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COMPUTER RECOGNITION OF CHEMICAL SUBSTANCES BASED ON THEIR ELECTROPHYSIOLOGICAL CHARACTERISTICS

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The objective of this review was to analyze the results of electrophysiological studies of some biologically active chemicals in order to determine relationships between their chemical structures and effects that they produce. It was proposed to use these relationships to improve the logical module of biotechnical expert system that works using a module principle based on object-oriented and regression analysis. The data of electrophysiological studies of glutamatergic receptor antagonists, phenol- and indole-derivatives, purified from the spiders' venoms of *Arthropodae* species were analyzed in this work. Some characteristics of receptor blocking by these toxins have been used to demonstrate empirical relationships between the chemical structures of antagonists and their electrophysiological effects. Possibilities to apply such relationships for monitoring of harmful environmental pollutants, phenol- and indole derivatives, as well as for developing new methods of their qualitative and quantitative analysis are discussed.

Key words: toxins, receptor antagonists, transmembrane electric current, biological expert systems, electronic informational systems, bioinformatics.

Devices with automatic recognition of different objects are very actual in contemporary world [1]. Among them there are the large group of biotechnical information systems (IS) and devices for such tasks solution [1–12]. In our publication our developed electronic expert system (ExpS) was described for the registration and identification of the toxic organic substances, for example, in polluted environment with anthropogenic pollution: industrial, agricultural, military, due to the disasters and so on [11]. Here the continuation of these works is suggested. The works done were devoted to further perfection of logical unit of this biotechnical expert IS. In computer sciences the expert systems were studied usually together with knowledge bases as models of experts' behavior in a certain field of knowledge using the procedures of logical conclusion and decision making [1]. Knowledge bases, consequently, were seen as a set of facts and rules of logical conclusion in the chosen subject area of activity.

The suggested expert system obtains input signals with characteristics of different chemical substances and its logical unit solves the task of these substances identification (Fig. 1) [11]. This information expert system is aimed at distinguishing and identifying chemicals in contact with the detectors of this system that was patented [7, 11]. The architecture of this device was developed, the framework of the information expert system was created, and

its logic module identifies chemicals using the developed algorithm. The base of such algorithm may be formed by the following groups of methods: 1) statistical analyses [1, 2]; 2) methods of cluster analyses [1, 4]; 3) artificial neuronal network methods [1, 3]; 4) images processing [1, 5] and analyses as well as 5) substances identification using established qualitative empirical dependencies including ones based on the use of regression analyses methods. Such methods are successful likewise for biological objects analyses despite all the difficulties — their complexity, necessity of preliminary statistic processing of results, etc.

If necessary, the logic unit algorithm can be changed (or modified) without changing the system framework. From the point of view of software development, a software module was developed basing on approaches of object-oriented analysis. In the present work, it is proposed to pick up the last way as the basis of the logic module algorithm — using the empirical dependencies registered by Klyuchko O. As a demonstration of the developed method, the article presents its application to a number of active chemical agents.

Indeed, this is an attractive solution — to find a rule, a regularity which helps to "make a bridge" between "structure" and its "function", to find co-relations between characteristics of input signals in ExpS and chemical structures that have to be identified. Studying the experience of different chemicals electrophysiological investigations we had found a set of substances that may be suitable for such tasks solutions [13–107]. There are the experiments of electrophysiological investigations of some *Arthropodae* toxins. Indeed, some of these studied toxins had known chemical structures. Besides they have revealed electrophysiological activity, for

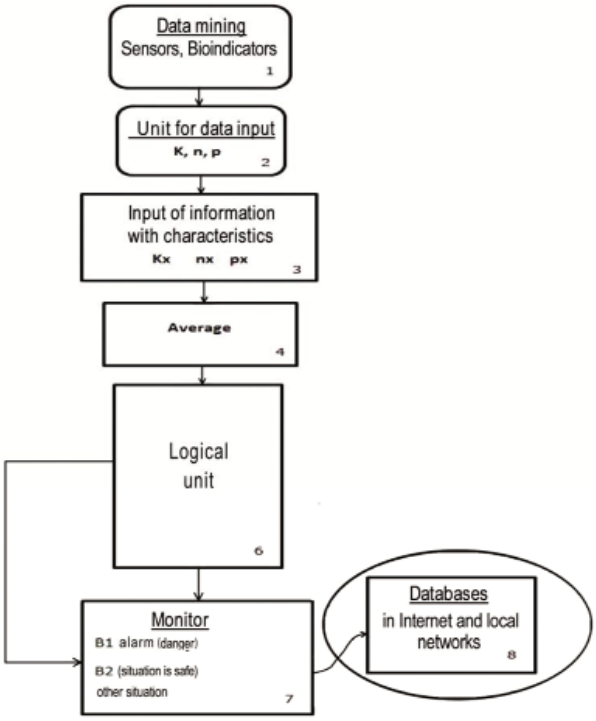


Fig. 1. **Biotechnical expert system**
Above: an algorithm of functioning of the expert system with the logical unit, the output data recording and the work of linked alarm subsystem. Below: interface for operator communication with expert analytic system; view of monitor screen (comments and instructions were written in Ukrainian for domestic use of device) [11]

example the toxins from the spiders *Nephila clavata* (JSTX-3 and others) and *Argiope lobata* (AR, ARN-1, ARN-2 and others) are known as antagonists of glutamate channel-receptor complexes (gCRC, CRC) in cell membranes. Chemical structures of these toxins are known: JSTX-3, AR are phenol derivatives, and ARN-1, ARN-2 are indole derivatives (Fig. 2) [33, 55–67]. The reactions of gCRC blocking by these toxins can be well studied using sodium salt of kainic acid (KK) that is agonist of gCRC causing non-inactivated transmembrane electric currents (KK-activated electric currents), so, the kinetic characteristics of blocking (modifying) effect can be studied well.

Electrophysiological effects of all studied toxins can be registered successfully on these non-inactivated KK-currents as exponential dependencies and they have well registered numerical characteristics. So, these substances have a set of their peculiarities that makes them attractive for the studying and our tasks solution.

Logical module: finding of empirical dependencies "effect" — "structure". In recent decades in neurophysiology, some researchers studed the influence of various chemical agents including organic compounds (derivatives of phenols and indoles) on transmembrane electrical chemo-activated currents. Huge amount of experimental results have been accumulated in this direction [13–107]. Among them there are the classic monographs by Prof. Kostyuk P. G. [68], Kryshtal O. A. [68], Magura I. S. [73], Skock V. I. [91], other researchers [13–67, 69–72, 74–107]. This huge experimental material allows us to make a number of generalizations that we will try to carry out basing on the results of the author's researches with colleagues [17, 48–67].

This enables us to solve the inverse problem, namely, the possibility to determine the approximate chemical structure of organic compound acting on the chemosensitive currents from the measured numerical characteristics of the currents *(*the "effect" — "structure" dependence study). And herein lies the difference from the direct problem — under the action of chemical compound with the known structure on the ionic currents the obtained effect is investigated (study of dependence "structure" — "effect"*).*

To find to find the inverse problem solution, we studied the characteristics of chemosensitive transmembrane ionic currents depending on the influence on them of phenols and indoles derivatives with known structure, namely the toxins of some *Arthropodae* species.

Empirical dependencies "effect" — "structure" were studied on the basis registered experimental data and their processing using the methods of regression analyses, other types of analyses. Empirical diagrams were done and they characterized such dependencies. In such a way the new methods of qualitative and quantitative analyses were developed [61–64].

Some of these works were described below. We suggested: 1) brief review of some known methods of qualitative and quantitative analyses for phenol and its derivatives identification [108–111]; 2) the review of investigations of toxins from *N. clavata* (JSTX-3 and others) and *A. lobata* (AR, ARN-1, ARN-2 and others); 3) results of these toxins studying that demonstrate the regularities between their "structure" and "function" [61–64]; and 4) conclusions — how to use these regularities for the perfection of logic unit in our biotechnical expert system. The author's results with inventions of new methods of qualitative and quantitative anaysis were supported by patents [46–57], as well as original biotechnical expert system [50]. Concerning the item of this article it is necessary to mention that in our previous publications we had written briefly about contemporary computer information systems [1–12] with expert subsystems [11], as well as about mathematic tools used for expert systems' construction [1–6]: methods of artificial neural networks [1, 3], methods of cluster analyses [1, 4], methods of images processing [1, 5]. Additional necessary data both experimental and theoretical were used for the work in [112–140].

Some methods of qualitative and quantitative analyses for phenol and its derivatives identification. A number of such methods for phenol and its derivatives identification is suggested in this sub-chapter.

A. Method of quantitative determination of mezaton, other phenol compounds was invented in Ukraine [108]. This method was applied for quantitative analyses of phenol compounds [108] in pharmacology with the use of diazole salts, as highly sensitive analytical color agents, by analyzing the optical characteristics of electronic absorption spectra, methods of spectrophotometric determination of medicinal substances.

