: the mechanism of equilibrium molecular distribution of BAS between two phases in the extraction system is explained and described by the classic Boltzmann distribution for discrete values of molecular energy (or quantum distribution according to Fermi and Dirac), equation (1). This hypothesis allows developing a mathematical model, which will describe the experimental data in the theoretically predicted coordinates, as shown by equations (2) and (3):

$$\frac{n}{n_0} = \frac{1}{1+\exp\left(\frac{\Delta G}{kT}\right)} \quad (1)$$

where n is quantity of BAS in the solvent with energy equals ΔG, mol;

n_0 is an overall quantity of BAS, mol;

ΔG is the difference of Gibb's energy for BAS molecules in the extraction system, J;

k is Boltzmann constant value, $1.38 \cdot 10^{-23}$ J/K;

T is absolute Kelvin's temperature, K.

$$\frac{1}{C} = \frac{1}{n_0} \cdot V + \exp\left(\frac{\Delta G}{kT}\right) \cdot \frac{1}{n_0} = \frac{M}{m_0} \cdot V + K_H \cdot \frac{M}{m_0} = a \cdot V + b \quad (2)$$

$$\ln K_H = \ln\left(\frac{b}{a}\right) = \frac{1}{T} \cdot \frac{\Delta G}{k} \quad (3)$$

where C is concentration of BAS in the extracting solution, g/ml;

m_0 is *an* overall (initial) content of BAS in the raw material, g;

V is the volume of the extracting solution, ml;

M is the weight of the raw material in the extraction system, g;

a is a constant that equals to the reversed value of overall BAS content in the raw material (M/m_0);

b is a constant that equals to multiplicity of Henry's constant and (K_H) and the reversed value of overall BAS content in the raw material (M/m_0), ml/g.

To determine the degree of adequacy of the proposed hypothesis, the authors used the regressive analysis of the experimental data in the theoretically predicted coordinates $1/C=f(V)$ and $\ln(b/a)=f(1/T)$. The obtained data were processed with preset for the data analysis in MS Excel 2010.

Table 1 – The main validation parameters of analytical method and suitability of RP HPLC system for the assay of isosalipurposide, salipurposide, and chlorogenic acid

Parameter	Pharmacopoeia criteria [22]	Isosalipurposide	Salipurposide (sum of isomers)	Chlorogenic acid
1. Retention time, min*	–	20.1±0.2	11.9±0.2 and 12.8±0.2	6.3±0.3
2. Separation coefficient	≥1.5	3.0	3.1 and 3.2	11.0
3. Number of theoretical plates	≥1,000	103,458	39,541 and 29,267	12,282
4. Relative Standard Deviation, RSD, %	≤2.0	1.2	1.2	0.8
5. LOD, g/mL	–	$4.1\cdot10^{-5}$	$5.5\cdot10^{-6}$	$2.2\cdot10^{-5}$
6. LOQ, g/mL	–	$1.3\cdot10^{-4}$	$1.7\cdot10^{-5}$	$6.5\cdot10^{-5}$
7. Determination coefficient, r^2	≥0.98	0.9999	0.9999	0.9999
8. Linear regressive equation, C(g/ml) = $f(S$(mAU·sec))	-	$C=(2.79\pm0.06)\cdot10^{-7}\cdot S$	$C=(3.94\pm0.01)\cdot10^{-7}\cdot S$	$C=(2.92\pm0.04)\cdot10^{-7}\cdot S$

** Note. The average value and its error (X±ΔX) were calculated on the basis of the repeat count n = 3 and the significance level P = 0.95.*

Table 2 – Constant values for BAS from Helichrysum arenarium flowers

BAS	Constant value		
	ΔG, J/mol	g	m_p, % wt.
1. Isosalipurposide	5,390±380	–1.5±0.2	22.0±3.0
2. Salipurposide	19,930±1,030	–7.2±0.4	0.075±0.004
3. Chlorogenic acid	18,640±2,160	–5.4±0.9	0.45±0.08

Note. The number of experiments: n=4, the significance level: P=0.95.

Table 3 – Values of overall (initial) BAS content in the raw material (m_0) found by the experiment and theoretical calculation

BAS	Theoretically calculated content, m_0/M, % wt.*	Experimental values, m_0/M, % wt.**
1. Isosalipurposide	1.58±0.06	1.46±0.07
2. Salipurposide	0.47±0.02	0.43±0.02
3. Chlorogenic acid	0.19±0.04	0.19±0.01

*Note. * The number of experiments: n=4, the significance level: P=0.95.*
*** The number of experiments: n=3, the significance level: P=0.95.*

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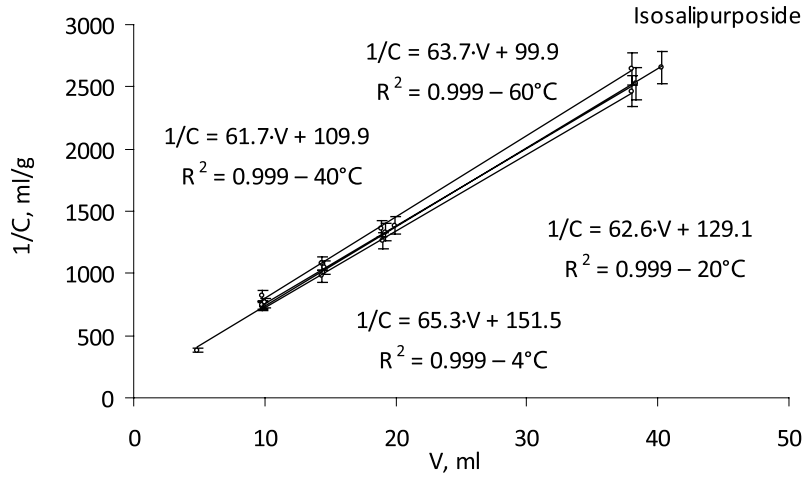

Figure 1 – Regressive equation of isosalipurposide concentration dependence on the volume of the extracting solution in coordinates 1/C=f(V)

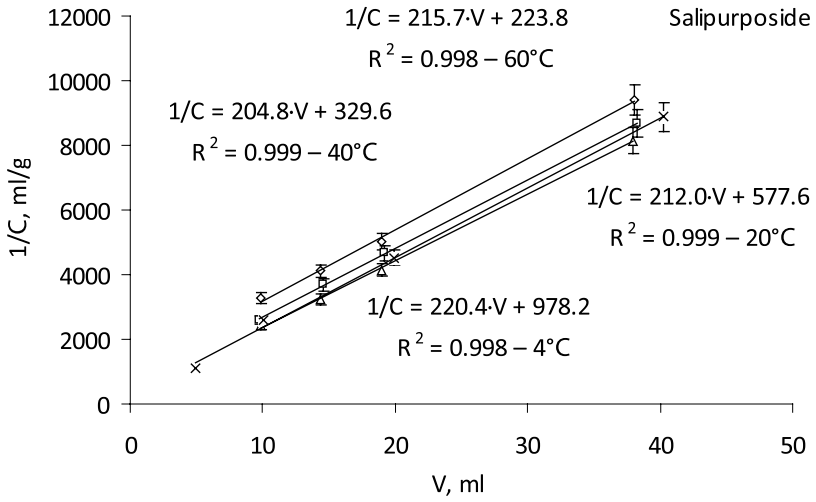

Figure 2 – Regressive equation of salipurposide concentration dependence on the volume of the extracting solution in coordinates 1/C=f(V)

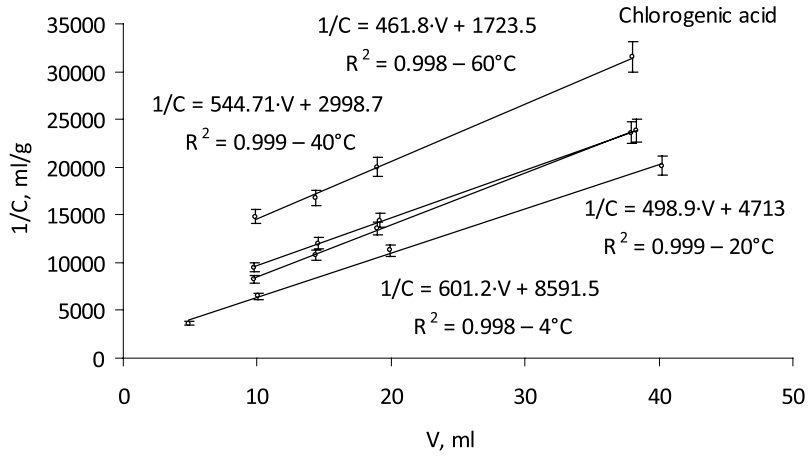

Figure 3 – Regressive equation of chlorogenic acid concentration dependence on the volume of the extracting solution in coordinates 1/C=f(V)

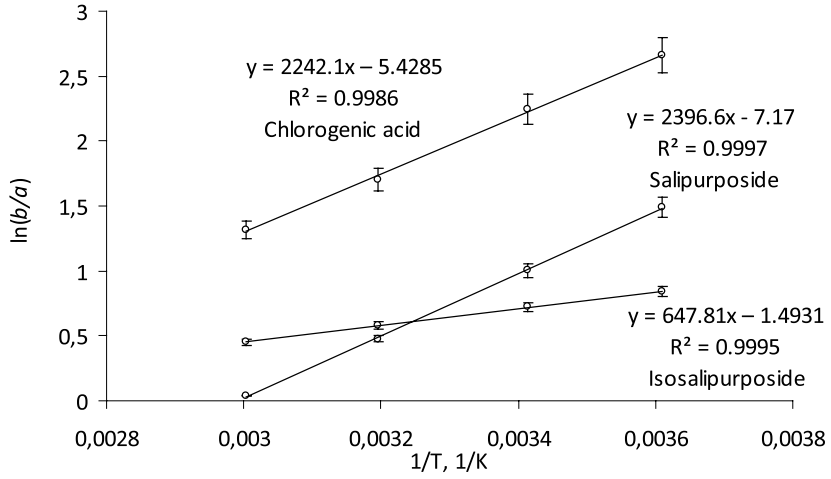

Figure 4 – Regressive equations of empiric constant values' (a, b) dependence on temperature in coordinates ln(b/a)=f(1/T) for BAS from Helichrysum flowers

RESULTS AND DISCUSSIONS

The experimental data and regressive linear equations of isosalipurposide, salipurposide, and chlorogenic acid concentration dependence on the volume of the extracting solution in the theoretically predicted coordinates are shown in Figures 1, 2, and 3.

As Figures 1, 2, and 3 show, the experimental points are well-approximated by regressive linear theoretically predicted coordinates $1/C=f(V)$. Wherein, the coefficient of determination of the regressive equations was $R^2 \geq 0.998$. It determines the functional dependence between the studied parameters and confirms the adequacy of the equation (2).

Thereafter, the obtained results were used for building up the regressive linear equations of dependence of Henry's constant for isosalipurposide, salipurposide and chlorogenic acid in the theoretically predicted coordinates. They are shown in Figure 4.

As Figure 4 shows, the dependence of Henry's constant values on the temperature is well-approximated for isosalipurposide, salipurposide, and chlorogenic acid by regressive linear equations in the theoretically predicted coordinates $\ln(b/a)=f(1/T)$. Wherein, the coefficient of the determination of the regressive equations was $R^2 \geq 0.998$. It proves the functional dependence between the studied parameters and confirms the adequacy of the equation (3). However, the obtained results have identified the additional constant value (g) in the equation (3), which was not predicted by the theory, hence, this requires to add this experimentally found constant value ($g=ln(m_p/100)$) into the initial equation (1).

The constant values (ΔG, g and m_p) for BAS from *Helichrysum arenarium* flowers, found in accordance with the proposed theoretical equations (2) and (3) and also the experimental outcome, are summarized in Table 2.

As Table 2 shows, constant value ΔG, which expresses the energy of the BAS interphase distribution, is found at 5-20 kJ/mol level. It is well-complied with the values of the physical adsorption of the substances on the adsorbents [24]. Judging by this fact, BASes in the medicinal plant raw material are found to be in a bound or even adsorptive status, as it was discovered by M.S. Tsvet at the beginning of the XXth century [25].

The conclusive test of the proposed hypothesis was performed, comparing the experimentally obtained and theoretically calculated values of the overall (initial) content of BAS in the raw material (m_0/M), which are shown in Table 3.

As Table 3 shows, the experimentally found and theoretically calculated values of the overall (initial) content of BAS in *Helichrysum arenarium* flowers (m_0/M), do not differ from each other. This fact additionally confirms the adequacy of the equation (2).

Hence, the obtained experimental results are well-complied with theoretically developed mathematical models via equations (2) and (3). However, the experiment identified the necessity of adding constant (g) into the mathematical model, wherein equation (1) transforms to the following:

$$\frac{n}{n_0} = \frac{1}{1+\exp\left(\frac{\Delta G}{kT} - g\right)} \quad (4)$$

Hence, the proposed hypothesis concerning the mechanism of the interphase distribution of BAS in the extraction system, is explained and described by the classic Boltzmann distribution for discrete values of the molecular energy (or quantum distribution according to Fermi and Dirac), is not denied.

Thus, the proposed hypothesis and the developed mathematical model based on it, explain the mechanism of the BAS distribution in the extraction system between the phases; make it possible for us to find the constants required; forecast the equilibrium (limiting) concentration of BAS in the extract; and choose/calculate

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the optimal values of the volume and temperature of the extractant to achieve certain values of the exhaustive degree of the plant raw material for BAS.

CONCLUSION

A new hypothesis was proposed to explain and quantitatively describe the distribution of BAS in the extraction system of *Helichrysum arenarium* flowers and 80% ethanol. With this hypothesis, the mathematical models have been developed, their adequacy has been proved using a regressive analysis in the theoretically predicted coordinates. The constant values have been found, they are present in the mathematical model. The necessity of the addition of a new constant value has been identified experimentally. The obtained results do not deny that the mechanism of BAS distribution between the phases is explained and described by the classic Boltzmann distribution for discrete values of the molecular energy (or quantum distribution according to Fermi and Dirac).

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AUTHORS’ CONTRIBUTION

All authors have equally contributed to the research work.

CONFLICT OF INTEREST

Authors state there’s no conflict of interest between them.

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MORPHOLOGICAL, TECHNOLOGICAL AND BIOPHARMACEUTICAL STUDIES OF ALGINATE-CHITOSAN MICROCAPSULES WITH VINPOCETINE

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The aim of the investigation is to study morphological, technological and biopharmaceutical properties of alginate-chitosan microcapsules with Vinpocetine.