B. Method for preparing phenolic compounds and their identification was protected by USA patent [109] A method for preparing a phenolic compound has been invented. The method includes providing a lignin depolymerization product, and hydrogenating the lignin depolymerization product under iron oxide and hydrogen gas to prepare a phenolic compound. The prepared phenolic compound is a crude phenolic composition including phenol, methylphenol, dimethylphenol or a combination thereof. The methods of gas chromatography — mass spectroscopy (GC-MS) were used for quantitative determination of phenol compounds.

C. Another USA patent for industry "Clear tobacco aroma oil, a process for obtaining it from a tobacco extract, and its use" is in [110]. Ther method of qualitative analyses of phenolic compounds is known. The invention relates to a process for obtaining aromatic materials from a tobacco extract (primary extract) obtainable by means of solvents, by mixing this tobacco extract with an adsorbent, treating the mixture obtained with CO_2 in a pressure extraction vessel under extraction conditions (secondary extraction) and isolating a clear tobacco aroma oil in a downstream separating vessel. The invention also relates to a new tobacco aroma oil which is free of resins, waxes and polyphenols and has a considerably reduced nicotine content. The invention further relates to the use of the obtainable tobacco aroma oil for aromatizing tobacco or tobacco products. To determine the compounds of phenol (nicotine) in the formed mixtures, spectrophotometric methods were used.

D. Method for phenol determining in aqueous media was invented in Russia. There is a method for determining of phenol in aqueous media [111]. This invention relates to the determination and sanitary-and-epidemiological control of the content of phenol in drinking, natural and sewage waters, as well as in atmospheric rainfalls. The method includes chemical modification of phenol in 2,4,6-threebromophenol, and further extraction concentration of 2,4,6-threebromophenol and subsequent gas chromatographic detection, and before chemical modification from the aqueous sample the humus acids on aluminum oxide are removed in the presence of cuprum sulfate in quantities of 0.05–0.25% of the weight of the water sample. The invention relates to the analytical chemistry of organic compounds (concentration and determination).

The disadvantages of all these above described methods are that they all can not be applied to such compounds of phenol whose chemical structure is destroyed at significant deviations from living conditions (temperature, pH, humidity, etc.), for example, to study the phenol compounds in living organisms. Our methods had no these disadvantages, they are grounded and described below, and they were protected by patents of Ukraine [61–64]. As chemical substances for logical module programming in our biotechnical expert system following substances (toxins) were used: JSTX-3 (from *N. cavata* venom) and AR, ARN-1, ARN-2 (from *A. lobata* venom).

The reason of used toxic organic substances selection. For logic module programming in developed expert system we needed in substances with physical (or biophysical) properties that depend on chemical structures or organic molecules detected by the sensor of this system. Taking the neuronal membrane as the element of sensor we registered transmembrane electric currents at input of this system. The sensitivity of glutamate CRC (gCRC) with kainat (KK) as agonist gave a bright possibility to register the kinetic and other characteristics of toxins blocking action at the stationary KK-activated currents. Than it gave a possibility to calculate further all other parameters, to find the relations and regularities between them necessary for logic unit functioning. This scheme suggests following advantages.

1. Electric currents — responces in described biophysical system are well combined, fit into the electrical circuit of the electronic recording system, which is important for the normal its functioning.

2. Kainat (KK) as gCRC agonist gives a bright possibility to register toxins' blocking (or modificatory) kinetics and other biophysical characteristics well at the stationary KK-activated currents.

3. Studied *Arthropodae* toxins are organic substances with relatively small molecular weight (in comparison with snakes' and some other toxins); they have known chemical structure; they all are phenol or indole derivatives with polyamine radicals (substituents) — linear or branched.

4. Main mechanisms of their interaction with the molecules of membrane CRC have

been already studied; they all have irreversible or sightly reversible type of the action.

5. Due to 2, 3, 4 it is possible to reveal satisfactory co-relation between obtained experimental data (and related calculated characteristics) and molecular structures of studied substances.

6. Finally, basing on the abovelisted, we tried to find the regularities between "chemical structures" and their "effects" necessary for our logical module good functioning.

So, below in the next sub-chapters there is a review of the studies of electrophysiological properties as well as chemical structures of JSTX-3, AR, ARN-1, ARN-2 [13–107].

Basic studies of blocking effects of Atrhropodae venoms and toxins. In the next few sub-chapters we would like to concentrate our attention on toxins and venoms of two spider species — *N. clavata* and *A. lobata*, they become known due to the properties of their venoms as antagonists of glutamatergic synapses, they were used successfully for electrophysiological experiments, for investigations of membrane structures from the late 1980th. In our previous publications we had written already about different Arthropods' venoms and toxins [17, 46, 49, 50, 51, 58–67]. Let's observe so important electrophysiological properties of *N. clavata* and *A. lobata* products in details. In present publication we would like to give more information on experimental studies of glutamate receptors antagonists from *Araneidae*, namely spider species *N. clavata* and *A. lobata*; these spiders are known as good producers of venoms and toxins for laboratory practice. Further we would like to compare some electrophysiological properties for the pairs: venom from *N. clavata* (JSTX-V) with toxin JSTX-3, and venom from *A. lobata* (AR-V) with toxin argiopin (AR). It is known that the studying of different natural toxins for the purposes of neurophysiologic investigations were demonstrated in details in classic monographs by professors Kostyuk P. G., Krishtal O. A., Magura I. S., Skock V. I., and others [68, 73, 91]. Later these investigations were continued by representatives of their scientific schools in collaboration with foreign colleagues [17, 46]. For today the results of the studying of some toxins from *Arthropodae* (including *Araneidae* toxins) as well as other similar phenol and indole derivatives were applied in agriculture [16, 20, 29, 59], and in methods of ecological monitoring of environment [38–57]. Because of importance of results of *Arthropodae* venoms and toxins studying and their applications [31–47, 70–107] in our review below the data from fundamental works of different authors who studied such venoms and toxins were given. In some of these works the results of arthropods' toxins chemical structures studying have been described [32, 33, 38, 45, 47, 50].

A. Basic studies of blocking effects of Nephila clavata venom and its active components — toxins of JSTX family. The first studies of the actions of *N. clavata* venom and obtained from it active component — toxin JSTX were done in Japan on 1982. For today glutamate receptors antagonists from *N. clavata* — venom and its elements — toxins from JSTX family — are seen as excellent tools for laboratory investigations in the whole world; these toxins include fragment of 2,4-dihydroxyphenyl acetic acid (DHPA) or its derivative binded with asparagin (DHPA-Asp) [50, 51]. But at the beginning, in early 1980-th, the chemists and biochemists obtained from *N. clavata* venom only one active toxic fraction that was called JSTX; later few such electrophysiologically active toxic fractions were subdivided from this venom [51]. The first experiments with the use of microelectrodes have demonstrated that JSTX-3 blocks specifically glutamatergic synapses in lobster muscles [40, 41], stellate squid ganglia [42–44, 88], and in the central nervous system (CNS) of mammals [42, 43, 88]. In all these experiments toxins of JSTX family blocked both excitatory postsynaptic potentials (EPSP) and potentials caused by ionophoretic application of glutamate (Glu) without the influence on inhibitory potentials (IPSP). The rest potential of presynaptic membrane remained constant until and after the action of toxin. The washing of JSTX even for a long time did not caused the EPSP restoring, that means that the toxin in postsynaptic membrane binds irreversibly and strongly to the glutamate channel-receptor complex (CRC).

The results of experiments had demonstrated that JSTX acts on the post-synaptic membranes. Antidromic potentials of action, registered on the squid giant axon [42, 43, 88] were insensitive to JSTX.

In addition, JSTX did not act on presynaptic potentials in a giant squid synapse [42, 43, 88]. Intracellularly registered spikes in lobster neuromuscular terminals also were insensitive to JSTX [13, 40, 41] and antidromic action potentials registered on pyramidal neurons of the hippocampus [88]. The quantum composition of mediator released in neuromuscular lobster junction

and its change under the action of JSTX were studied. It was revealed that under the action of JSTX it was not changed [80, 81]. During the intracellular registration from the nerve terminals of neuromuscular lobster junction, the changes in the membrane potential were recorded under the action of glutamate. However, these responses also were insensitive to JSTX [80, 81]. Based on these studies, it was concluded that JSTX blocks the transmission in glutamatergic synapses being bonded to glutamate CRCs in the postsynaptic membrane.

Later it has been found that JSTX affects not only the glutamate receptors of the postsynaptic membrane, but also the mechanism of glutamate reuptake. Both JSTX and a fragment of its molecule DHPA-Asp inhibited both sodium dependent and sodium-independent binding of marked glutamate to synaptosomes in rat brain [84–86]. The inhibition and binding of glutamate of both types was practically complete and depended on toxin concentration.