Materials and Methods: Alginate-chitosan microcapsules with different concentrations of sodium alginate (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) and a medium viscosity chitosan solution (0.25–0,5%), as well as microcapsules not treated with a solution of chitosan, were obtained. The surface morphology was studied by methods of atomic-powered microscopy with the use of an NT-MDT Corporation probe scanning microscope (model Solver P47 Pro). To study biopharmaceutical properties of the obtained microcapsules, the "Rotating Basket" apparatus was used.

Results: It has been found out that the microcapsules not treated with a chitosan solution, have a smooth, transversely striated surface with large heights and deep cavities. With an increase in the concentration of sodium alginate, the surface becomes smoother, the peaks become larger, higher and wider, the cavities get deeper and more sinuous. The microcapsules treated with a chitosan solution, on the contrary, have a rough surface, low heights and shallow cavities, and with an increase in the concentration of sodium alginate, the surface becomes rougher, the heights are evenly distributed along the microcapsule. The spectrophotometry method was used to determine the efficiency of microencapsulation and the release rate of Vinpocetine from the microcapsules per unit time. When the concentration of a sodium alginate solution is 2.5%, the efficiency of microencapsulation is maximum (86.8%). At this concentration, saturation occurs and with its further increase, the efficiency decreases. The maximum release rate of Vinpocetine from microcapsule samples is observed when the concentration of a sodium alginate solution is 1%: it amounts to 41.17%.

Conclusion. The amplitude parameters of the microcapsules surface are different at different concentrations. There is a pattern of alternating signs of asymmetry and excess in the samples with chitosan. With a change in the scale of scanning, the surface characteristics of the microcapsules change. The most distinctive details of the structure are visible at the scale of 2 × 2 μm^2. At the concentration of sodium alginate of 2.5%, the efficiency of microencapsulation is maximum (86.8%). Studying the effect of the concentration of a sodium alginate solution on the release rate of Vinpocetine from the microcapsule samples has shown that at the concentration of 1%, the release rate is 41.17%, and at the concentration of 2.5% it is 4.5%. These microcapsules can be used in order to produce capsules with modified release.

Keywords: sodium alginate, chitosan, alginate-chitosan microcapsules, atomic-powered microscopy, amplitude parameters, microencapsulation efficiency, release rate

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RESEARCH ARTICLE

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МОРФОЛОГИЧЕСКИЕ, ТЕХНОЛОГИЧЕСКИЕ И БИОФАРМАЦЕВТИЧЕСКИЕ ИССЛЕДОВАНИЯ АЛЬГИНАТ-ХИТОЗАНОВЫХ МИКРОКАПСУЛ С ВИНПОЦЕТИНОМ

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Цель: изучение морфологических, технологических и биофармацевтических свойств альгинат-хитозановых микрокапсул с винпоцетином.

Материалы и методы. Получены альгинат-хитозановые микрокапсулы с различной концентрацией натрия альгината (0,5%, 1%, 1,5%, 2%, 2,5% и 3%) и раствором хитозана средней вязкости (0,25–0,5%), а также микрокапсулы, не обработанные раствором хитозана. Исследования морфологии поверхности проводились методом атомно-силовой микроскопии с помощью сканирующего зондового микроскопа корпорации NT-MDT модели Solver P47 Pro. Для изучения биофармацевтических свойств микрокапсул использовался аппарат «Вращающаяся корзинка».

Результаты. Установлено, что микрокапсулы, не обработанные раствором хитозана, имеют гладкую, поперечно исчерченную поверхность с крупными высотами и глубокими впадинами. С увеличением концентрации натрия альгината поверхность становится более гладкой, пики – крупнее, выше и шире, впадины – глубже и более извилистыми. Микрокапсулы, обработанные раствором хитозана, напротив, имеют шероховатую поверхность, небольшие высоты и неглубокие впадины, и с увеличением концентрации натрия альгината поверхность становится более шероховатой, высоты равномерно распределяются в микрокапсуле. Методом спектрофотометрии определена эффективность микрокапсулирования и степень высвобождения винпоцетина из микрокапсул в единицу времени. При концентрации раствора натрия альгината 2,5% эффективность микрокапсулирования максимальна (86,8%). При данной концентрации происходит насыщение и при её дальнейшем увеличении эффективность снижается. Максимальная степень высвобождения винпоцетина наблюдается из образцов микрокапсул с концентрацией раствора натрия альгината 1% и составляет 41,17%.

Заключение. Амплитудные параметры поверхности микрокапсул имеют отличия при разных концентрациях. Существует закономерность чередования знака асимметрии и эксцесса у образцов с хитозаном. При изменении масштабов сканирования происходит изменение характеристик поверхности микрокапсул. Наиболее чётко отличительные детали структуры видны при масштабе 2×2 мкм^2. При концентрации натрия альгината 2,5% эффективность микрокапсулирования максимальна (86,8%). При изучении влиянии концентрации раствора натрия альгината на степень высвобождения винпоцетина из образцов микрокапсул установлено, что при концентрации 1% степень высвобождения составляет 41,17%, а при 2,5–4,5%. Данные микрокапсулы можно использовать для изготовления капсул с модифицированных высвобождением.

Ключевые слова: альгинат натрия, хитозан, альгинат-хитозановые микрокапсулы, атомно-силовая микроскопия, амплитудные параметры, эффективность микрокапсулирования, степень высвобождения

INTRODUCTION

In recent years, more and more significance has been attached to the complexes with chitin and chitosan throughout the world. Studies of their quantitative and qualitative analyses have been conducted, as well as the production of these polymers, their physicochemical properties and the possibility of expanding the use of chitin and chitosan in medicine [1]. One of the most promising and actively developing areas in pharmacy and pharmacology is the development of controlled delivery of drugs [2, 3].

Studies on the formation of a film coating based on chitosan with the inclusion of antibiotics, are also being conducted [4].

In addition to the studies of drug complexes with chitosan, the use of chitosan itself as a medicine is of scientific interest. In the course of studying the effect of chitosan on a model of contact allergic dermatitis, it has been found out that by using photophoresis, chitosan is able to reduce the concentration of metal in the skin of the experimental animals. These results indicate the effectiveness of chitosan in the treatment of skin diseases [5–8].

Chitosan is a natural polycationic, linear polysaccharide, a derivative of chitin. It is a universal biomaterial due to the lack of toxicity and good biodegradability and biocompatibility. Mixtures of high purity chitosan mono-, poly- and oligomers act as regenerative, wound healing

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and antitumor drugs. Chitosan exhibits a wide range of positive properties, which makes it possible to use it in various fields of biomedical science [9–13].

The use of chitosan as a nanocarrier of drugs is a promising area of science, since chitosan-drug complexes are more resistant to destruction under the influence of the internal environment of the body, and also increase the delivery of the drug to the target unchanged.

A water-soluble and biodegradable polymer, sodium alginate is often used as a coat [14]. As a polymer for microcapsules, sodium alginate is widely used. In one of the studies, sodium alginate was used in the form of a 2% solution to obtain microcapsules with a bacteriophage. An innovative enteric-soluble dosage form that can be used as an antibacterial drug, has been obtained [15–17].

Atomic-powered microscopy (APM) is one of the most advanced methods for studying surface properties. Traditionally, this method is used to determine the surface morphology of various objects with high spatial resolution. The study of the roughness of microcapsules is carried out to prove that the true surface area is most often more geometric, since it is affected by the structure of the microrelief. Processing the data on the surface relief makes it possible to deeply analyze its various characteristics [18–20].

THE AIM of the investigation is to conduct morphological, technological, and biopharmaceutical studies of alginate-chitosan microcapsules with Vinpocetine.

MATERIALS AND METHODS

Alginate-chitosan microcapsules with various concentrations of sodium alginate (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) and a medium viscosity chitosan solution (0.25–0.5%), as well as microcapsules not treated with a solution of chitosan, have been studied. Obtaining the samples of microcapsules was carried out by extrusion.

The surface morphology was studied by methods of atomic-powered microscopy with the use of an NT-MDT Corporation probe scanning microscope (model Solver P47 Pro) (Zelenograd, Russia).

Scanning was performed by HA_NC cantilevers (for microcapsules without chitosan with 0.5% and 1% concentrations of sodium alginate), by HA_FM (for microcapsules without chitosan with 2% and 3% concentrations of sodium alginate) and by NSG03 (for microcapsules without chitosan with 1.5% and 2.5% concentrations of sodium alginate for microcapsules with chitosan with all used concentrations of sodium alginate).

The length was 90 ± 5 μm, the resonant frequency was (260 ÷ 630) kHz and the radius of the curvature of the probe tip was 10 nm. The experiments were carried out in air at the temperature of 25 ± 1°C. The scanning fields reached (5 × 5) μm^2 with the difference in the elevation of the relief of no more than 2.5 μm. Using a probe and an atomic-powered microscope scanner, it was possible to obtain surface images with the lateral resolution up to 10 nm and the vertical one up to 1 nm.

Visualization of the measurement results consisted in representing the relief in the form of three-dimensional images. Processing of the obtained APM images was carried out with the use of ACM Solver P47 Pro Nova RC1 software and consisted in the analysis of the amplitude average statistical parameters of the surface roughness in accordance with international standards:

1) R_a – arithmetic average roughness;
2) R_q – quadratic mean roughness;
3) R_z – maximum profile height;
4) R_{sk} – asymmetry;
5) R_{ku} – excess.

To determine the true and geometric surface areas of the microcapsules, processing of the obtained APM images was performed using Gwyddion 2.11 software.

The efficiency of microencapsulation was determined by the “direct” method. For the direct determination, the actual content of Vinpocetine in microcapsules after microencapsulation was calculated. For this, a sample of microcapsules was dissolved in 0.0 1 M HCl, heated for 20 minutes, then cooled down, and the volume was adjusted to the mark with the same acid.

Using the spectrophotometry method, the optical density of the resulting solution was determined at the absorption maximum of 312 nm.

After determining the amount of Vinpocetine released during the dissolution, knowing its initial concentration, the microencapsulation efficiency was calculated taking into account the amount of $m_{caps.}$ included in the microcapsules, the initial amount of the substance that was dissolved – $m_{init.}$ according to the formula:

$$E = m_{caps.} / m_{init.} * 100\% \quad (1)$$

The study of biopharmaceutical properties was carried out in accordance with General Pharmacopoeial Monograph 1.4.2.0014.15. Using the spectrophotometry method, the optical density of the obtained solutions was determined with an absorption maximum of 314 ± 2 nm at each sampling stage.

RESULTS AND DISCUSSION

Microcapsules are medium-weight large opaque isodiametric (equiaxed) yellowish-white or yellow crystals. They have good flowability, which is the basic feature for the process of capsules manufacturing.

A comparison of the microcapsules surface and their microprofiles with different concentrations of sodium alginate, treated with a solution of chitosan and without this treatment, is shown in Figs. 1–6.

In Fig. 1a, the surface has a characteristic longitudinal striation, protrusions and cavities of various heights and depths, respectively. The structure is homogeneous, there are no visible inclusions, the outer surface is smooth. In Fig. 1b the surface is cellular, wrinkled, rough. There are small protrusions**.** On the microprofile with chitosan (Fig. 1c), a pronounced surface relief with a

wavy surface is visible, the peaks are smoothed. There is no microprofile without chitosan, since the surface does not have any characteristic relief features.

In Fig. 2a, the surface is slightly rough, it has a rare longitudinal striation, deep cavities and gently-sloping wrinkled heights. In Fig. 2b microcapsules have a transversely wrinkled structure. The surface is smooth, there are deep cavities, pits and voluminous pointed protrusions. The surface micro profiles are similar. Both are sharply scattered at maxima and minima of heights. The peaks are slightly serrated. There are practically no visible differences in the relief structure.

In Fig. 3a, the surface is very rough, with protrusions of different heights and widths. The cavities and pits are small. At the top of the heights there are rare small spherical inclusions. On the ups and downs, the surface is longitudinally wrinkled. In Fig. 3b, the surface is smooth, sporadically it has a longitudinal striation. There are single rocky and spherical inclusions of different sizes, as well as groups of such inclusions. The cavities are practically absent, but there are small protrusions and ups. The microprofile of the surface with chitosan (Fig. 3c) has a less smoothed surface, but smaller elevation differences, as well as serrated peak tops, than the microprofile without chitosan (Fig. 3d).

In Fig. 4a, the surface is slightly rough, there are characteristic high peaks found in the groups with a wide base and a pointed apex. The pits are shallow, but long. In Fig. 4b, the surface is uneven, rough, with numerous spherical protrusions and inclusions. The microprofile of microcapsules with chitosan (Fig. 4c) has an abrupt relief, which differs significantly from the microprofile without chitosan (Fig. 4d) characterized by a sawtooth surface.

At the given concentration of sodium alginate, the differences in the surface of microcapsules with and without chitosan begin to come out.

In Fig. 5a, the surface is wrinkled, slightly cellular, and there are no visible inclusions. In Fig. 5b, the surface of the microcapsule is smooth, with long, winding, shallow pits. The protrusions are large, gentle, with a slight transverse striation. There are no inclusions. Microprofiles (Figs. 5c and 5d) have characteristic differences. The surface with chitosan has a coarse-toothed profile with narrow and finely serrated peaks, and without chitosan, on the contrary, it is more even and smoother, and has also a wavy smooth appearance. The amplitude profile parameters for the samples (see Table 1), vary greatly with respect to each other, which makes it possible to consider the differences between microcapsules with and without chitosan.

In Fig. 6a, the microcapsule is highly wrinkled, covered with numerous pits. The surface is unevenly rough. There are gently-sloping heights of various shapes.

In Fig. 6b, the surface of the microcapsule is smooth, deeply sinuous, the protrusions are big, gently-sloping, transversely striated. There are no inclusions in them. In Fig. 6c, the microprofile has a coarse toothed appearance with elongated serrated peaks. The microprofile without chitosan is wavy, smoothed.