Already in the early work a number of quantitative characteristics of JSTX blocking action were obtained. On the neuromuscular lobster junction it was shown [13, 42, 43] that this toxin blocked the EPSP irreversibly in concentration that exceeded 10^3 units/l. At lower concentrations it could be removed by washing. Within the limits of concentrations 10^{-4}–10^{-2} units/l this toxin acted in dose-dependent manner, and the degree of EPSP suppression was the greater, the higher its concentration was. The speed of blocking depended on the concentration of toxin. The constant rate of EPSP amplitude decrease was directly proportional to the concentration of toxin: the higher toxin concentration was, the faster EPSP was blocked. The process of EPSP amplitude reducing could be described by one exponent [88].

The results of all experiments were analyzed to determine whether JSTX blocks ion channels of glutamate CRC (1987) [80, 81]. The effective JSTX concentrations were found to be slightly lower than those for channel type blockers. The phase of EPSP decrease in lobster muscle was described by one exponent and it does not depend on JSTX. Other channel blockers affect the EPSP decreasing them, evidently changing the dipole moments of the groups in the channel. Unlike other channel blockers, the JSTX's action was potentially dependent. The dependence of the peak amplitudes of EPSP on the potential was linear and it was not changed under the influence of JSTX. Otherwise, the toxin did not affect the electromotive forces in the synapses. Finally, the irreversibility of toxin action is also considered by the authors as proof that JSTX is not an antagonist of the channel type

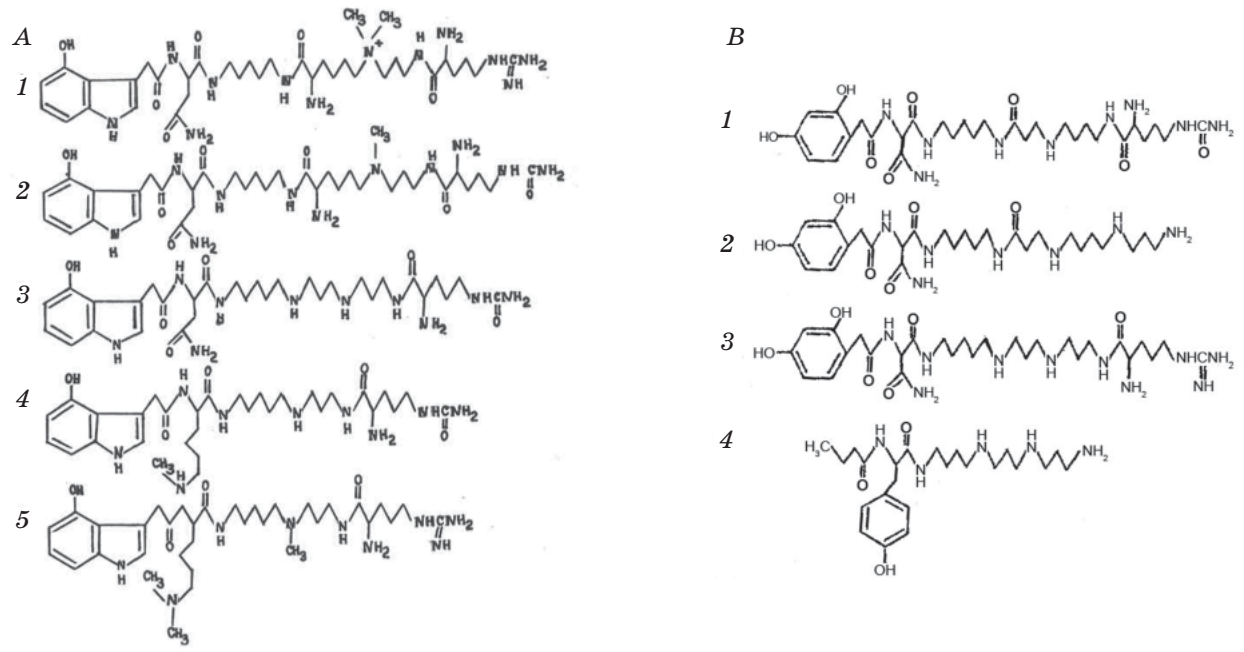


Fig. 2. **Chemical structures of some toxins from *Arthropodae* [33, 50, 51]:**
A — Family of toxins from *A. lobata* — argiopinines (ARN);
B — *1* — JSTX-3; *2* — NSTX-3; *3* — argiopin AR; *4* — PTX 433

[81]. In several publications the results of research of JSTX influence on the response of excitatory membranes were demostrated after the application of aspartate (Asp) and glutamate analogs — kainat (KK) and quisqualat (QL). The depolarization caused by aspartate in neuromuscular junction of the lobster was not sensitive to JSTX [13, 40–43].

Toxin JSTX did not block postsynaptic potentials caused by the application of aspartate in a giant squid synapse [42, 43]. He also did not affect the spikes caused by aspartate in the pyramidal neurons of hippocampus and neurons of brain cortex of guinea pig [42, 43, 88]. However, in some experiments on guinea pig hippocampal slices the same authors had shown that the depolarization of some pyramidal neurons caused by NMDA is reduced under the influence of JSTX-3, but lesser than that caused by glutamate [88].

Post-synaptic potentials caused by KK and QL in the neuromuscular lobster junction were blocked by JSTX [42, 43]. In squid giant synapse the depolarization caused by KK was blocked only partially [88]. In the pyramidal neurons of guinea pig hippocampus, the QL-receptors seemed to be more sensitive to JSTX than KK-receptors: smaller concentrations of toxin were required to block the responses induced by QL [88].

The results of experiments on various objects with blocking of glutamate receptors were very similar: irreversibility of JSTX action, blocking of responses to glutamate, but not aspartate, quantitative characteristics of blocking, and so on. Therefore, it was supposed that JSTX can be used as universal glutamate receptor "marker". In addition, it was concluded that there is a significant similarity of glutamate receptors in different phylogenetically distant objects: neuromuscular lobster junction [40–43] and some parts of guinea pig brain — hippocampus, olfactory bulb, *superior colliculus* [40–43, 88].

Therefore, JSTX was used to identify glutamatergic synapses. For example, until 1983 the question of whether glutamate performs a neuro-mediator function in giant synapses of star squid's ganglia remained open, because in previous experiments there were obtained contradictory data [42, 43]. The results of JSTX blocking role studying in this synapse were similar to those obtained in other sites where the role of glutamate as a neurotransmitter has already been proven [41–44, 88]. Here, the toxin also irreversibly and completely suppressed EPSP and miniature synaptic potentials, the rate of their blockage increased with toxin concentrations increasing, potentials caused by Glu and QL in post-synaptic membrane were completely blocked by JSTX, KK-activated potentials were blocked partially, Asp-activated potentials were not blocked at all. On the basis of this, the authors concluded that Glu in this synapse plays a mediator role by binding to a certain type of receptors [42–44].

However, further experiments have shown that the assertion that JSTX is a "universal marker" for glutamate receptors is not fully true. For example, in the presynaptic membrane of the lobster muscle were found the glutamate receptors that do not interact with JSTX [79]. Both Glu and QL-induced potentials in the membrane of presynaptic terminals were blocked by this toxin. Even more complicated case was registered in experiments on the membrane of the rod retina of the dogfish (shark eye) [89]. After the ionophoretic applications on these cells of glutamate, kainat and aspartate, the membranes were depolarized. The authors attempted, with the help of JSTX, to divide the population of studied receptors into types, but the toxin blocked the responses to all of these substances. The results of studies of chemoactivated single channels in the membranes of these cells can explain the reason of this failure. It has been shown that on this object all agonists interact with the same receptor molecule by opening only one ion channel [89].

The influences of glutamate receptors antagonists from *N. clavata* were studied also in Bogomolets Institute of Physiology of the National Academy of Sciences of Ukraine in scientific group of Prof. Krishtal O.O., Prof. Akaike N. (Japan) and young collaborators Drs. Tsyndrenko A., Kiskin N., Klyuchko O. using voltage-clamp technique in mode of holding potential at hippocampal membrane approximately at the mentioned period of these antagonists studying [17, 46, 50, 51]. Some of the results of these studying are presented on Figs. 3–9, and in the Table [17, 46, 55–67]. On Fig. 3, the blocking activity of the toxin JSTX-3 which is the main active element of the venom JSTX-V is presented.

B. Investigation of venom of spider Argiope lobata and toxins isolated from it. Usmanov and his co-authors published a paper devoted to the action of venom from spider *A. lobata* on glutamatergic and cholinergic synapses (1983) [101]. As elements of the venom, some factors have been identified that could

block postsynaptic processes in the nerve-muscle preparations of locusts and frogs. The venom was applied using ionophoresis, and synaptic potentials were recorded using glass microelectrodes. The venom influenced on the post-synaptic membrane, 75 μg/ml of the venom reduced the amplitude of the miniature potentials of terminal plate and the potentials of terminal plate up to their complete disappearance.