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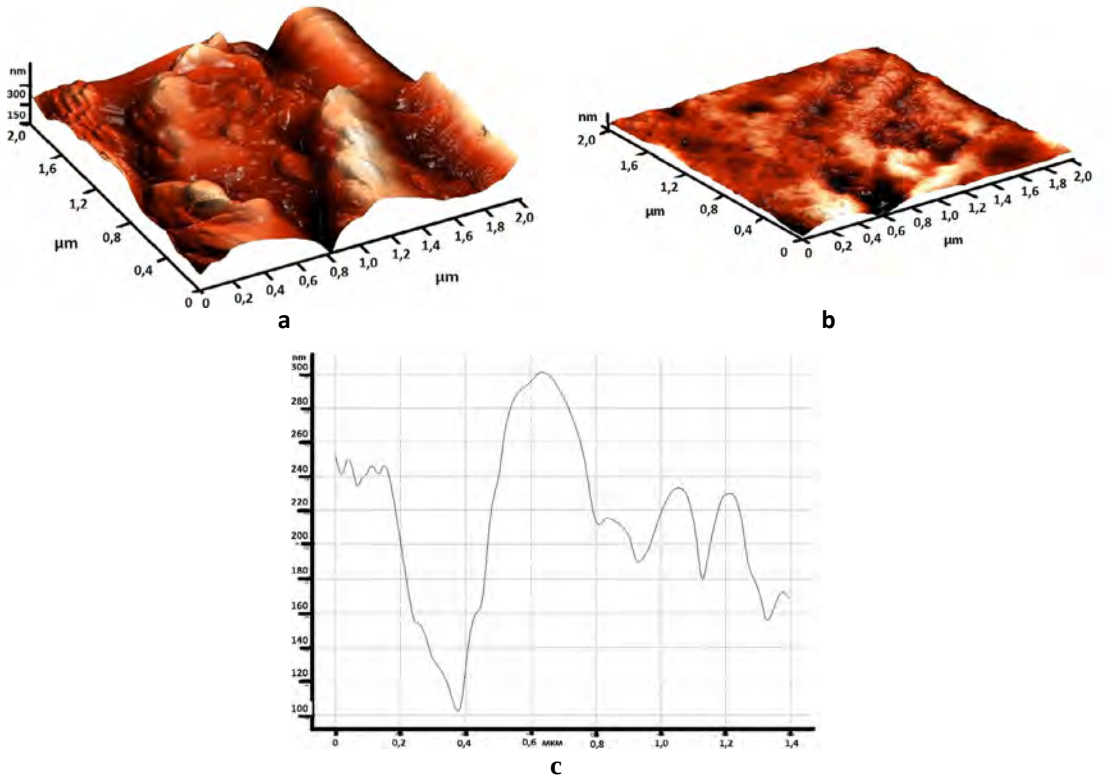
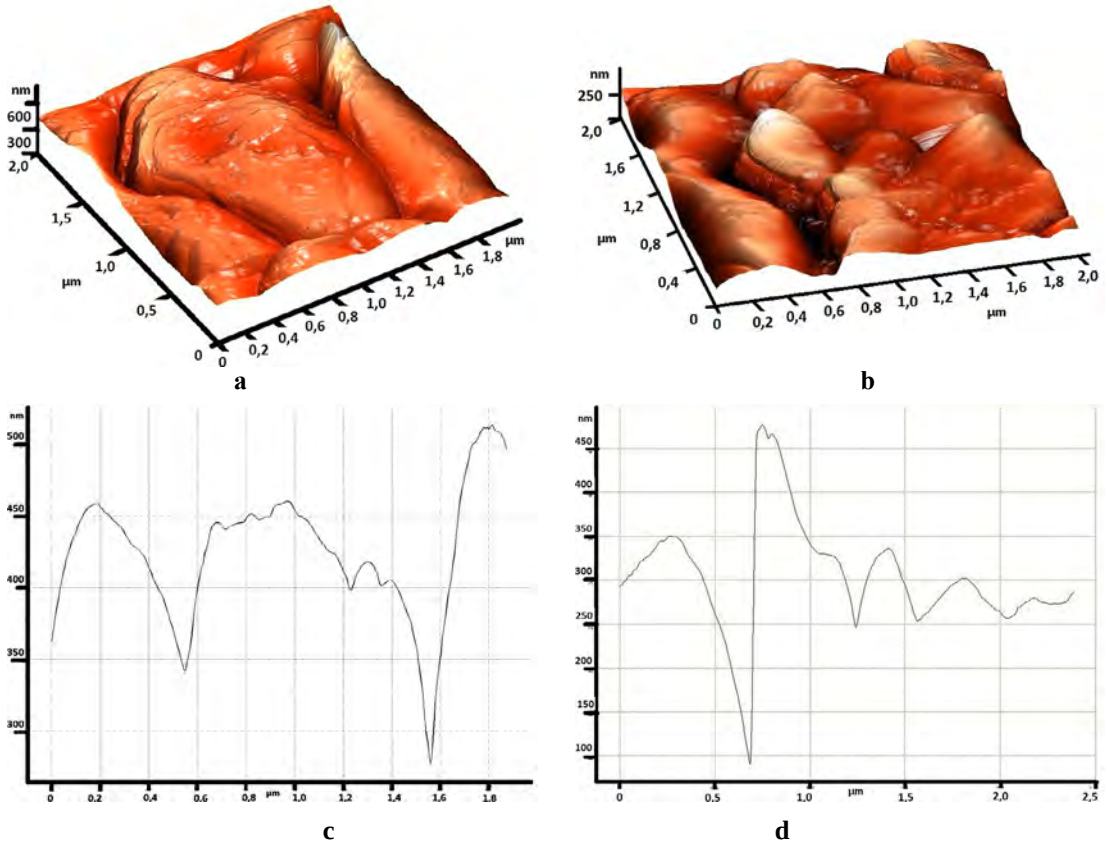


Figure 1 – Three-dimensional APM image of the microcapsules surface with a 0.5% concentration of sodium alginate treated with a chitosan solution (a), and its microprofile (c), and without chitosan (b), with a scanning area of 2 × 2 μm²



PFigure 2 – Three-dimensional APM image of the microcapsules surface with a 1% concentration of sodium alginate treated with a chitosan solution (a), and without chitosan, (b), and their microprofiles (c and d), with a scanning area of 2 × 2 μm²

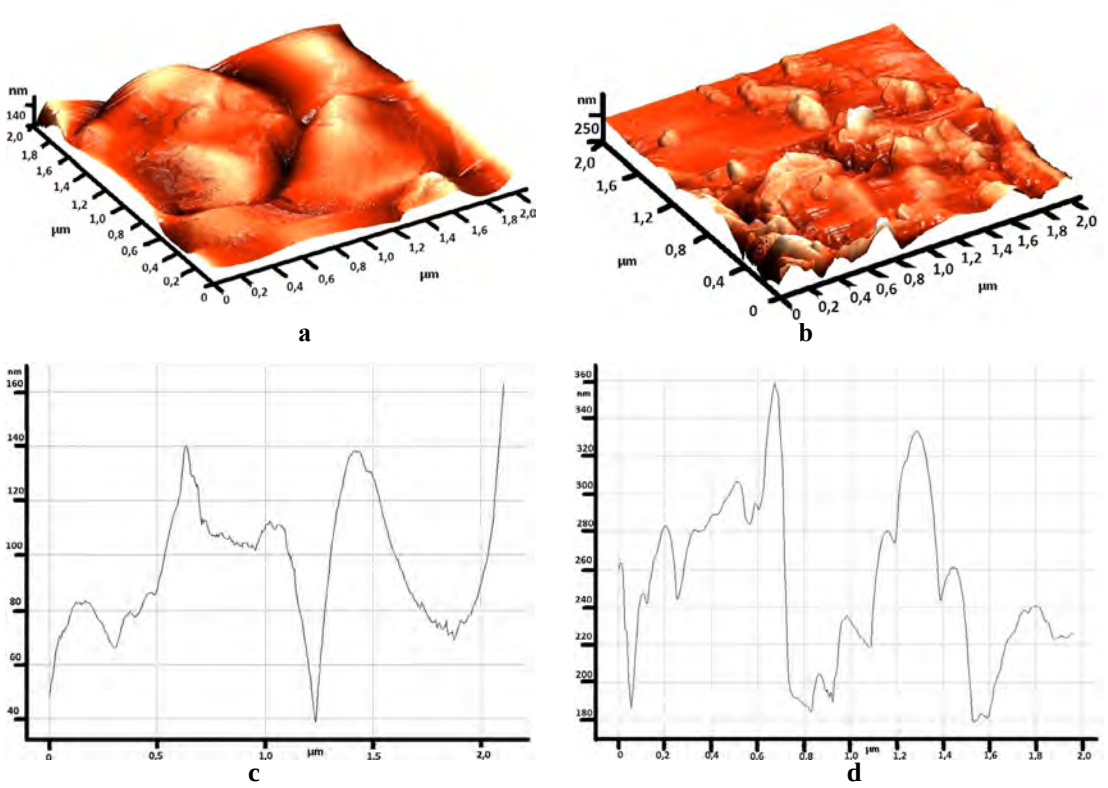


Figure 3 – Three-dimensional APM image of the microcapsules surface with a 1.5% concentration of sodium alginate treated with a chitosan solution (a), and without chitosan, (b), and their microprofiles (c and d) with a scanning area of 2 × 2 μm²

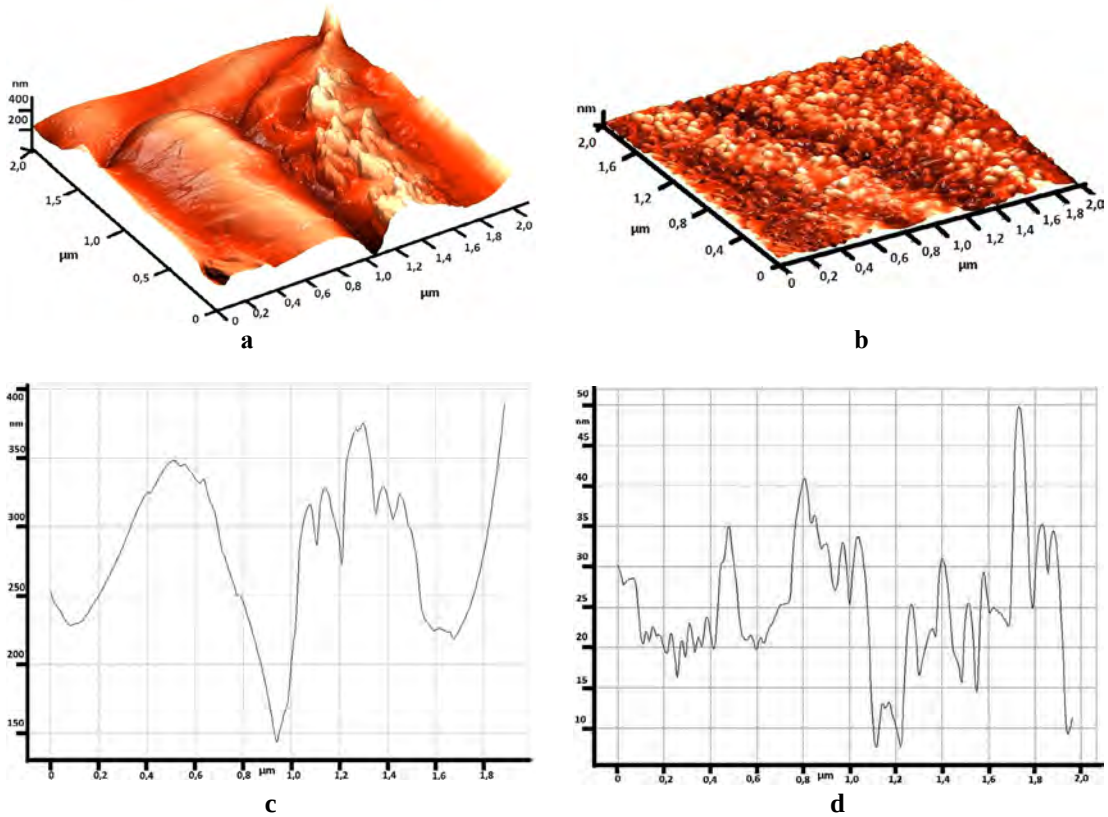


Figure 4 – Three-dimensional AFM image of the microcapsules surface with a 2% concentration of sodium alginate, treated with a chitosan solution (a), and without chitosan (b), and their microprofiles (c and d) with a scanning area of 2 × 2 μm²

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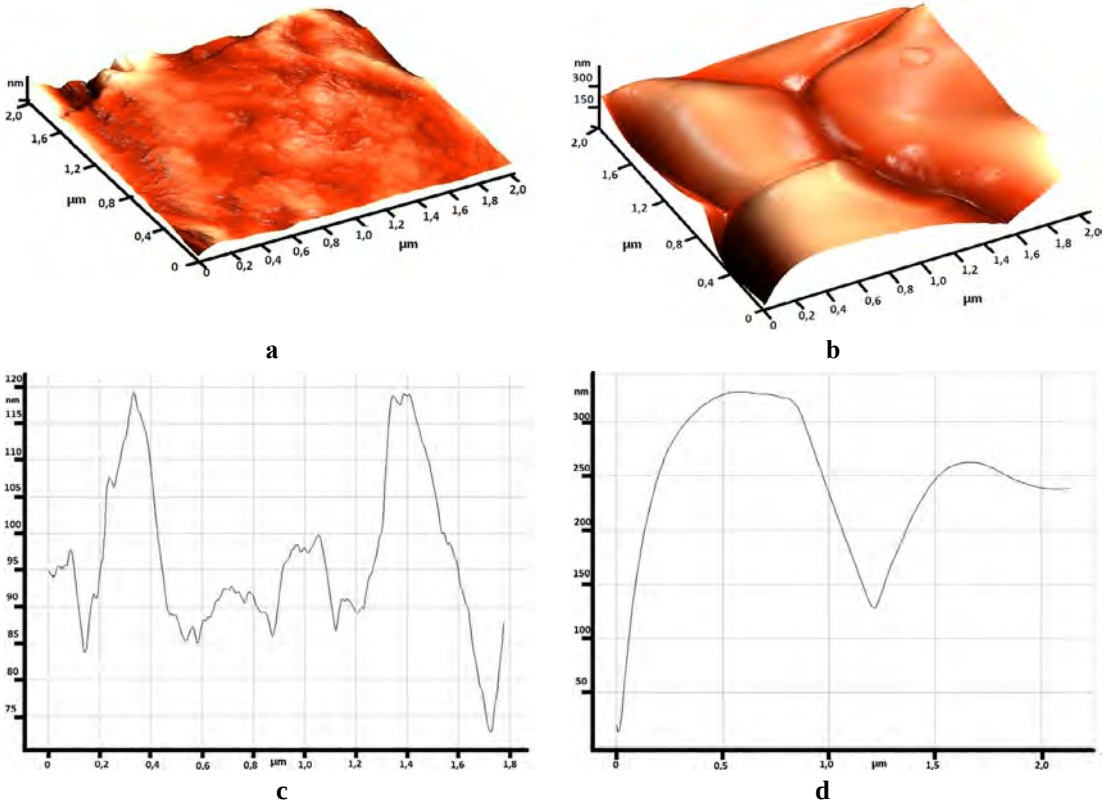

Figure 5 – Three-dimensional APM image of the microcapsules surface with a 2.5% concentration of sodium alginate treated with a chitosan solution (a) and without chitosan (b), and their microprofiles (c and d) with a scanning area of 2 × 2 μm²

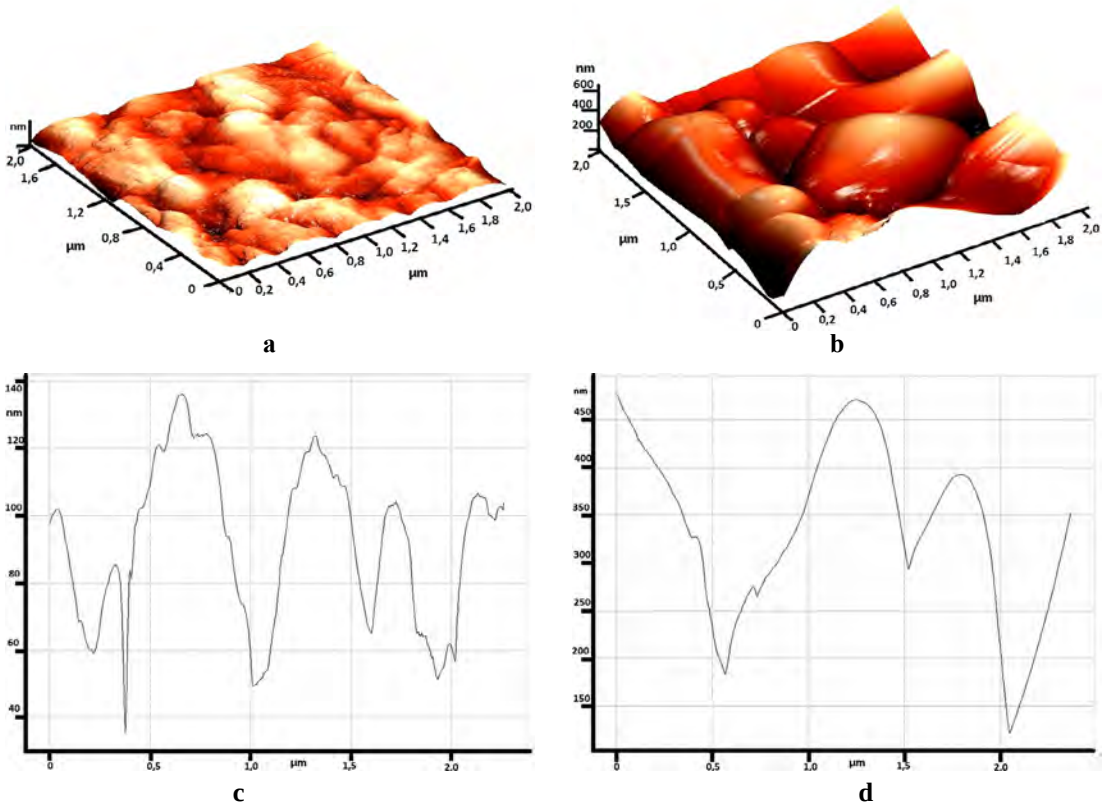

Figure 6 – Three-dimensional APM image of the microcapsules surface with a 3% concentration of sodium alginate, treated with a chitosan solution (a), and without chitosan (b), and their microprofiles (c and d) with a scanning area of 2 × 2 μm²

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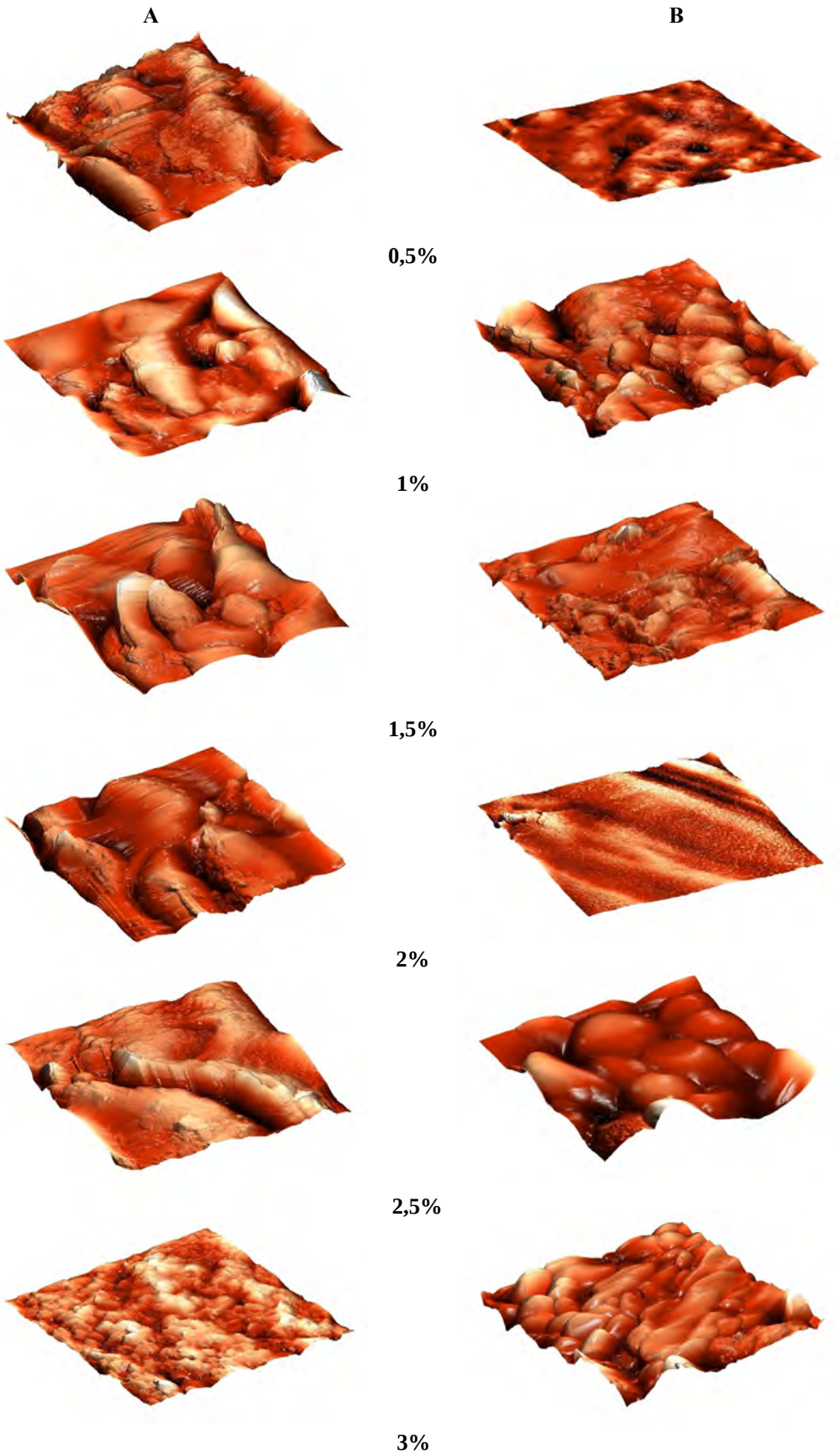

Figure 7 – Three-dimensional APM image of the microcapsules surface with different concentrations of sodium alginate treated with a chitosan solution (A) and without chitosan (B), with a scanning area of 5 × 5 μm²

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Table 1 – Parameters of the surface roughness of the microprofile with a scanning area of 2 × 2 μm²

Concentration of sodium alginate solution	Type of microcapsules	R_z, nm	R_a, nm	R_q, nm	R_{sk}, nm	R_{ku}, nm
0.5%	With chitosan	199	41,8	51.4	−0.3	−0.5
	Without chitosan	–	–	–	–	–
1%	With chitosan	240	31.9	46.7	−1.5	3.6
	Without chitosan	390	49.2	73.5	0.1	2.6
1.5%	With chitosan	126	19.4	23.9	0.3	1.2
	Without chitosan	180	39.4	48.4	0.6	−0.4
2%	With chitosan	232	47.0	56.0	−0.4	−0.3
	Without chitosan	42	6.3	8.2	0.5	0.6
2.5%	With chitosan	46.5	8.3	10.8	0.8	0.1
	Without chitosan	314	57.4	85.9	−1.5	2.4
3%	With chitosan	101	24.8	27.2	−0.2	−1.4
	Without chitosan	680	77.8	95.8	−0.2	−0.6

The amplitude roughness parameters based on the microprofiles, were calculated. The results are shown in Table 1.

On the basis of the carried out studies it was found out that microcapsules not treated with a chitosan solution have a smooth, transversely striated surface with large heights and deep cavities. With an increase of the sodium alginate concentration, the surface becomes smoother, the peaks become larger, higher and wider, the cavities get deeper and more sinuous. The microcapsules treated with a chitosan solution, on the contrary, have a rough surface, small heights and shallow pits, and with an increase in the concentration of sodium alginate, the surface becomes rougher, the heights are evenly distributed along the microcapsule. At the sodium alginate concentrations of 0.5%, 1%, and 1.5%, the differences between microcapsules with and without chitosan are implicit, but at the concentrations of 2.0%, 2.5% and 3%, the characteristic features become clear. The microprofiles made it possible to calculate the necessary roughness parameters. The calculated data confirmed the differences in the surface character of the microcapsules. The samples with chitosan and 0.5%, 1%, 2% and 3% concentrations of sodium alginate, are characterized by negative asymmetry, which indicates that the distribution has a long left “tail” and a negative excess (except the 1% sample). Microcapsules without chitosan with a 1–2% concentration of sodium alginate have a positive asymmetry with close indicators, i.e. a long right “tail”. The samples with chitosan and without chitosan at the 1.5% concentration have different positive asymmetries at different excesses, and at the 3% concentration they have the same negative asymmetry with different but negative excesses. Some alternation of the asymmetry sign and excess is observed in the samples with increasing concentrations.

Below, Fig. 7 shows the surface of microcapsules with different concentrations of sodium alginate treated with and without a chitosan solution with a scanning area of 5 × 5 μm².

With a scanning area of 5 × 5 μm², one can also observe the manifestation of differences in the surface structure of microcapsules with and without chitosan, but clearly visible differences are noticeable only at 2.5% and 3% sodium alginate concentrations. At the concentration of 2%, vague characteristics are observed.

Besides, as far as the given scanning area is concerned, the characteristic and structural features of the microcapsules surface described above at different concentrations of sodium alginate, are less noticeable. As a result of that, it is difficult to distinguish microcapsules with and without chitosan at the concentration of 2%.

At the next stage of the research, the efficiency of microencapsulation was determined by spectrophotometry. Based on the data obtained, a graph of the dependence of the Vinpocetine microencapsulation efficiency on the concentration of sodium alginate has been constructed (Fig. 8).

So, at the 2.5% concentration of sodium alginate, the efficiency of microencapsulation is maximum – 86.8%. At this concentration, saturation occurs and with its further increase, the efficiency decreases.

As a result of the biopharmaceutical studies, carried out in the process of the microcapsules preparation, the effect of the concentration of a sodium alginate solution on the Vinpocetine release has been shown (Fig. 9).

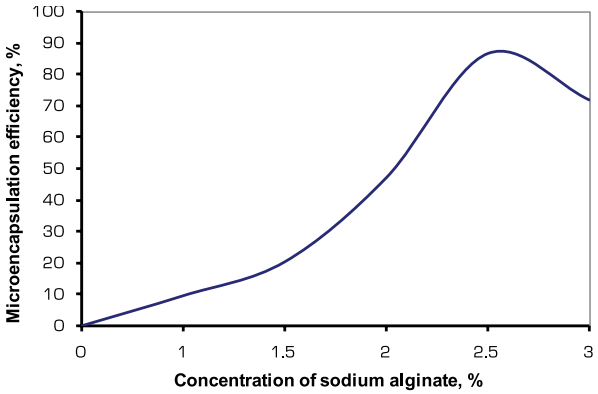

Figure 8 – Dependence of microencapsulation efficiency on the concentration of sodium alginate

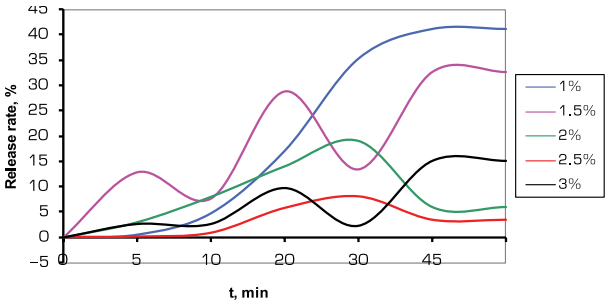

Figure 9 – Release rate of Vinpocetine from microcapsules

Table 2 – Technological properties of microcapsules

Test indicator	Methods of determination	Experimental values	Reference values
Microcapsules size	General Pharmacopoeial monograph 1.1.0015.15	1.0–2.0 mm 0.5–1.0 mm 0.2–0.5 mm (Medium, large and very large)	Very large: >1.4 mm Large: 0.355–1.4 mm Moderately fine: 0,18–0,355 mm Medium: 0.125–0.18 mm Very small: 0.09–0.125 mm
Microcapsules shape	General Pharmacopoeial monograph 1.2.1.0009.15	Equlaxial	Elongate: >3:1 Tabulate: 3:1 Equiaxed: 1:1
Flowability	General Pharmacopoeial monograph 1.4.2.0016.15	Loose weight density: 714.3 kg/m^3 (Medium)	Rather heavy: >2000 kg/m^3 Heavy: 1100–2000 kg/m^3 Medium: 600–1100 kg/m^3 Light: < 600 kg/m^3
		Flowability: 14.17 g/sec. (Good)	Excellent: 8.6–12.0 g/sec. Good: 6.6–8.5 g/sec. Satisfactory: 3.0–6.5 g/sec. Acceptable: 2.0–3.0 g/sec. Bad: 1.0–2.0 g/sec. Very bad: <1.0 g/sec.
		Angle of natural slope: 34° (Good)	Very good: 25–30° Good: 31–35° Satisfactory: 36–45° Poor: 46–55° Bad: 56–65° Very bad: >66°

The release rate of Vinpocetine from microcapsule samples with a 1% concentration of a sodium alginate solution is maximum and amounts to 41.17%. A slower release is observed from the microcapsules with a 2.5% concentration of a sodium alginate solution: by 45 minutes of the experiment it has been 4.5%.