In these locust preparations the venom also reduced effectively the amplitude of both EPSP and miniature EPSP, and also blocked irreversibly the appearance of glutamate potentials after the application of mediator, and it was impossible to remove it by "washing" even with prolonged perfusion of the preparation with normal physiological solution. Potentials caused by application of acetylcholine at the same preparation were blocked by the venom, but in this case, the responses were restored after washing. Six months later, this group of authors tried to obtain the cockroach glutamate receptors using this venom [95, 96]. From the venom *A. lobata* there was isolated the fraction that blocks glutamatergic synapses.

The ligand fraction was bound to the affinity column, and it was incubated with membrane fragments of cockroaches containing glutamate receptors. The properties of the total protein fraction removed from the sorbent were studied using the technique of bilayer phospholipid membranes (BLM). The injection of protein fraction into the experimental block by itself caused a slight increase in BLM conductivity. The conductivity for sodium ions increased by two orders and depended on the mediator's concentration followed by glutamate adding. In presence of 10^{-4} M calcium ions, glutamate could increase conductivity to three orders. The complex of these proteins on BLM was inactive followed by neurotoxins adding.

Similar experiments on the isolation of glutamate receptors from neuromuscular crab synapses were carried out on 1985 [93, 94]. Characteristics of this protein on BLM did not differ from those described above [95, 96]. In addition, it has been shown that in the absence of glutamate, the increase in conductivity of BLM caused concanavalin A, wich prevents desensitization of glutamate receptor. Glutamate-induced BLM conductivity was blocked effectively by diethyl ether of glutamic acid, a glutamate receptor blocker [17, 46]. And, finally, an activity of single channels of glutamate complex receptor-ionophore in-built in BLM, was registered. The disadvantage of these works, however, is the use as ligand of "active fraction" of the venom during the isolation of rough mixture of toxins. In subsequent years, the composition of venoms and properties of the components isolated from them were investigated.

It was found that the protein — peptide fraction of the venom consists of several components — toxins, acting on signal transmission in glutamatergic synapses. The main active component of *A. lobata* venom was called argiopin [33, 72]. According to the Tashmukhamedov's works [95, 96] polypeptides with a molecular weight of 6–7 kDa are responsible for the activity of *A. lobata* venom. Subsequently, these data were related to argiopin with 636 kDa molecular weight [33]. Argiopin blocked effectively the glutamatergic transmission in locust neuromuscular preparations [95, 96], larvae of meat fly, frog muscle [72], frog spinal cord [14].

The blocking properties of argiopin were the same for all preparations. They were studied using intracellular microelectrodes from the region of synaptic contact under the voltage-camp conditions on the membrane [32, 90]. Like JSTX, argiopin acted on a post-synaptic membrane, but its action was reversible [14, 33, 72]. The decrease of registered postsynaptic currents was exponential and was approximated by two exponents [72]. The blocking action of argiopin depended on potential. In muscles of frog and fly larvae, it decreased strongly the amplitudes of excitatory postsynaptic currents near the resting potential and decreased them worse in case of hyperperpolarization [72]. On Fig. 4 the blocking activity of the toxin argiopin, the main active element from this venom is shown. In our experiments both, venom AR-V and argiopin, acted in reversible manner on glutamate receptors in hippocampal membranes but the rates of their reversible effects were different.

On locust neuromuscular preparations it was found that the venom *A. lobata* blocked not only glutamate, but also cholinoreceptors. Cholinoreceptors were blocked by the fraction of venom, which did not contain argiopin [101]. Contrary, according to other researchers, the active fraction of the venom influenced the transmission, both in glutamatergic and in cholinergic synapses, although in the latter — its effect was 40–70 times weaker [72] and argiopin blocked both glutamate and cholinergic receptors as well.

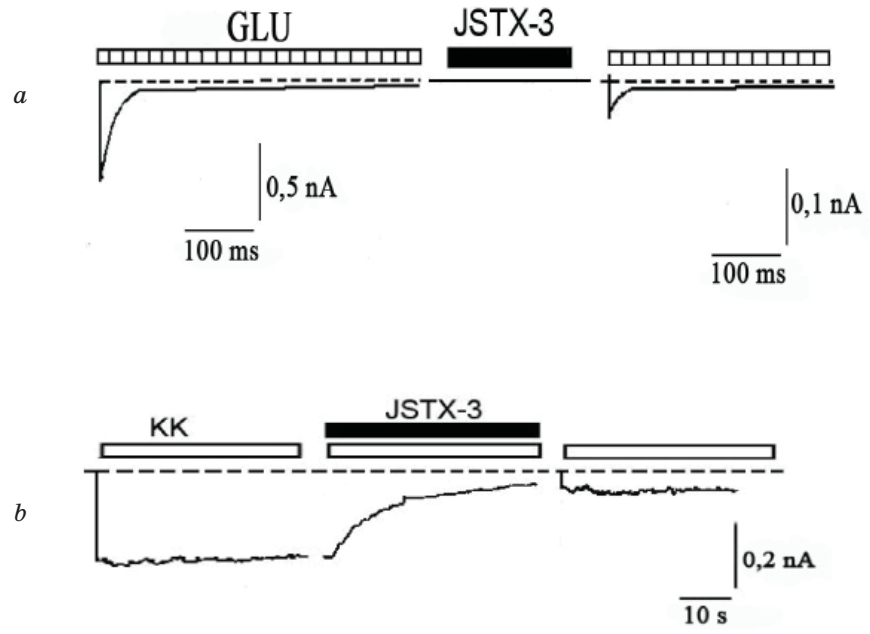


Fig. 3. **Blocking of chemo-activated transmembrane electrical currents by toxin JSTX-3:**
a — glutamate-activated; *b* — kainat-activated ionic currents. After the receiving of the control response to KK, toxin JSTX-3 was applied against the background of KK-activated current. Concentrations Glu and KK were 1 mmol/l; JSTX-3 was 10^{-4} mol/l; V_{hold} is — 50 mV.
Records *a* and *b* were done on different neurons [61, 62]

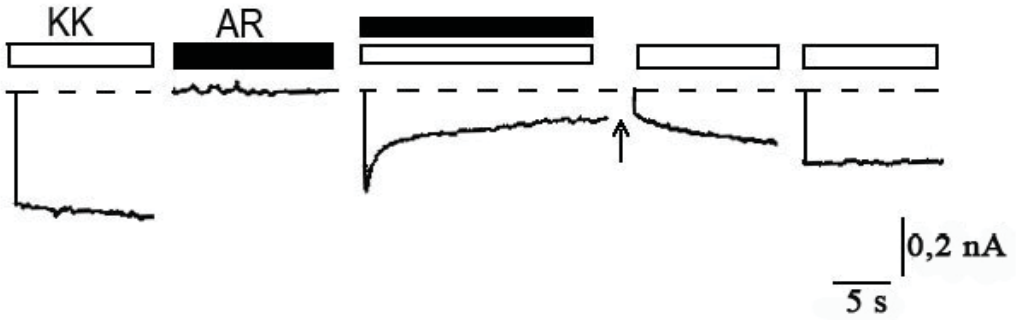


Fig. 4. **Argiopine causes blockage of the open state of kainat- activated ion channels**
After receiving of control response, the neuron was maintained in AR during 3 min, than on background of AR -KK was added. Concentrations: KK 1 mmol/l, AR 1.6×10^{-2} mol/l, V_{hold} — 100 mV.
Toxin removing by "washing" lasted 15 s [63]

According to the results of experiments, the rate constants of argiopin binding to the open channel and the dissociation of this complex for glutamate and cholinergic synapses were calculated. Under the normal conditions, they differed 36 times due to the fact that the rate of argiopin binding with the glutamate receptor in activated state was 5.3 times higher, and its dissociation was 7.4 times lower than with cholinoreceptor [72].

In synapses of the frog spinal cord, argiopine did not interact with NMDA receptors. Argiopin suppressed the depolarization of motor neurones caused by glutamate, and did not affect depolarization caused by aspartate [14].

Argiopin acted mainly on the opening of ion channels activated by glutamate [72]. But due to the fact that the ability of argiopin to reduce excitatry postsynaptic currents was more pronounced near the resting potential, it was concluded that arginipin also binded to closed channel. The calculated values of the dissociation constants (K_d) of argiopin for the open and closed channels practically coincided respectively [72]:

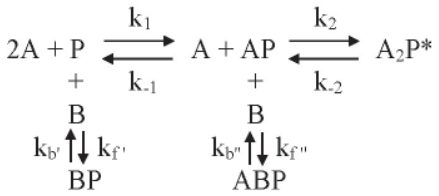
$(6.7\pm1.5)\times10^{-7}$ M and $(4.4\pm1.4)\times10^{-7}$ M.

The effect of argiopin on the kinetics of activation of glutamate-activated channels was demonstrated by analyzing the fluctuations in the membrane conductivity under the action of glutamate. The energy spectrum of these fluctuations was approximated by two Lorentz functions under the action of argiopin and by one Lorentz functions in control [72]. K_d value for argiopin in these experiments was 8 times lower than other one during the analyses of excitatory postsynaptic currents; perhaps, the toxin was more effective at application of glutamate than with its normal secretion. The set of received data allowed authors to describe the process of blocking from the point of view of model of consequent blocking of open channels:

$$2A + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A + AP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A_2P^* + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} A_2P^*B$$

where A is the activating molecule of mediator; P, P^* is the receptor corresponding to the closed and open states of channel; B is molecule-blocker, K_1–K_3 and K_{-1}–K_{-3} are the rate constants of corresponding reactions.

To describe the process of argiopin blocking of the closed channel, another scheme was proposed. According to it the antagonist can interact with P in a state of the rest or with inactivated (AR) receptor, which prevents further activation and transition to the state P^* [72].



In addition to argiopin, a number of other substances were isolated from the venom *A. lobata*. Grishin and his co-authors isolated at least three different compounds with similar biological activity from *A. lobata*. They all were able to block glutamatergic synapses. They all belong to family of toxins with homologous chemical structures [50, 51]. Like argiopin, they acted on glutamate receptors of the post-synaptic membrane, but the effectiveness of their action was different for various toxins [32].

It has been shown that the venom *A. lobata* contains at least two components differing in their effect on the binding of marked [^{3}H]-L-glutamate to locusts muscle membranes. One — with molecular weight of less than 5 kDa — suppresses effectively the synaptic potentials, but does not affect binding of [^{3}H]-L-glutamate, and thus does not interact with the glutamate-binding site of receptor. Another one, with molecular weight more than 5 kDa, interacts with the site of glutamate binding, suppressing competitively both mediator binding and synaptic potentials [101–104].

In addition to glutamate receptor antagonists, *A. lobata* venom contained high molecular weight components of presynaptic action. The activities of these components were aimed on the process of mediator release due to excitation and caused a decrease in quantum composition of mediator, but the rate of spontaneous mediator release was constant [71, 72].

Characteristics of *A. lobata* venom are determined by the sum of characteristics of all these components and combining 2 effects: 1) the presynaptic effect — is suppression of mediator release stimulus due to the excitation and 2) post-synaptic effect, the most important component of which is the ability to block the opening of postsynaptic ion channels [71, 72]. In the experiments of some authors the properties of integral venom (AR-V) differ from the properties of its main component (argiopin). Thus, some authors demonstrated that argiopin blocked glutamate receptor in reversible manner [72]. The action of integral venom in different experiments was less reversible [72], or irreversible [95, 96, 101].

C. Investigations of venoms and toxins of N. clavata and A. lobata in Ukraine. The influences of glutamate receptors antagonists from *N. clavata and A. lobata* were studied also in Bogomolets Institute of Physiology the National Academy of Sciences of Ukraine in scientific group under the supervision of Prof. Krishtal O.O. by his collaborators Drs. Tsyndrenko A., Kiskin N., Klyuchko O. The experiments were done using voltage-clamp technique in mode of holding potential at hippocampal membranes [50, 51]. IThe author analyzed some *Araneidae* toxins with the following known chemical structures: JSTX-3, AR are derivatives of DHPA-Asp, and ARN-1, ARN-2 are derivatives of indole-acetat-asparagin. Some of the obtained experimental results are presented on Figs. 3–9 and in the Table.

Among all of these substances JSTX-3 has the simplest structure: the chain of its polyamine is the shortest one, there is no

branching. In AR molecule the chain is slightly longer and 2 chemically active amino-groups are linked with it. ARN-1 and ARN-2 have the same length of polyamines, and amino groups are linked with them. The characteristic feature of ARN-1 is cationic group presence that is able to dissociate easily. This group contains pentavalent nitrogen in its polyamine chain. These substances can be arranged in a line depending on the length of their polyamine chains:

JSTX-3<AR<ARN-1=ARN-2.

The action of all studied toxins on the glutamate receptor was characterized by the number of similar characteristics. They all blocked GLU, KK, and QL (quisqualate)-activated currents in the membranes of rat hippocampal neurons in varying degrees and they all were able to be removed ("washed") to different degrees by normal Ringer solution (Fig. 7).

They did not act on electrically excitable membranes, glycine- and GABA-activated currents in membranes of these neurons. Their blocking effect depended on the membrane holding potential — it become less visible with depolarization of the membrane. All these substances could block the open glutamate channel-receptor complex (gCRC). It is natural to assume that all these toxins' properties are due to the common for all toxins fragments of their molecules, namely the phenolic or indole group, linked with asparagine.

Figs. 8, 9 demonstrate other experimental data on the development of new methods for qualitative analyses: the characteristics of transmembrane electrical currents, by which various substances can be identified according to dose-effect dependencies. K_d values were different for the toxin JSTX-3 and for the venom JSTX-V, from which this toxin was obtained. Together with JSTX-3 this venom containes other toxins and substances that caused registered shift [50–51]. K_d values for studied venoms and toxins were presented in the Table below.

Our experiments did not show significant differences in properties of toxins — phenol or indole-derivatives. There is only a little insignificant difference: the dissociation of AR derivatives is going a little bit slower (Table) [64].

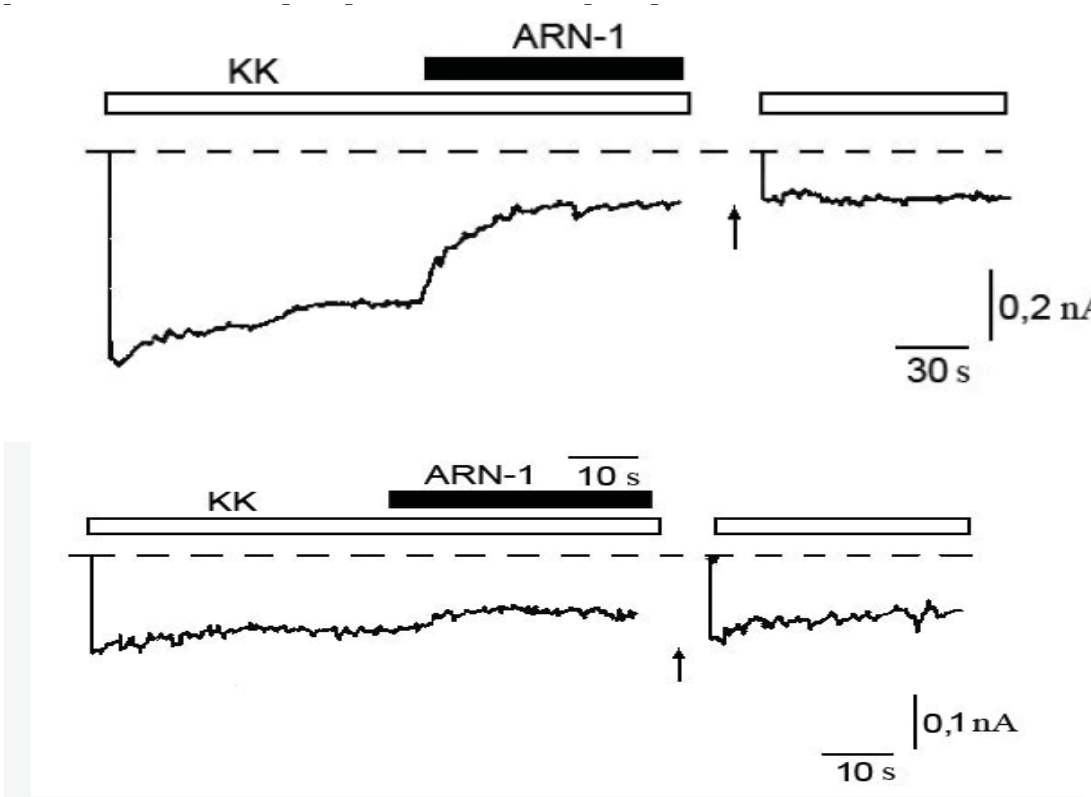


Fig. 5. **Irreversible blocking of KK-activated currents by argiopinin1(ARN-1) [61–64]**