Next, the technological properties of microcapsules were studied (Table 2).

The data obtained indicate that microcapsules are opaque, medium-weight large opaque isodiametric (equiaxed) yellowish-white or yellow particles with good flowability, which makes it possible to be used as fillers for encapsulated forms.

Thus, as a result of the carried out studies, model samples of Vinpocetine microcapsules were obtained. The method of atomic-powered microscopy was used to study morphological features of alginate-chitosan microcapsules. In the surface structure, the microcapsules treated with chitosan, have characteristic differences from the microcapsules without chitosan. The differences are most pronounced at the 2.5% and 3% concentrations of sodium alginate. Amplitude parameters are different at different concentrations. Negative asymmetry prevails in the sam-

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ples with chitosan (0.5%, 1%, 2%, 3%), the excess is evenly distributed. The samples without chitosan are dominated by positive asymmetry (1%, 1.5%, 2%) and positive excess. There is a certain pattern of alternating the sign of asymmetry and excess in the samples with chitosan.

With a change in the scale of the scan, the surface characteristics of the microcapsules change, too. The most distinctive details of the structure are visible at the scale of 2 × 2 μm^2.

When the concentration of asodium alginate solution is 2.5%, the efficiency of microencapsulation is maximum (86.8%).

While studying the effect of the concentration of a sodium alginate solution on the release rate of Vinpocetine from the microcapsule samples, it was found out that at the concentration of 1%, the release rate is 41.17%, and at the concentration of 2.5% it is 4.5%. These microcapsules can be used to make modified release capsules.

CONCLUSION

Thus, it has been established that with an increase in the degree of microcapsules roughness, an increase in the efficiency of microencapsulation occurs. With an increase in the concentration of a sodium alginate solution, the release rate of the substance decreases. This can be explained by the fact that Vinpocetine has an affinity for sodium alginate, which binds it to the complex, and with an increasing concentration of a sodium alginate solution in an aqueous medium, the binding strength increases.

The results obtained during the study are promising for a further detailed study of microencapsulated vinpocetine in order to create its dosage forms.

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AUTHOR CONTRIBUTIONS

All authors have equally contributed to the research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CORRECTION OF PSYCHOLOGICAL AND NEUROLOGICAL SIGNS OF ALCOHOL HANGOVER IN RATS WITH ACETYLCYSTEINE

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The development of medications to treat alcohol hangover is important for a large number of people, especially those, who perform responsible and dangerous types of work. The severity of the hangover syndrome is determined by the toxic effect of acetaldehyde and its metabolic rate, which decreases with the depletion of glutathione, worsening and prolonging the hangover.

The aim of the article is to provide an experimental basis for use of acetylcysteine as a precursor of glutathione in treatment of psychological, neurological and cognitive symptoms of ethanol hangover.

Materials and methods. The study was performed in male Wistar rats. The ethanol hangover was modeled via an acute ethanol injection (i.p., 3 g/kg). After awakening, the animals were divided into 2 groups, receiving acetylcysteine (p.o., 1 mg/kg) or saline. The intact group of the animals received saline only. Before and after acetylcysteine or saline administration, the animals were assessed according to Combs and D'Alecy scale. The adhesive test, the open field test, the passive avoidance test and Morris water maze test were also performed twice. The liver glutathione level was assessed in sacrificed animals.

Results. The control group animals showed signs of neurological deficits and cognitive impairment, including a decreased locomotion, motor deficits and a memory decline. The rats administered with acetylcysteine after the ethanol intoxication, had a less severe impairment associated with an improved performance in the adhesive test, Morris water maze test and the passive avoidance test, and demonstrated an increased locomotion in the open field test. The liver glutathione content in the animals treated with acetylcysteine, was comparable to the glutathione content in the liver of the animals not exposed to the ethanol intoxication.

Conclusion. Against the background of an acute ethanol intoxication, an oral administration of acetylcysteine in the rats, promoted an increase in liver glutathione levels and led to a decrease in severity of neurological and cognitive deficits in the animals.

Keywords: ethanol, post-toxic state, hangover, acetylcysteine, rat, preclinical study

Abbreviations: ATP – adenosine triphosphate; WHO – World Health Organization; NAD – nicotinamide adenine dinucleotide; OP – Open field.

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КОРРЕКЦИЯ ПСИХОНЕВРОЛОГИЧЕСКИХ ПРОЯВЛЕНИЙ АЛКОГОЛЬНОГО ПОХМЕЛЬЯ У КРЫС АЦЕТИЛЦИСТЕИНОМ

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Разработка средств для устранения алкогольного похмелья важна для большого числа людей, в том числе связанных с выполнением ответственных и опасных видов работ. Тяжесть похмельного синдрома определяется токсическим действием ацетальдегида и скоростью его метаболизма, которая снижается при истощении запасов глутатиона, что приводит к утяжелению и пролонгации похмелья.

Цель. Экспериментальное обоснование применения ацетилцистеина в качестве предшественника глутатиона для снижения выраженности психоневрологических и когнитивных нарушений, возникающих на фоне алкогольной интоксикации.

Материалы и методы. Исследование проведено на крысах-самцах линии Wistar. Состояние «похмелья» моделировали путем однократного введения этанола (3 г/кг, в/б). После пробуждения животных разделяли на 2 группы: опытной группе однократно перорально вводили раствор ацетилцистеина (1 мг/кг), контрольной группе – физ. р-р. Интактной группе вводили физ. р-р. До лечения и спустя 3 часа поочередно оценивали уровень неврологического дефицита по шкале «Combs and D'Alecy», а также проводили тесты: Адгезивный тест, Открытое поле (ОП), УРПИ и лабиринт Морриса. После эвтаназии определяли уровень глутатиона в гомогенатах печени.

Результаты. У животных контрольной группы отмечались выраженные признаки психоневрологических и когнитивных нарушений, проявляющихся в низкой двигательной активности, нарушении мелкой моторики и когнитивных функций.

У животных, которым вводили ацетилцистеин, нарушения психоневрологических и когнитивной функций были менее выражены, что проявлялось в улучшении мелкой моторики в Адгезивном тесте, повышении двигательной активности в тесте ОП. В тестах лабиринт Морриса и УРПИ, в которых оценивают сохранность памятного следа, большее количество животных успешно решили задачу, а уровень глутатиона в печени отвечал физиологической норме.

Заключение. На фоне острой алкогольной интоксикации введение ацетилцистеина, способствует восстановлению содержания глутатиона в печени и приводит к снижению выраженности нарушений психоневрологических и когнитивной функций у экспериментальных животных.

Ключевые слова: этанол, постинтоксикационное состояние, похмелье, ацетилцистеин, крысы, доклинические исследования

Список сокращений: АТФ – аденозинтрифосфат; АЦЦ – ацетилцистеин**;** ВОЗ – Всемирная организация здравоохранения; НАД – никотинамидадениндинуклеотид; ОП – Открытое поле; УРПИ – условная реакция пассивного избегания.

INTRODUCTION

An acute consumption of large quantities of ethyl alcohol, causes profound negative socio-economic consequences. According to the CDC data for 2010, economic losses due to the reduced labor productivity because of the alcohol consumption, amounted to $ 179 billion. Despite the large socio-economic costs and the costs of medical services spent on the treatment of the hangover syndrome and its consequences, this problem is of relatively little interest among the researchers. As a result, this extremely widespread symptom complex, still has many unexplained aspects in its pathogenesis. To improve the methods of its prevention or pharmacotherapy, a deeper understanding of the pathological processes that occur in the body during the development of a similar condition is necessary [1, 2].

Currently, this syndrome is defined as a general discomfort that occurs after the complete metabolism of previously taken alcohol (i.e. in the situation where alcohol cannot be detected in the blood). Neuropsychiatric disorders that occur after drinking large doses of alcohol, are not a steady state of the body, but a developing and slowly running process, which is especially important to consider by the people whose professional activities require attention and a high speed of decision-making at the risk of threatening situations (for example, drivers and operators of complex mechanisms) [3]. For an outsider, the presence of a hangover is dif-

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ficult to detect and distinguish from the symptoms of certain diseases (a tension headache, food poisoning, arterial hypertension), which, under certain conditions, can pose a threat to others. In particular, professional drivers who are in a state of post-alcohol intoxication, make significantly more serious errors when driving in simulation conditions (on a car simulator). They more often and by a larger amount exceed speed, ignore traffic signs, do not notice abrupt interference, take notice of emergency situations slower and/or inappropriately [4, 5]. It is important to note that in this state, irritability and aggressiveness often increase, which can be associated with a severe pain and/or increased levels of testosterone [6, 7]. Its metabolism occurs in the liver and can change with the depletion of intracellular substrates caused by an intensive biotransformation, first of the alcohol, and then of its metabolites. A hangover is characterized by the development of a complex of negative symptoms, the most common of which are: a headache, nausea, vomiting, diarrhea, chills, fever, drowsiness, tremor, irritability, aggressiveness [8]. In the groups whose employees, on condition of anonymity, reported performing their duties in the morning after drinking large doses of alcohol, interpersonal conflicts occurred more often, and the overall labor productivity was low [9].

Ethanol intake is an example of a voluntary, largely uncontrolled, consuming of the most common and probably the most studied psychotropic drug [10]. With a lot of stressful factors in modern life, antidepressant, psychostimulating, disinhibit and hedonic effects of ethyl alcohol, often become a cause of abuse. Although alcohol is often consumed in the doses that cause a hangover, there are no universal signs to determine a risk group. For this reason, negative phenomena associated with its use, can occur in absolutely any person (regardless of their social, financial or legal status, mental and physical level of development).

According to the results of some sociological studies, 75% of the people who had been consuming alcoholic beverages to the state of intoxication, had a hangover at least once, 15% of the respondents noted that the latest episode of this kind had taken place a month before. About 50% of the people who regularly take ethanol (those who consume one or two drinks per day; one drink is an equivalent dose of 50 g of pure alcohol [11]) have a habitual hangover. 40% of the people who openly report the fact of their frequent use of ethyl alcohol say, that they have neuropsychiatric post-toxic effects every month or more often. In patients with alcoholism, their prevalence is lower and amounts to 20–25%. Up to 87% of university students experience one or more hangover episodes during a year, while numerous studies have shown a negative impact of this symptom complex on their cognitive activity [12].

Pharmacotherapy is the only effective way to eliminate the effects of an acute excessive consumption of alcoholic beverages, but the modern pharmaceutical market has a limited number of drugs for the prevention and treatment of this state [13]. The situation is complicated not only by the lack of the general consensus of the professional community regarding the pathogenesis of a hangover, but also by the difficulties encountered in initiating and conducting preclinical studies.

One of the promising directions in the treatment of hangover is the development of the agents that accelerate the metabolism of acetaldehyde. Among the compounds with a similar effect, acetylcysteine, which is the precursor of glutathione, the most important regulator of the redox processes in cells, including hepatocytes, can be distinguished. Ethyl alcohol is metabolized to acetaldehyde, which has a general toxic effect, the manifestation of which is a hangover. Glutathione inactivates free oxygen radicals formed in the mitochondria of hepatocytes in the processes of their functioning, the formation of which depends on the intensity of the course of the redox reactions. Since the alcohol metabolism proceeds in several stages (alcohol – acetaldehyde – acetic acid), hepatocytes stay in conditions of intensification of metabolic processes for a long time when a large amount of them enters the human body, which is followed by the formation of a large number of reactive oxygen species and reactive metabolites inactivated by glutathione. In such a situation, a slowdown in the metabolism of acetaldehyde due to the depletion of the reserves of reduced glutathione is not excluded. Therefore, the use of substances that can increase the content of the latter, can promote the activation of natural detoxification systems, accelerate the metabolism of acetaldehyde and alleviate the hangover syndrome. The use of acetylcysteine, able to increase the content of reduced glutathione, may become promising for this purpose [14].

This study was planned to provide an experimental basis for the use of acetylcysteine as a precursor of glutathione in treatment of psychological, neurological and cognitive symptoms of ethanol hangover.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the animal research standards defined by the law of the Russian Federation and EASC technical standards for Good laboratory practice (GOST R 53434-2009 and GOST R 51000.4-2011). The study design and the protocol were reviewed and approved by the Department of the ethical, legal, and sociological expertise in medicine of the Volgograd Medical Research Center [registration number: IRB 00005839 IORG 0004900 (OHRP)] on May 20, 2019 (protocol number: 132).

The experimental study was performed on 30 male Wistar rats (300–350 g, obtained from mouse bank of

"Rappolovo"). In the vivarium, the animals were acclimated for 14 days before starting the experiment where the rats were kept during the experiment. They were housed at 20 ± 2°C and 40–60% humidity in a standard 12/12-h light–dark cycle with food and tap water *ad libitum*.

Study design

Before the acute ethanol administration, all the animals were trained in Morris water maze task and in the passive avoidance reflex conditioning test (for 4 and for 2 days, respectively). The animals able to find a platform in Morris water maze task and showing avoidance of dark compartment in the passive avoidance test, were divided into 3 groups (n = 10 in each). The groups were the following:

1. Intact group – normal saline (15 ml/kg, i.p.) + saline (5 ml/kg, p.o.);
2. Control group – ethanol (3 g/kg, i.p.) + saline (5 ml/kg, p.o.);
3. Experimental group – ethanol (3 g/kg, i.p.) + acetylcysteine (1 g/kg, p.o.).

The rats of the first (intact) group were administrated with saline (i.p. and p.o.). The rats of the control and experimental groups were given a single dose of 20% ethanol solution (3 g/kg, i.p.). Then (after awakening and fixing the initial parameters), a single dose of a physiological solution (LLC "Hematec", Russia) or an aqueous solution of acetylcysteine (CJSC "Sandoz", Russia) was administered (1 g/kg, per os). The volumes of the solutions for intraperitoneal and intragastric administration were 15 and 5 ml / kg, respectively.

After the ethanol administration, the latent period of loss of the righting reflex (ability to stand up and lean on the limbs from the upside-down position) was recorded in the animals [15]. The duration of sleep in animals was 8 h ± 30 min.

Within 30 min after awakening the animals were assessed. Combs and D'Alecy scale, the adhesive test, the open field test, the passive avoidance test and Morris water maze test were performed. Then the rats (in accordance with the group) were administered with acetylcysteine or saline intragastrically. The animals, which did not wake up 8.5 hours after the ethanol administration, were excluded from the experiment.

3 hours later, all the tests were performed again: the signs of neurological deficits were assessed according to Combs and D'Alecy scale [16]. The adhesive test was used to assess the ability of the animals to feel and remove a duct tape applicated on the upper paws within 3 min of the observation [17]. The open field test was used to study locomotor and exploratory activities of the animals [18]. Cognitive functions were assessed in the passive avoidance test [18] and in Morris water maze test [19].

After euthanasia (decapitation under anesthesia with zoetil / xylazine at the dose of 20/8 mg / kg), liver tissue samples were taken from the animals for the subsequent analysis. The concentration of the reduced glutathione was assessed in the reaction with 5.5-dithiobis-(2-nitrobenzoic acid) [20]. All the reactions were performed in triplicates.

Statistical processing

The statistical processing was performed by methods of descriptive and analytical statistics. The distribution of quantitative values was evaluated using the Shapiro-Wilk test. The intergroup differences were assessed by a one-way analysis of variance using Newman-Keuls *post hoc* test. All the data were presented as the mean and standard error of the mean (unless otherwise indicated). The differences in the categorical data were evaluated by χ^2 test.

RESULTS

An intraperitoneal ethanol administration in rats resulted in the general anesthesia which lasted in average for 8 ± 0.5 hours. With regards to the latent period of anesthesia and to the total duration of this condition, the rats included in this study, were comparable.

The ethanol hangover in the rats was characterized by an inhibition of the total activity, a decrease of locomotion and somnolence (the animals spent more time with shut eyes, and their reactivity to mechanic stimuli was decreased). The results of the open field test reflected the general trends: the ethanol hangover significantly decreased the exploratory and motor activities in all the animals.

The decrease in the motor activity was also observed in the control animals 3 hours afterwards, while in the acetylcysteine-treated rats their motor and exploratory activities in the open field test increased (Figure 1). Thus, acetylcysteine promoted a restoration of the total activity in the rats exposed to the acute ethanol intoxication.

The signs of neurological deficits, assessed according to Combs and D'Alecy scale, were symptoms of severe neuropsychiatric deficiency after awakening in both ethanol-treated groups. The severity of neurological deficits decreased in 3 hours after the oral administration of saline and decreased significantly after the administration of acetylcysteine (Figure 2A).

In the adhesive test, the animals neglected a duct tape applicated on the volar side of the upper limbs. In 3 hours after the first testing and treatment of the animals undergoing acute alcohol poisoning (the oral administration of saline did not improve their sensory-motor function), only 2 of 10 animals were able to remove a duct tape (at least from one paw). In the group of acetylcysteine-treated rats, 7 of 10 animals were able to remove a duct tape ($p < 0.05$) using a shorter period of time ($p < 0.05$; Figure 2B).

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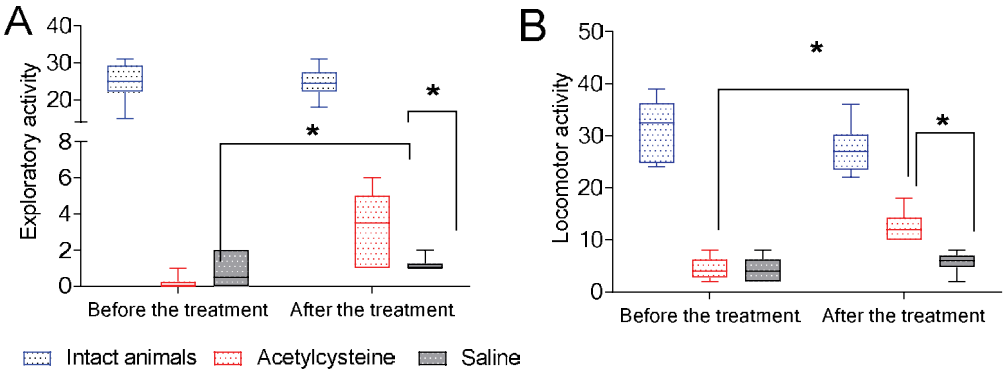

Figure 1 – Effect of acetylcysteine on exploratory (A) and locomotor (B) activities in the open field test performed by the rats exposed to acute ethanol intoxication

*Note: * – p < 0.05 (one-way ANOVA with Newman-Keuls post-test); the compared groups are connected with horizontal lines; the exploratory activity was defined as a sum of vertical postures and nose-poking acts; the locomotor activity was defined as a number of crossed sectors of the open field.*

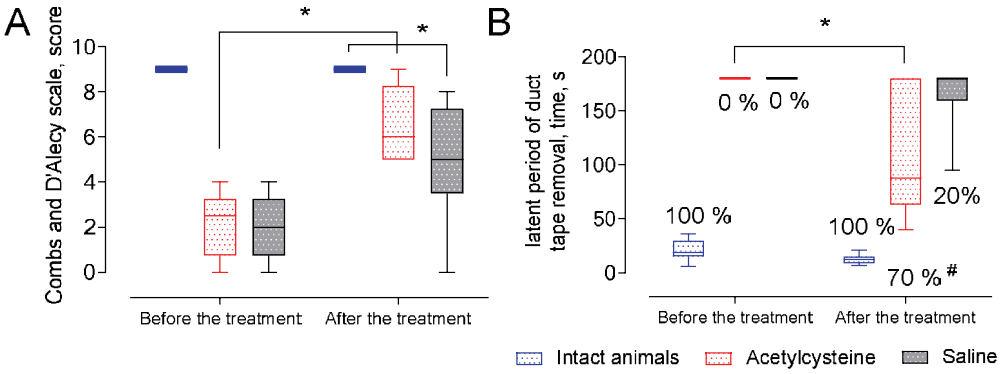

Figure 2 – Effect of acetylcysteine on neurological deficits assessed according to Combs and D'Alecy scale (A) and a latent period of the duct tape removal in the adhesive test (B) in the rats exposed to acute ethanol intoxication

*Note: * – p < 0.05 (one-way ANOVA with Newman-Keuls post-test); # – p < 0.05 (χ^2); % – a number of the animals in the group, which removed the duct tape from one paw; the compared groups are connected with horizontal lines.*

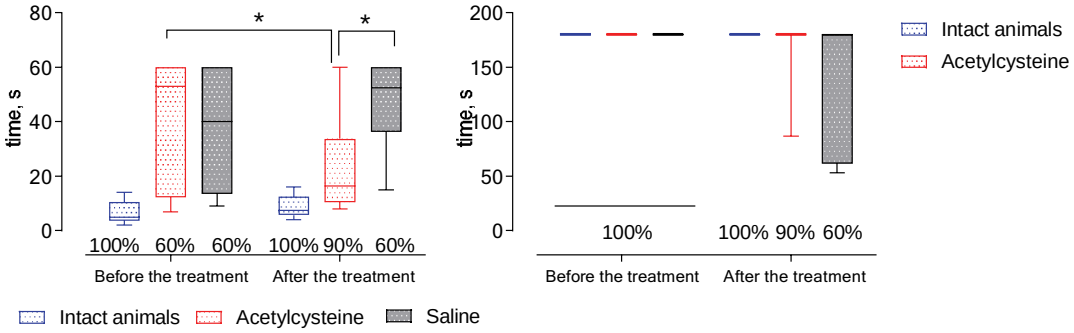

Figure 3 – Effect of acetylcysteine on the latent period of platform finding in Morris water maze (A) and on the latent period in the passive avoidance test (B) in the rats exposed to acute ethanol intoxication

*Note: * – p < 0.05 (one-way ANOVA with Newman-Keuls post-test); % – a number of the animals able to find a platform (A) or avoided a dark compartment of the passive avoidance test apparatus (B); the compared groups are connected with horizontal lines.*

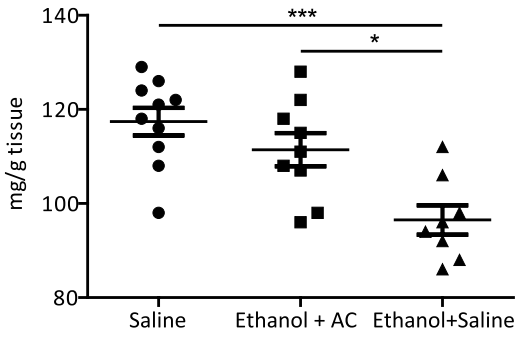

Figure 4 – Effect of acetylcysteine on the liver glutathione content in the rats exposed to acute ethanol intoxication

*Note: AC – acetylcysteine; data shown as means of triplicates, grand mean and standard error of mean; *, *** – p < 0.05, p < 0.0001 (one-way ANOVA with Newman-Keuls post-test); the compared groups are connected with horizontal lines.*

Before the ethanol administration, all the animals were trained in Morris water maze for 4 days to find a platform, and 2 days before the ethanol administration they passed a fear conditioning procedure based on the passive avoidance reflex. All the rats exposed to the ethanol intoxication ~~had~~ increased the period of platform finding in Morris water maze (Figure 3A). In the passive avoidance test, the animals demonstrated decreased locomotion without any exploratory activity, thus the assessment of the avoidance reaction could not be performed.

No changes in Morris water maze task and in the passive avoidance test performance were observed in the saline-treated animals within 3 hours after awakening. The rats treated with acetylcysteine, showed a better performance in both tests (Figure 3A, 3B). The observed results suggest that acute ethanol intoxication is associated with memory decline which could be partially rescued with the acetylcysteine administration.

At the end of study, the animals were sacrificed, and liver tissues were sampled for glutathione content detection. In the animals administered with ethanol and saline, the liver glutathione content reached 96.5 ± 3.11 mg/g tissue (*vs* 117.4 ± 2.95 mg/g tissue in intact rats; p < 0.0001). In the acetylcysteine-treated rats, the liver glutathione content reached 111.4 ± 3.52 mg/g tissue, which was higher than in the animals treated with ethanol and saline (p < 0.05; Figure 4). Thus, an oral administration of acetylcysteine led to an increase in liver glutathione content, which could reduce the severity of ethanol intoxication.

DISCUSSION

In modern society, alcohol abuse is a widespread problem due to the increase in stress load and the availability of alcohol. Although a hangover is a frequent adverse event of ethanol intake, this condition is not well understood in terms of current medical knowledge. Negative consequences of ethanol hangover include economic costs associated with inefficient work or study [1, 2].

An oxidation of ethanol by alcohol dehydrogenase leads to the formation of acetaldehyde, which turns into acetate. Both of these sequential and then parallel reactions could affect the balance between nicotinamide adenine dinucleotide (NAD) and NADH, indirectly decreasing the amount of reduced glutathione [21]. An increase in NADH causes a number of metabolic disorders, including hyperlactacidemia, which promotes acidosis and reduces uric acid excretion. This leads to secondary hyperuricemia, which is aggravated by alcohol-induced ketosis and enhances the acetate-mediated breakdown of ATP. An increase in NADH also prevents gluconeogenesis, thereby promoting hypoglycemia, increase in α-glycerophosphate levels, inhibition of the Krebs cycle. This is followed by inhibition of fatty acid oxidation, which promotes steatosis and hyperlipidemia. Acetaldehyde is a cytotoxic substance which inhibits the recovery of alkylated nucleoproteins, inhibits the activity of key enzymes and significantly decreases the efficiency of the oxygen metabolism in mitochondria. In addition, acetaldehyde contributes to cell death by depleting the level of reduced glutathione, causing lipid peroxidation, and an increase in the toxic effect of free oxygen species. By binding to microtubule tubulin, acetaldehyde blocks the secretion of proteins. The result of an increase in protein, lipids, water and electrolyte cell content is an increase in the size of hepatocytes. Acetaldehyde-protein adducts promote collagen production and can act as neoantigens that stimulate the immune response. The decrease in reduced glutathione, caused by ethanol, also contributes to damage to hepatocyte organelles and decreases the utilization of xenobiotics [21, 22].

Acetylcysteine was originally patented in 1960 and licensed for use in 1968. It is included in the WHO List of Essential Medicines, the most effective and safe medi-

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cines needed in the health system, as a drug used in case of paracetamol (acetaminophen) overdose and for mucolytic therapy [23]. This compound acts as the precursor of L-cysteine, from which the antioxidant glutathione is formed. Glutathione contains a peptide bond between the amino group of cysteine and the carboxyl group of the glutamate side chain. The value of glutathione in a cell is determined by its antioxidant properties. In fact, glutathione does not only protects the cell from toxic free radicals, but also generally determines the redox characteristics of the intracellular environment. In the cell, thiol groups are in a reduced state at the concentration of about 5 mmol/L. Such a high concentration of glutathione in the cell, leads to the fact that it restores any disulfide bond formed between the cysteine residues of intracellular proteins. In this case, the reduced form of glutathione turns into oxidized. Oxidized glutathione is restored under the action of the enzyme glutathione reductase, which is constantly in the cell in an active state and is induced by oxidative stress. The ratio of the reduced and oxidized forms of glutathione in the cell is one of the most important parameters that determines the level of oxidative stress, which increases during alcohol consumption and the development of an ethanol hangover [22, 24].

Acetylcysteine administration contributes to the replenishment of glutathione, which plays an important role in neutralizing reactive oxygen species and, together with oxidized glutathione and S-nitrosoglutathione, binds to the NMDA and AMPA receptors (through their γ-glutamyl fragments) and can be endogenous neuromodulator. At millimolar concentrations, they can also modulate the redox state of the NMDA receptor complex [25].

Glutathione modulates the NMDA receptor by acting on the redox site. L-cysteine also serves as a precursor of cystine, which is a substrate for antiportheracin glutamate on astrocytes. Therefore, cysteine could increase the release of glutamate into the extracellular space, where it acts on mGluR 2/3 receptors, and at higher doses of acetylcysteine – on mGluR 5 [26]. With regards to this fact, that hangover is accompanied by depression of neurotransmitters, the restoration of neurotransmitter balance can contribute to a faster restoration of neurocognitive functions. Acetylcysteine also exerts some anti-inflammatory effects, which could be due to inhibition of NF-κB transcription factor and due to modulation of cytokines synthesis [27].