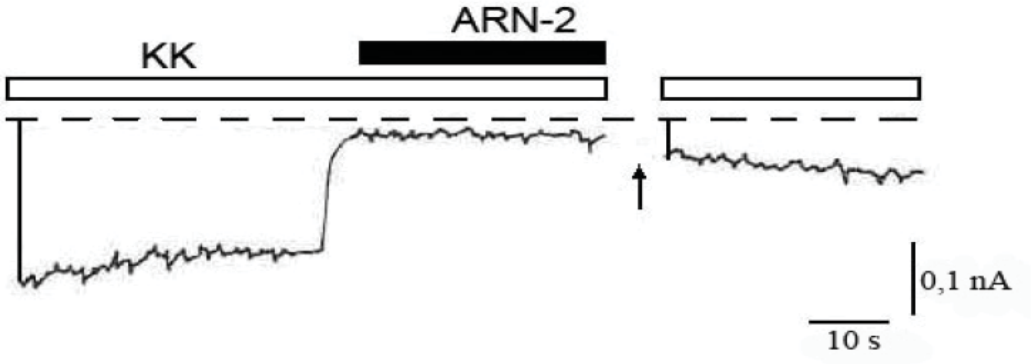


Fig. 6. **Irreversible blocking of KK-activated currents by argiopinin 2 (ARN-2) [61–64]**

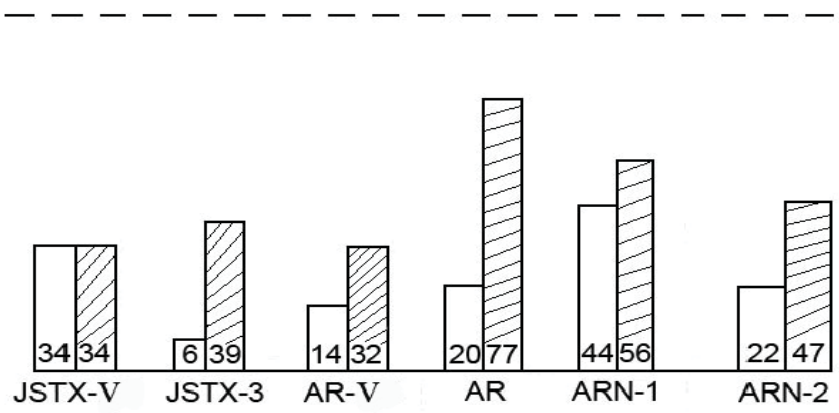


Fig. 7. **The degree of blocking of kainat-activated currents by various antagonists (white columns) and the degree of recovery of currents' amplitudes after the antagonists removing in Ringer's solution (shaded columns)**
The values are given in percent of the amplitude of control response, the value of which was taken for 100% (top straight dotted line). Diagram was done for 6 different antagonists of glutamate CRC [61–64]

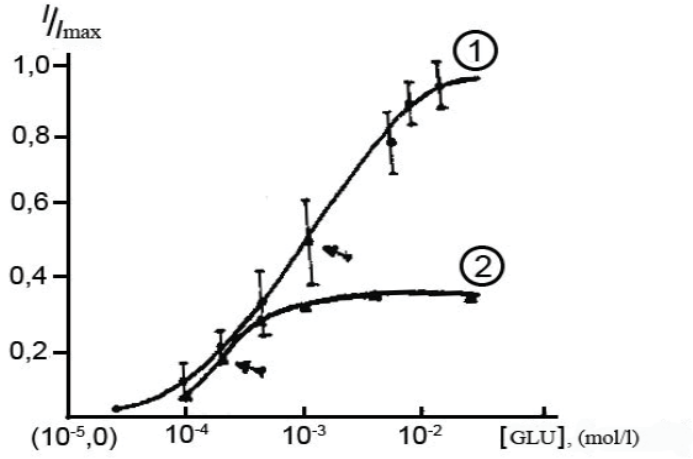


Fig. 8. **Dose-effect dependence of the glutamate-activated currents in the control (*1*) and during the action on the cell of 5×10^{-5} units/μl JSTX-V**
The currents' values at the points of peaks were normalized to the maximum. The curves represent a single-binding isotherm with $K_d = 1.1\times10^{-3}$ mol/l (1) and $K_d = 2.35\times10^{-4}$ mol/l (2) [61–63]

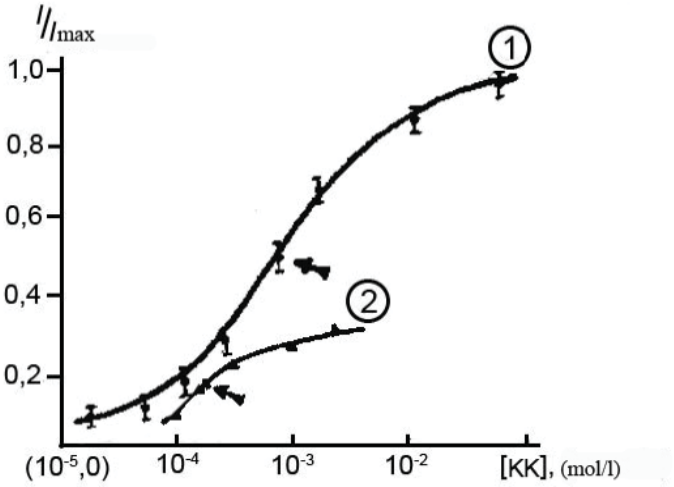


Fig. 9. **Dose-effect dependence of the KK-activated currents in the control (*1*) and during the action on the cell of 10^{-5} mol/l AR (*2*)**
Both curves are single-bonded isotherms with dissociation constants $K_d = 5.0\times10^{-4}$ mol/l (*1*) and $K_d = 2.4\times10^{-4}$ mol/l (*2*) [61–63]

Kinetic characteristics of KK-activated ionic currents blocking
Comparative analysis of the properties of various *Araneidae* toxins as result of their chemical structure

Antagonist	Constant rate of blocking (direct reaction)		Velocity of electrical current amplitude recovering
	K_1	K_2	I
JSTX-V	4.4×10^3 μl/(un.s)	–	0
JSTX-3	2.1×10^3 l/(mol.s)	–	1.3×10^{-2} s^{-1}
AR	1.6×10^3 l/(mol.s)	0.85×10^4 l/(mol.s)	4.9×10^{-2} s^{-1}
ARN-1	3.3×10^3 l/(mol.s)	1.6×10^4 l/(mol.s)	7.9×10^{-2} s^{-1}
ARN-2	2.9×10^3 l/(mol.s)	0.59×10^4 l/(mol.s)	3.1×10^{-2} s^{-1}

However, the direct constant rates of reactions between toxins and AR derivatives didn't get the significant differences. Such characteristics of toxins — phenol and indole derivatives — permitted to make conclusions.

Firstly on the basis of gCRC blocking mechanism by toxins, it should be the reaction of the interaction of toxin aromatic groups with membrane. This conclusion coincides with the Japanese authors opinion [86]. Secondly, this reaction should be common to indole and phenolic groups, so, the membrane should not "distinguish" them. And, finally, probably exactly these groups (or in connection with asparagine) determine the blocking effect of gCRC and its main features (potential-dependence, and others).

Toxins influence on activated and non-activated receptor. The main difference in the effect of toxins is that JSTX-3 interacts with the gCRC, regardless of whether it is in the activated state or inactivated. The main mechanism of action of AR, ARN-1, ARN-2 is the blocking of GLU- and KK-activating channels in the open state [33, 38].

What may cause the difference in the effects of toxins? The only structural difference between JSTX-3 and others studied toxins is simpler structure of its molecule. Perhaps slightly more complex structure of toxin molecule of *A. lobata* (lengthening of

polyamine chain, presence of amino-groups branched off the main chain) increases the selectivity of their interaction with gCRC so, that they lose their ability to bind with gCRC conformations corresponding to inactivated state. Thus, more simple JSTX-3 molecule, probably less "legible" and "does not distinguish between" receptor conformations corresponded to activated and inactivated state. In both cases, ion currents are blocked almost completely. In future it would be nice in similar experiments to study even more simple fragments of molecules: DHPA and DHPA-Asp, that also block gCRC [86] and the selectivity of which is not known well. Such ideas [86] stimulated the discovery of new methods of qualitative and quantitative analyses based on the registered differences in electrophysiological effects of toxins with known structure. So we tried to find regularities in effects of toxins with unknown structures.

An interesting regularity is that the removing of toxins AR and JSTX-3 from membrane (their "washing") was the same in Ringer's solutions, both in the presence of agonists (GLU, KK), and without them. According to our preliminary data, for the "washing" of some argiopines in solutions, contrary, the presence of agonists (GLU, KK) was necessary. For these substances, activation of the gCRC improved both the formation and dissociation of toxin-receptor complex. In compliance with the literature, there is known another toxin with similar property — gCRC antagonist: δ-phylantototoxin [18]. However, a significant difference in its molecule structure (instead of DHPA or DHPA-Asp it has oxyphenol) and not so much data on this issue allow to make any conclusions about the relationship of properties with the structure of its molecule.

According to some authors, the necessary condition for AR removing by "washing" was the presence of an agonist in washing solutions [18, 38]. This contradicts our data. However, this effect is easy to explain taking into account that in these works the roughly purified AR preparations were used, and this effect might be caused be the mixtures of argiopinins.

A. *The degree of blocking of KK-activating currents*. Any of studied toxins blocked chemoactivated currents completely even at high concentrations (10^{-4} mol/l). JSTX-3 decreased the amplitudes of KK-activated currents the most effectively — up to 6% of the initial value. The effectiveness of KK-activating currents depression decreased from left to right in the line of toxins:

JSTX-3 > AR > ARN-2 > ARN-1.

As one can see, the degree of blocking of currents by toxins can depend on the length of their polyamine: the shorter the toxin the more effectively it closes the ion channel. However, this statement should be reinforced by studies of other analogs with different lengths of polyamine chains.

B. *Irreversibility of toxins' effects*. For analogues of JSTX-3 with different lengths of polyamine chain, it has been found that elongation of polyamine chain is accompanied by the formation of more stable toxin-receptor complex [36, 38]. However, in our experiments, the most pronounced reversed action had AR, although its length is slightly longer than that of JSTX-3, Perhaps this is due to the fact that two amino groups are coupled with AR polyamine chain, which are easy to react, and the length of the polyamine appears to be functionally less important? To answer this question, let's analyze how molecular structure is linked the degree of "washing" of other *A. lobata* toxins. In our experiments there were registered that among *A. lobata* toxins AR has peculiar characteristics: all its analogues were "washed" much worse, regardless of their structure. However, during studying of the properties of AR analogues separately, it also appeared that it was easier to remove the substances with longer fragment of polyamine. This was true within groups of argiopinins 1-5 and pseudo-arhiopinins 1-3 (own in print data, as well as the data given in [17, 46]). Thus, our conclusion is completely opposite to other one one that was made by other authors for JSTX-3 analogues earlier. For AR analogues the longer is the toxin molecule the better it is washed off. The argiopinin 1 was the exception because it acted practically irreversibly. However, unlike other toxins, its polyamine includes easily dissociated cationic group containing pentavalent nitrogen. This group is likely to contribute to the formation of a more stable toxin-receptor complex.

C. *Kinetic characteristics of blocking effects of the toxins*. Basing on the results of calculations of the values of binding constant rates and the rates of "washing" of toxins (given in the Table), as well as basing on the preliminary data obtained during the argiopins 1-5 and pseudo-argiopinins 1-3 studies, the following conclusion can be made. When lengthening the chain of polyamine, the value of the first binding constant rate of toxins

with gCRC increases, and the rate of toxin "washing" respectively. Otherwise, the longer toxin molecule is, the faster it binds to the receptor and faster breaks the links with gCRC when dissociating. This rule is not true for the molecules having long side chains, namely ARN-4, whose binding constant is higher than expected, and this is exclusion. Perhaps this regularity should be transformed as follows: the longer and the branched molecule of the toxin is the sooner it binds and faster breaks the links with the gCRC.

Some regularities in biophyscal effects of JSTX-3, AR, ARN-1, ARN-2. Basing on the observed data following regularities between these molecules "chemical structures" and their "effects" can be presented.

A. The presence of phenol- or indole acetate-fragments in the toxin molecules is the "key" phenomena of the interaction of the toxin with gCRC. So the reaction of interaction of aromatic groups with the membrane groups should lie at the heart of the blocking mechanism. These fragments define the basic and common for all properties of these antagonists (potential-dependence of blocking, and others).

B. The length and structure of polyamine determine the individual differences in toxins' properties. Thus, with the complication of polyamine structure, the selectivity of toxins action increases and the toxins don't bind more with gCRC in inactivated state. Shortening of polyamines' chains leads to the effect that the toxin more "tightly closes" the ion channel, and the degree of blocking increases. At the same time, the shorter the molecule of the toxin, the worse it is "washed" off. And, finally, the longer and branched the molecule of the toxin, the faster the reaction of formation toxin-receptor complex is going, and the sooner this complex dissociates.

C. Among all the tested toxins, JSTX-3 really has unique characteristics. Having the simplest structure it causes maximal hysiological effect. This observation coincides with the data from the literature [28]. Among all known for today glutamate receptor antagonists, JSTX-3 has an optimal structure in terms of physiological effect. Toxin AR is distinguished by its properties among *A. lobata* toxins (the highest degree of "washing" and others). We can assume that these particular features in the process of evolution have selected the toxins JSTX-3 and AR as the main active components of the *Araneidae* venoms among the large families of other toxins — antagonists of glutamate receptors.

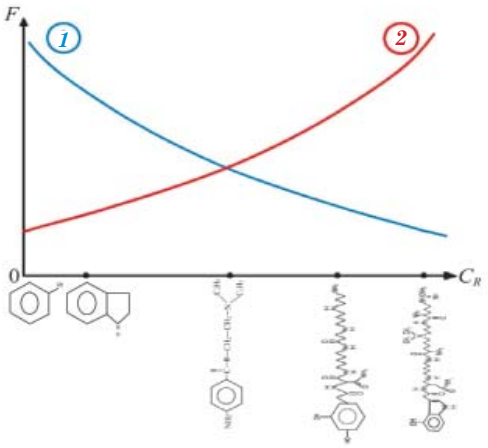


Fig. 10. **The qualitative regularities of the damaging toxic effect of studied substances [61–64]**

Empiric qualitative dependence demonstrates that with elongation and complication of polyamine:
1 — Toxin (Tx) molecules close the ion channel less densely;
– ability to depress the amplitudes of ion currents by Tx decreases;
– Tx molecules loss the ability to bind with gCRC in inactivated state.
2 — reversibility of Tx action increases;
– complexes Tx-gCRC are forming better;
– complexes Tx-gCRC are dissociating better;,
– Tx molecules can be better removed by "washing" in normal Ringer solution;
– selectivity of Tx action increases (Tx molecules loss the ability to bind with gCRC in inactivated state)

D. All toxins, studied in this work, were received both from the natural sources — venoms as well as ones that were synthesized in laboratory conditions. The effect of synthetic analogues was completely identical to the action of natural toxins. This confirms again the correctness of the decoded toxins' structures and the identity of the synthetic toxins with the corresponding natural analogs.

Above said can be illustrated by the simple qualitative graph (Fig. 10). Axis OX means the chemical structures (phenol or indole derivatives). Their structure complication is going from the left to right. Axis OY — qualitative representation of the the following effects described above. Circles 1 and 2 mean the effects: 1 — decrease with chemical structure complication, and 2 — increase with chemical structure complication [61–64].

Further development of this task suppose also further complication of molecular structures with the registration