Available preclinical and limited clinical data suggest that acetylcysteine is able to normalize glutamate neurotransmission to the *nucleus accumbens* and other brain structures, in part due to an increased expression of excitatory amino acid transporter 2, known as glutamate transporter 1, in addicted individuals. In adults with cocaine dependence, acetylcysteine modulates the neurotransmission of glutamate, which is not observed in people without dependence [28]. This study, in conjunction with the data presented, suggests that acetylcysteine could have a double effect related to the hangover symptoms: facilitation of the metabolism of xenobiotics and a neuroprotective effect, which helps to reduce alcohol craving. Acetylcysteine, acting as a modulator of glutamate and dopamine neurotransmission with a pronounced antioxidant effect, is a promising candidate for further development of a medication for correction of ethanol hangover.

CONCLUSION

An oral administration of acetylcysteine in rats suffering from acute ethanol intoxication promoted an increase in liver glutathione levels and led to a decrease in severity of neurological and cognitive deficits in animals. With regards to the obtained results, acetylcysteine could be used as a perspective medication for ethanol hangover treatment.

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AUTHOR CONTRIBUTIONS

All authors had equally contributed to the research work.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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RESEARCH ARTICLE

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COMPLEX COMPOUND OF PRO-GLY-PRO-LEU WITH HEPARIN: HYPOGLYCEMIC, FIBRINOLITIC AND ANTICOAGULANT EFFECTS IN RATS WITH HYPERGLYCEMIA

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Previously it was shown that the use of regulatory peptides of the glyprolin family helps to normalize the hemostasis system and blood glucose levels in experimental resistant hyperglycemia in rats, similar to type 2 diabetes mellitus in humans. It is also known that the anticoagulant heparin inhibits blood coagulation and exhibits a hypoglycemic effect in the body.

The aim of the study is to obtain a complex of the Pro-Gly-Pro-Leu (PGPL) peptide and the unfractionated heparin, to study its effect on glucose and anticoagulant fibrinolytic properties and show its ability to restore the impaired functions of the insular and coagulating blood systems in experimental hyperglycemia in rats.

Materials and Methods. Laboratory Wistar male rats, intact and with experimentally induced hyperglycemia, were used in the experiment. A complex compound of PGPL and heparin was created with a component ratio of 1:1 (mol/mol), which was administered intranasally to hyperglycemic rats once a day for 5 days at the dose of 1 mg/kg. Similarly, the constituent parts of the complex were administrated in equivalent amounts. The anticoagulant activity was determined by the test of activated partial thromboplastin time, fibrinolysis parameters – by tests of total, enzymatic and non-enzymatic fibrinolytic activities, as well as the activity of a tissue plasminogen activator. In addition, blood glucose was measured using special test strips.

Results. The use of the PGPL-heparin complex in the animals with hyperglycemia led to normalization of blood glucose levels, an increase in the anticoagulant and fibrinolytic background of blood plasma. These effects persisted for 6 days after the cancellation of the peptide-heparin complex administration to rat.

Conclusion. In the development of experimental hyperglycemia, the PGPL complex with heparin exhibits a combined hypoglycemic, anticoagulant and fibrinolytic enzymatic and non-enzymatic nature of the effect. In the future, the studied peptide-heparin complex can be used for the prevention and treatment of type 2 diabetes mellitus, complicated by increased blood coagulation.

Key words: anticoagulant activity; blood glucose level; complex compound; fibrinolysis; glyprolin peptides; hyperglycemia; type 2 diabetes mellitus (T2DM)

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КОМПЛЕКСНОЕ СОЕДИНЕНИЕ PRO-GLY-PRO-LEU С ГЕПАРИНОМ: ГИПОГЛИКЕМИЧЕСКИЙ, ФИБРИНОЛИТИЧЕСКИЙ И АНТИКОАГУЛЯНТНЫЙ ЭФФЕКТЫ У КРЫС С ГИПЕРГЛИКЕМИЕЙ

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Ранее показано, что применение регуляторных пептидов семейства глипролинов способствует нормализации системы гемостаза и уровня глюкозы крови при экспериментальной стойкой гипергликемии у крыс, подобной сахарному диабету 2 типа у человека. Также известно, что антикоагулянт гепарин подавляет свертывание крови и проявляет гипогликемическое действие в организме.

Цель исследования**.** Получить комплекс пептида Pro-Gly-Pro-Leu (PGPL) и нефракционированного гепарина, изучить его влияние на уровень глюкозы и антикоагулянтно-фибринолитические свойства и показать его способность восстанавливать нарушенные функции инсулярной и свертывающей систем крови при экспериментальной гипергликемии у крыс.

Материалы и методы. Использовались лабораторные крысы-самцы линии Wistar, интактные и с экспериментально вызванной гипергликемией. Было создано комплексное соединение PGPL и гепарина при соотношении компонентов 1: 1 (моль/моль), которое вводили один раз в сутки в течение 5 дней интраназально в дозе 1 мг/кг гипергликемическим крысам. Подобным образом вводили составные части комплекса в эквивалентных количествах. Определяли антикоагулянтную активность по тесту активированного частичного тромбопластинового времени, параметры фибринолиза по тестам суммарной, ферментативной и неферментативной фибринолитической активности, а также активности тканевого активатора плазминогена. Кроме того, проводилось измерение уровня глюкозы крови с использованием специальных тест-полосок.

Результаты. Применение комплекса PGPL-гепарин у животных с гипергликемией приводило к нормализации уровня глюкозы крови, повышению антикоагулянтного и фибринолитического фона плазмы крови. Эти эффекты сохранялись в течение 6 дней после прекращения введения крысам пептидно-гепаринового комплекса.

Заключение. Комплекс PGPL с гепарином проявляет комбинированное гипогликемическое, антикоагулянтное и фибринолитическое ферментативной и неферментативной природы действие при развитии экспериментальной гипергликемии. Исследуемый пептидно-гепариновый комплекс в перспективе может применяться для профилактики и лечения сахарного диабета 2 типа, осложняющегося повышенной свертываемостью крови.

Ключевые слова: антикоагулянтная активность; уровень глюкозы крови; комплексное соединение; фибринолиз; глипролиновые пептиды; гипергликемия; сахарный диабет 2 типа

INTRODUCTION

It is known that the incidence of type 2 diabetes mellitus (insulin-independent, T2DM) is constantly growing. Diabetes mellitus is a complex of physiological dysfunctions characterized by a persistent increase in blood glucose (hyperglycemia) due to insulin resistance, insufficient secretion of insulin and excessive secretion of glucagon into blood [1, 2]. Hyperglycemia and the development of diabetes mellitus are usually accompanied by anticoagulant system (ACS) dysfunction and hypercoagulation, which is manifested by increased platelet aggregation and a decreased anticoagulant and fibrinolytic blood activity [3, 4].

There is evidence that amino acids such as arginine, leucine play an important role in the prevention of diabetes mellitus. Leucine stimulates the production of insulin by pancreas, which leads to normal blood glucose levels in patients with diabetes mellitus [5, 6].

Short regulatory proline- and glycine-containing peptides (glyprolines) are constantly produced in the body during the synthesis and degradation of collagen [7]. Some glyprolines (Arg-Pro-Gly-Pro, Gly-Pro-Arg, Leu-Pro-Gly-Pro, Pro-Gly-Pro-Leu) exhibit a protective antidiabetogenic effect, meanwhile stabilizing hemostasis in metabolic disorders.

It has been shown that repeated intranasal admin-

istration of these peptides to rats with persistent hyperglycemia (similar to the initial stage of human T2DM) was leading to positive changes in the insular and coagulation systems of the body. At the same time, a decrease in the glucose level, an increase in the anticoagulant and fibrinolytic potential of plasma, as well as inhibition of platelet aggregation in the animal blood plasma, have been noted [3, 4, 8, 9].

One of the natural anticoagulants of the hemostatic system – heparin, is a component of the internal environment of the body, which is synthesized by mast cells of the liver, basophilic leukocytes and other cells. It belongs to fast-acting anticoagulants, inhibits a thrombin-coagulating activity, and also exhibits a hypoglycemic effect in the body. Heparin is able to form complexes with amino acids and peptides due to the presence (in its molecule) of structural zones of binding to these ligands [10, 11]. It has been experimentally shown that the administration of heparin complexes with glyprolines to the animals led to an increase in the anticoagulant, fibrinolytic and antithrombotic activity of the blood [12, 13].

It has been established that intranasal administration of a complex of heparin with the peptide Arg-Pro-Gly-Pro to the animals with hyperglycemia, increases the anticoagulant properties of blood plasma, fibrinolytic activity of an enzymatic and non-enzymatic nature, reduces platelet aggregation and protects rats from developing diabetes mellitus [13].

In this study, the hypoglycemic, anticoagulant and fibrinolytic effects of the complex compound Pro-Gly-Pro-Leu (PGPL) with unfractionated heparin were studied. That complex had been created and repeatedly administrated by intranasal way to the animals with persistent hyperglycemia similar to T2DM in humans.

THE AIM of the study is to obtain a complex of the Pro-Gly-Pro-Leu (PGPL) peptide and unfractionated heparin, to study its effect on the level of glucose and on the anticoagulant and fibrinolytic properties and show its ability to restore the impaired functions of the insular and coagulating blood systems in experimental hyperglycemia in rats.

MATERIALS AND METHODS

Obtaining the PGPL-heparin complex

In this study, unfractionated heparin (Serva, Germany) was used. The PGPL peptide was synthesized at the Institute of Molecular Genetics, Russian Academy of Sciences (Moscow, Russia). A complex compound of this peptide with heparin at the component ratio of 1: 1 (mol / mol) was created according to the previously described protocol with some modifications [12].

Heparin and the PGPL peptide were dissolved in distilled water, mixed up and incubated for 1 h at 37° C, pH 7.2. The reaction product was precipitated with 1% acetic acid, pH 5.0–5.2, and centrifuged for 30 min. at 3000 × g. The precipitate was dissolved in 0.85% NaCl (physiological saline), pH 7.2–7.4.

Bond formation between heparin and peptide was assessed by cross-sectional electrophoresis at 20° C in 0.053 M phosphate buffer, pH 7.0, with a potential gradient of 7.3 w/cm for 2 hours. Then, the electrophoregrams were stained with a 0.033% solution of Azur II, which reveals the acid groups of heparin [12].

Animals, study design and drug administration

50 male Wistar rats (aged 10 months, weighing 320–350 g) were obtained from the Scientific Center for Biomedical Technologies, Stolbovaya branch (Moscow, Russia). The animals were kept in plastic cages under standard vivarium conditions at the ambient temperature of 21–24°C, a 12-hour day/night cycle and a relative humidity of 60 ± 10% with free access to food and water during the experiment.

All the experimental procedures were carried out in accordance with the ethical principles for the use of laboratory animals and were approved by the local ethics committee (Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia).

After a 10 days' adaptation, all the animals were divided into 5 groups, each of 10 rats (Fig. 1).

Group 1 – rats with hyperglycemia, treated with 0.85% saline.

Group 2 – rats with hyperglycemia treated with the PGPL-heparin complex (1 mg/kg body weight).

Group 3 – rats with hyperglycemia treated with PGPL (20 μg/kg body weight).

Group 4 – rats with hyperglycemia treated with heparin (0.98 mg/kg body weight).

Group 5 consisted of healthy animals.

The preparations dissolved in a 0.85% sodium chloride solution (the PGPL-heparin complex compound, PGPL peptide and heparin), were prepared daily before the administration to the animals. The dose of the PGPL-heparin complex was selected on the basis of previous studies [13]. The doses of the components of the complex, i.e. the PGPL peptide and heparin, were equivalent to their content in the complex compound.

Experimental persistent hyperglycemia (HG) in the rats of groups 1-4 was induced by a daily single intragastric administration of a 40% glucose solution at the dose of 2.5 ml/kg body weight for 7 days, after which the rats of these groups continued to be treated with a glucose solution throughout the experiment. After the development of HG, the rats of groups 2–4 were intranasally injected with the studied drugs in the volume of 25 μl once a day for 5 days. At the same time, the rats of the 1st and 5th groups were treated with 0.85% NaCl as a placebo intranasally.

On the 22nd day of the experiment, 1 hour after the last injection of the drugs, the blood was taken from each animal for biochemical analyzes. At the end of the experiment (Day 28), repeated blood sampling was carried out 1 hour after the last glucose administration against the background of the cancellation of the drug administration.

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	Daily administration of a 40% glucose solution per os (Groups 1–4)			
Day 1	Day 11	Day 18	Day 22	Day 28
Adaptation to vivarium conditions		Intranasal administration: Group 1 – 0,85% saline Group 2 – Complex Group 3 – PGPL Group 4 – Heparin Group 5 – 0,85% saline		
			1st blood drawing	2nd blood drawing

Figure 1 – Scheme of the experiment

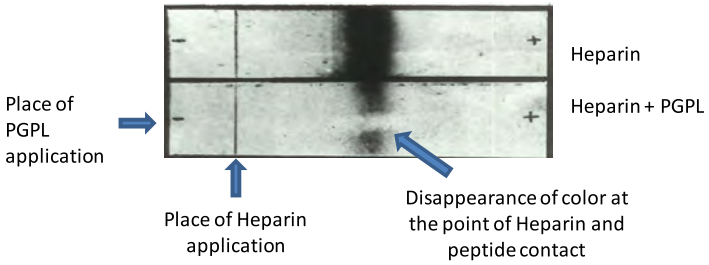

Figure 2 – Electrophoregrams of migration of heparin detached and heparin interacting with the peptide, in the electric field

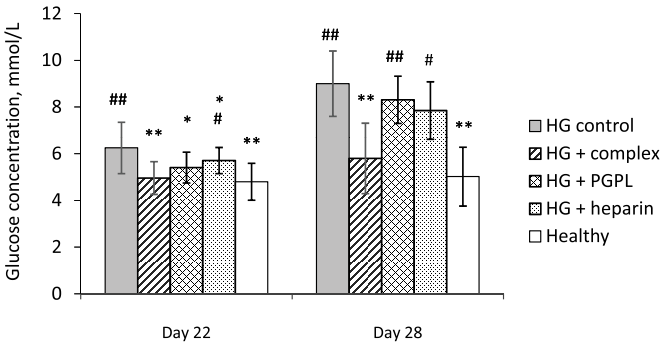

Figure 3 – Blood glucose level (mmol/l) on the 22nd day (1 hour after the 5th administration of drugs) and on the 28th day of the experiment (6 days after the cancellation of drug administration)

*Note: * P <0.05 and ** P <0.01– compared with the HG control (Group 1), # P <0.05 and ## P <0.01 – compared with healthy animals (Group 5).*

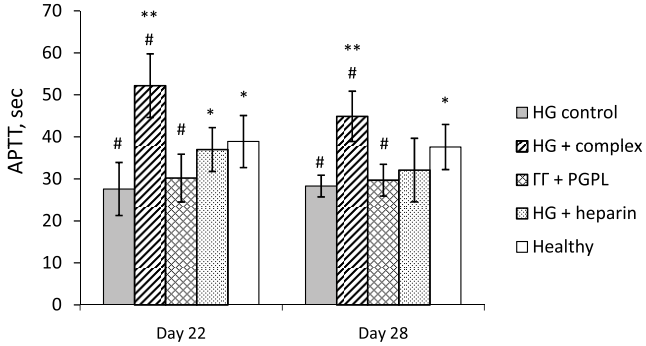

Figure 4 – Anticoagulant activity of blood plasma (according to the APTT test) on the 22nd day (1 hour after the 5th administration of drugs) and on the 28th day of the experiment (6 days after the cancellation of the drug administration)

Note: P <0.05 and ** P <0.01 – compared with the HG control (Group 1), # P <0.05 and ## P <0.01 – compared with healthy animals (Group 5).*

Conducting biochemical analyzes

For the analysis, the blood was taken from the jugular vein *(vena jugularis),* using 3.8% sodium citrate as a preservative in the ratio of blood : preservative as 9: 1. In the whole blood, glucose concentration was determined by an Accutrend GC analyzer (Roche, Germany) using special test strips. Then, the blood was centrifuged at 2000 × g for 15 min at room temperature to obtain platelet-poor plasma, in which an anticoagulant activity was assessed by the activated partial thromboplastin time (aPTT) test on an ASK 2-01 blood coagulation analyzer (Russia); fibrinolysis indicators according to tests of total (TFA), non-enzymatic (NFA) fibrinolytic activity on unstabilized fibrin, enzymatic fibrinolytic activity (EFA) and tissue plasminogen activator activity (PAA) in the euglobulin plasma fraction on stabilized fibrin [14].

All the obtained data were statistically processed and expressed as mean ± SD (standard deviation). The normality of the distribution was revealed by the Shapiro-Wilk test, the group differences were analyzed by the method of one-way Analysis of Variants (ANOVA) using the Newman-Keuls-test for multiple comparisons. The values of P <0.05 were considered statistically significant.

RESULTS

Preparation of the PGPL-Heparin Complex

The formation of a complex compound of the PGPL peptide with heparin in a pure system has been established. This compound was obtained with an optimal molar ratio of the components: 1: 1. The use of cross-electrophoresis showed the interaction and the occurrence of a chemical bond between the amino groups of the PGPL peptide and the acid groups of heparin.

As Fig. 2 shows, when the solutions of heparin and PGPL were perpendicular to the carrier with their further migration in the electric field, the color of the acidic carboxyl and sulfate groups of heparin (Azur II dye) disappeared at the meeting point of the substances.

Next, the hypoglycemic effect and the effect on the hemostasis system of the PGPL-heparin complex compound and its components – the PGPL peptide and heparin in equivalent doses under conditions of persistent GH in rats – were studied.

The effect of the PGPL-heparin complex on blood glucose

On the 18th day of the experiment, after the induction of hyperglycemia, the blood glucose level in rats with GH (group 1) was significantly higher than the one in the healthy control (group 5) (6.25 ± 0.35 mmol/L versus 4.8 ± 0.25 mmol/L, respectively; P < 0.01), which amounted to 130%. The administration of the PGPL-heparin complex compound to the rats decreased this indicator by 21% (4.96 ± 0.23 mmol/L; P <0.01). The administration of the complex components (PGPL or heparin) had a weaker hypoglycemic effect, which led to a lower decrease in blood glucose levels by 13 and 9%, respectively (to 5.41 ± 0.21 mmol/L with PGPL and 5.71 ± 0.56 mmol/L with heparin; P <0.05).

On the 28th day of the experiment (6 days after the treatment had been canceled), the blood glucose level in the rats of group 1 continued to increase and amounted to 179% relative to the healthy animals (group 5) (9.0 ± 0.42 mmol/L versus 5.02 ± 0.32 mmol/L; P <0.01).

Meanwhile, the blood glucose level in group 2 rats treated with the complex, was 5.8 ± 0.72 mmol/L (P <0.01), while the administration of both PGPL and heparin did not change this indicator (P> 0.05) (Fig. 3)

The effect of the PGPL-heparin complex compound on the anticoagulant activity of blood plasma by the APTT test

As Fig. 4 shows, on the 18th day of the experiment, in rats with HG (group 1) the anticoagulant activity of blood plasma was 27.6 ± 6.32 sec., i.e. it had been reduced by 29% compared with healthy normal rats (P <0.01). This conclusion confirms that in HG animals, the procoagulant activity of blood increases 12 days after the induction of HG.

In HG rats, after therapy by the complex and heparin, the anticoagulant activity significantly increased by 89% and 34%, respectively (52.2 ± 7.58 sec. – group 2; P <0.01 and 37.0 ± 5.22 sec. – group 4; P <0.05), while the PGPL administration did not changed the anticoagulant activity of blood plasma in the rats (P> 0.05).

On the 28th day of the experiment, i.e. 6 days after the cancellation of the drug administration, the anticoagulant blood activity significantly differed from the control group (28.3 ± 3.52 sec.) only in Group 2 after the administration of the PGPL-heparin complex (44.9 ± 5.01 s; P <0.01) by 58% (Fig. 4). The introduction of the complex components (PGPL and heparin) did not lead to a significant change in this parameter (P> 0.05).

The effect of the PGPL-heparin complex compound on fibrinolytic activity (TFA, NFA, EFA and PAA) of blood plasma

The carried out experiment showed that in rats with GH (Group 1), the fibrinolytic activity of blood plasma was inhibited in comparison with healthy animals of Group 5 (Table 1). Thus, the indicators of TFA, NFA and EFA in HG animals were reduced by 19, 28 and 32%, respectively, compared with those in healthy rats (P <0.05). In the rats of Group 1, PAA was also 36% lower than in the rats of Group 5 (P <0.01).

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Table 1 – Fibrinolytic activity (mm²) of blood plasma of animals on the 22nd day (1 hour after the 5th administration of drugs) and on the 28th day of the experiment (6 days after the cancellation of the drug administration)

Groups of animals	TFA	NFA	EFA	PAA
	1 hour after the 5th administration of drugs (22nd day)			
HG control (Group 1)	27.5 ± 5.05##	16.7 ± 3.09#	52.7 ± 11.3#	17.8 ± 6.51##
HG + complex (Group 2)	52.0 ± 10.1**,##	35.8 ± 6.29**,#	89.3 ± 8.54**,#	49.4 ± 10.1**, ##
HG + PGPL (Group 3)	33.4 ± 6.24*	21.8 ± 3.99	71.5 ± 5.25*	24.1 ± 4.43*
HG + heparin (Group 4)	31.8 ± 4.78	19.4 ± 4.6	68.7 ± 7.48*	27.3 ± 6.15**
Healthy (Group 5)	34.0 ± 5.98**	23.1 ± 2.56*	77.5 ± 12.4*	28.0 ± 7.2**
	6 days after the cancellation of drug the administration (28th day)			
HG control (Group 1)	28.7 ± 2.83##	17.3 ± 3.4##	37.1 ± 8.36##	9.4 ± 3.61#
HG + complex (Group 2)	38.7 ± 5.22**	25.8 ± 2.86**	60.6 ± 4.9**	22.6 ± 4.38**
HG + PGPL (Group 3)	32.2 ± 4.44	20.7 ± 3.74	45.2 ± 5.31*,#	15.7 ± 2.41*
HG + heparin (Group 4)	30.7 ± 6.27#	22.8 ± 4.18	42.1 ± 6.06##	12.5 ± 4.6
Healthy (Group 5)	39.6 ± 4.33**	25.0 ± 3.97**	65.8 ± 5.05**	19.0 ± 5.33*

*Note: * P <0.05 and ** P <0.01 – compared with group 1 (HG control), # P <0.05 and ## P <0.01 – compared with group 5 (healthy rats). The data are presented as M ± SD. TFA – Total fibrinolytic activity, NFA – Non-enzymatic fibrinolytic activity, EFA – Enzymic fibrinolytic activity, PAA – t-Plasminogen activator activity.*

After the intranasal administration of the PGPL-heparin complex, PGPL peptide and heparin, the fibrinolytic activity of blood plasma in the rats with resistant HG led to an increase of fibrinolysis in all types (TFA, NFA, EFA, PAA), but to different extents. So, on the 22nd day of the experiment, in rats of group 2 treated with the complex, these indicators increased by 89, 115, 69 and 177% (P <0.01), respectively, compared with the control group (Group 1). Under these circumstances, fibrinolysis parameters exceeded those in healthy animals (Group 5). At the same time, the use of peptide led to a significant increase in TFA by 21% (P <0.05), EFA – by 36% (P <0.05) and PAA – by 35% (P <0.05) in the blood plasma of rats of group 3, but it did not change NFA in comparison with the HG control (P> 0.05). The administration of heparin caused a significant increase in EFA alone – by 30% (P <0.05) and PAA – by 53% (P <0.01) in the rats of group 4 compared with the control group.

On the 28th day of the experiment, in rats with GH (group 1), a reduced background of fibrinolysis remained: TFA, NFA, EFA (P <0.01 in all cases) and PAA (P <0.05) were significantly lower (by 27, 30, 44 and 51%, respectively) than in healthy animals (Group 5). At the same time, in group 2, even after the cancellation of the complex administration, a statistically significant increase in fibrinolytic activity was noted: TFA – by 35%, NFA – by 49%, EFA – by 63% and PAA – by 140% compared with the HG control group (P < 0.01). These indicators corresponded to the values of healthy control. In addition, in group 3 (PGPL), there was a significant increase in only EFA (by 22%, P <0.05) and PAA (by 67%, P <0.05). Under these circumstances, in group 4, after the cancellation of the heparin administration, there were no significant differences in fibrinolytic activity compared with the HG control (P> 0.05).

DISCUSSION

Analyzing the obtained results, it should be noted that the progression of diabetes mellitus is accompanied by a suppression of inhibition of insular [15] and anticoagulation systems. The depression of ACS is characterized by increased platelet aggregation, a decreased anticoagulant and fibrinolytic activity of plasma hemostasis, which leads to an increase in blood thrombogenic potential, and also often causes blood clots [8].

According to the literature data, in HG rats the suppression of the ACS function is accompanied by an increase in blood coagulability, a decrease in fibrinolysis, and a significant increase in blood glucose levels [3, 4, 13]. A similar effect was observed in our model of experimental hyperglycemia.

In this study, the effect of the PGPL-heparin complex compound and its components – heparin and PGPL peptide – on the hemostatic and insular systems in HG rats was evaluated. It was shown that in the situation where persistent hyperglycemia developed, the PGPL-heparin complex had a protective effect on the functional state of both the insular and hemostatic systems of the body, which had been identified for the first time.

Both PGPL peptide and heparin in the doses equivalent to their content in the complex, stimulated the anticoagulant and fibrinolytic activity of blood and lowered glucose levels. It was established that the anticoagulant effects of the complex exceeded the action of its constituent parts: as a result of the activation of NFA and a tissue plasminogen activator, TFA and anticoagulant activities of the blood got increased in the blood plasma. In addition, the effect of the PGPL-heparin complex was prolonged, since it persisted 6 days after the cancellation of its administration.

Previously, the effect of the peptides PG, PGP and RPGP, heparin and their complex compounds were

evaluated in healthy and hyperglycemic rats. In the rats treated with heparin intranasally, only an anticoagulant activity increased, and when the peptides were administered, both anticoagulant and fibrinolytic properties of the blood increased [12, 13, 16]. A repeated intranasal administration of the PGPL peptide at the higher dose (1 mg/kg) than in the present study, to rats with persistent GH, protected the body from the development of hypercoagulation. At the same time, there was an increase in total and non-enzymatic fibrinolysis of the animals' blood. Positive changes in the insulin and anticoagulation systems were observed within 5 days after the cancellation of the peptide administration [9].

Considering the mechanism of the effect of the complex on the normalization of blood glucose levels, it can be assumed that the PGPL-heparin had this effect and prevented the development of T2DM due to the presence of the amino acid leucine in its structure, which stimulates the production of insulin, providing a normoglycemic effect [6].

The mechanism of the anticoagulant effect of heparin is due to the blockade of the activity of thrombin and other coagulant proteins [17]. At the same time, heparin increased the activity of the tissue plasminogen activator, which led to the increase of enzymatic fibrinolysis [10].

Some peptides are effective thrombin inhibitors that have anticoagulant properties, can activate enzymatic fibrinolysis and prevent forming blood clots. It was established that proline and glycine-containing peptides PG and PGP can induce dissolution of the unstabilized fibrin [16]. The presence of glyproline receptors on the endothelium has not been established yet, but specific PGPL binding sites on the cytoplasmic membrane of the rats' brain basal nuclei have been reported [18]. Perhaps this peptide, administered by the intranasal route, passes through the blood-brain barrier, penetrating the structures of the brain and exerting its action through specific receptors. According to the literature data [19, 20], as well as according to the results of this study, individual peptides are able of exerting a hypoglycemic effect and inhibit the progression of T2DM.

CONCLUSION

The PGPL-heparin complex studied by the carried out experiment, enhanced the hypoglycemic and anticoagulant effect of its constituent components, heparin and peptide, due to its structural features. It prevented the formation of fibrin due to the presence of its fibrinde-polymerization activity, determined by its anticoagulant effect on the thrombin activity. The appearance of both PGPL and the complex in the bloodstream, stimulates the release of the tissue plasminogen activator from the vascular endothelium, which leads to an increase in the enzymatic fibrinolytic properties of the blood, and the effect of the complex is more effective. It has also been shown that the complex significantly improves functioning of the insular system in hyperglycemic rats, and its component heparin modulates the function of the anticoagulant system, participating in the prevention of the hypercoagulation process that accompanies the development of T2DM.

In the future, the obtained results on the model of the rats with persistent hyperglycemia, may be applicable in clinical practice for patients with type 2 diabetes who are predisposed to the development of thrombotic complications.

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AUTHOR'S DEPOSIT

All the authors have equally contributed to the research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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