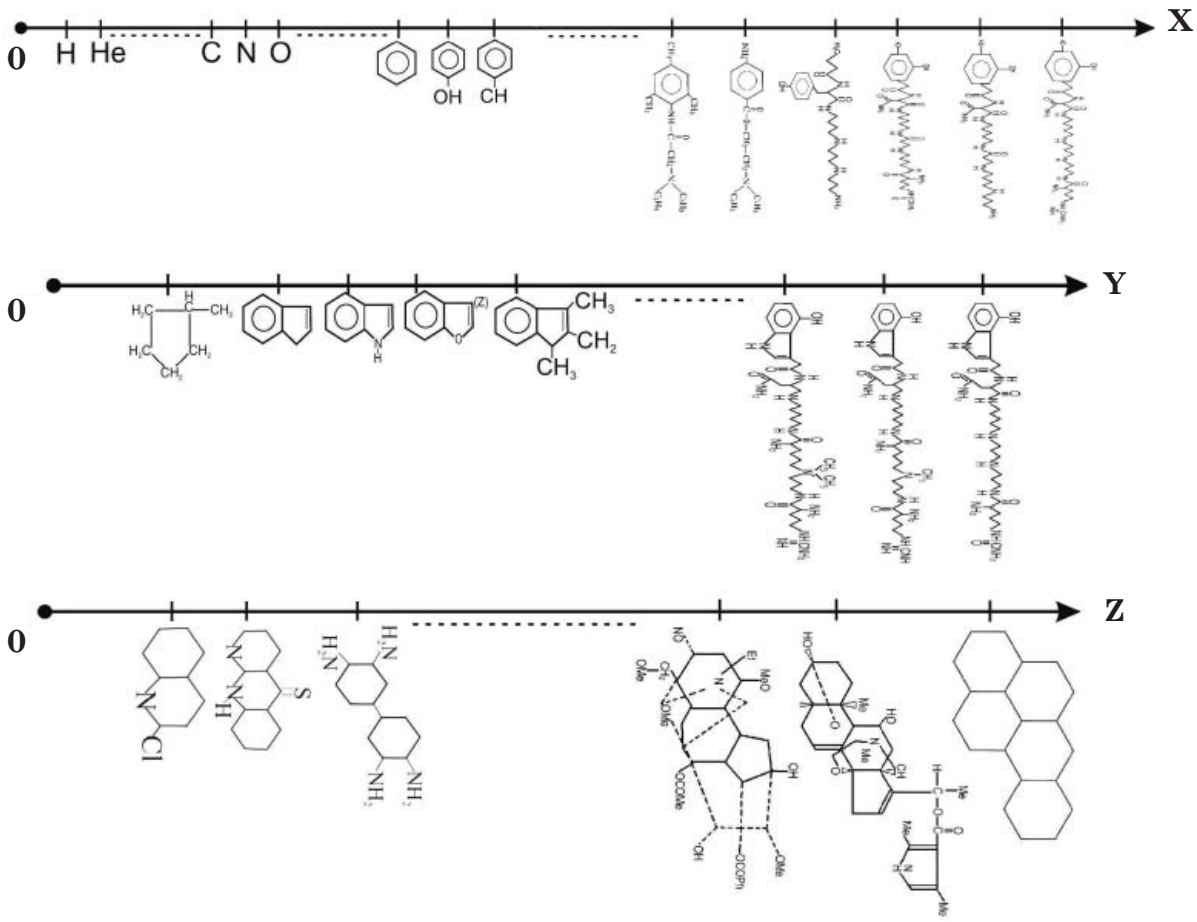


Fig. 11. **Observed and analyzed chemical substances that influence the living organisms [63]**

There are harmful, toxic substances — derivatives of phenol, indole, etc., combined with radicals of different length and complexity. In terms of the chemical structure, all of the compounds are represented by molecules with a «head» formed by cyclic compounds (phenol, indole, or polycyclic structures) that is coupled to polyamine (s) radical (s) of varying length and complexity.

Axis *X*: phenol and its derivatives with polyamine radicals of varying length and complexity. Length and complexity of chemical structure of radicals increase from the left to right, accordingly, their biophysical properties are gradually changed;

Axis *Y*: indole and its derivatives with polyamine radicals of varying length and complexity. Length and complexity of chemical structure of radicals also increase from the left to right, their biophysical properties are gradually changed;

Axis *Z*: some hydrocarbons that have several cycles in their composition and affect the biophysical processes in living organisms (and, consequently, their physiological effects including the expressed harmful and toxic effects). The chemical structure of these substances is complicated from the left to right, their biophysical properties also varying gradually.

of relative biophysical effects for perfection of possibilities of the qualitative and quantitative analyses of organic structures. For example, in computer databases all studied chemical substances can be arranged according to their complication along to hypothetical axes (Fig. 11). The axes CR on Fig. 10 could be substituted by any of the axes from Fig. 11 (*OX*, *OY* or *OZ*). In this case when somebody plots toxin blocking characteristics along the axes F (Fig. 10) theoretically it is possible to find related chemical structure along the axes CR. Having united all together the axis F, OX, OY, OZ, a virtual space "structure"-"function" that had been laid in a base of the novel methods of substances' computer analysis was formed. If necessary, it is possible to use other axis where other groups of substances are ordered in the framework of this model.

Today we have no enough standardized information for the construction of such computer expert system for qualitative and quantitative analyses, but with time with further databases completing this idea may be realized.

Thus, in this publication it was demonstrated further development of electronic expert system logical module, a powerful tool in contemporary biotechnology that also can be used as analytical system for quantitative and qualitative analyzis of organic chemical substances, derivatives of phenol and indole with polyamine radicals (substituents) — linear or branched. Fundamental observation of the results of some *Arthropodae* toxins and venoms studies was done to determine the regularities in chemical structures and electrophysiological properties they have.

The results from numerical literature sources of electrophysiological investigations (including own ones) were presented: analyses of chemosensitive transmembrane electric currents obtained in voltage-clamp mode under the influence of phenol and indole-derivatives from two spider species. They are known as antagonists of glutamatergic receptors and were used successfully for investigations of membrane structures. In such a way JSTX-3 (from *N. clavata* venom) and AR, ARN-1, ARN-2 (from *A. lobata* venom) were studied. These substances were selected due to their relatively small molecular weights and known chemical structures.

All described above gave us a possibility to find the regularities between "chemical structures" and "effects" of these substances influence necessary for our logical module. Also this gave us potential opportunity to solve the inverse problem to traditional one. Previously the direct problem was solved in such a way: under the action of chemical compound with known structure on the ionic currents, the researchers investigated obtained effect (study of dependence "structure" — "effect"). And now the possibility of inverse problem solution was suggested — to determine the approximate chemical structure of organic compounds through their effects on chemosensitive currents from the measured numerical characteristics of electrical responces (the "effect" — "structure" dependence study). On the base of experimental data in our work we tried to solve inverse problem: knowing electrical reaction of cell membrane on the influence of unknown substance to decipher its structure — structure of acting molecule.

We would like to emphasize the practical value of these data. Empirical dependencies "effect" — "structure" were found on the basis of the registered experimental data, their processing using the methods of regression analyses, other types of analyses. Empirical diagrams were constructed, which characterized such dependencies. In such a way the new methods of qualitative and quantitative analyses were developed and patented [61–64].

These regularities may be laid in base of logical module functioning. The observed chemical substances — toxins JSTX-3, AR, ARN-1, ARN-2 and other from this family were really good for such purpose. Obtained regularities have been described and used for further development of logical module in biotechnical expert system for automatic identification of organic substances.

Logical unit programmed on the base of such data, found regularities may be really useful for identification of organic environment pollutants in industrial regions, places of accidents, and etc. Among such pollutants there are many organic substances (including derivatives of phenol or indole); their identification, studies and analyses are really difficult for today [137]. Our developed methods show a way to qualitative and quantitative analyses conducting not only in stationary laboratory conditions but also for mobile methods of organic pollutants revealing and identification in environment.

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КОМП'ЮТЕРНЕ РОЗПІЗНАВАННЯ ХІМІЧНИХ РЕЧОВИН НА ОСНОВІ ЇХНІХ ЕЛЕКТРОФІЗІОЛОГІЧНИХ ХАРАКТЕРИСТИК

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Метою огляду був аналіз результатів електрофізіологічних досліджень деяких біологічно активних хімічних речовин для визначення закономірностей між їхньою структурою та ефектами, які вони спричиняють. Запропоновано також використовувати ці закономірності для вдосконалення логічного модуля біотехнічної експертної системи, яка працює з використанням модульного принципу на основі об'єктно-орієнтованого та регресійного аналізу. В роботі проаналізовано дані електрофізіологічних досліджень антагоністів глутаматергічних рецепторів: фенол- та індолпохідних, отриманих з отрут павуків видів *Arthropodae*. Деякі характеристики блокування рецепторів цими токсинами було використано для демонстрації емпіричних закономірностей між хімічними структурами антагоністів та їх електрофізіологічними ефектами. Обговорено можливість практичного застосування подібних закономірностей для моніторингу шкідливих забруднювальних речовин навколишнього середовища — фенол- та індолпохідних, а також розроблення нових методів їх якісного та кількісного аналізу.

Ключові слова: токсини, антагоністи рецепторів, трансмембранний електричний струм, біологічні експертні системи, електронні інформаційні системи, біоінформатика.

КОМПЬЮТЕРНОЕ РАСПОЗНАВАНИЕ ХИМИЧЕСКИХ ВЕЩЕСТВ НА ОСНОВАНИИ ИХ ЭЛЕКТРОФИЗИОЛОГИЧЕСКИХ ХАРАКТЕРИСТИК

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Целью обзора был анализ результатов электрофизиологических исследований некоторых биологически активных химических веществ для определения закономерностей между их химической структурой и эффектами, которые они оказывают. Предлагается также использовать эти закономерности для совершенствования логического модуля биотехнической экспертной системы, работающей с использованием модульного принципа на основе объектно-ориентированного и регрессионного анализа. В работе проанализированы данные электрофизиологических исследований антагонистов глутаматэргических рецепторов: фенол- и индолпроизводных, полученных из ядов пауков видов *Arthropodae*. Некоторые характеристики блокирования рецепторов данными токсинами были использованы для демонстрации эмпирических закономерностей между химическими структурами антагонистов и их электрофизиологическими эффектами. Обсуждается возможность практического использования подобных закономерностей для мониторинга вредных загрязняющих веществ окружающей среды — фенол- и индолпроизводных, а также разработки новых методов их качественного и количественного анализа.

Ключевые слова: токсины, антагонисты рецепторов, трансмембранный электрический ток, биологические экспертные системы, электронные информационные системы, биоинформатика.

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METABOLIC ENGINEERING OF SOLVENTOGENIC *Clostridia*

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Literary data on the organization of the genome, the connection of genes with the production and regulation of solvents, and the metabolic engineering of solventogenic clostridia are presented. The transition from the phase of the formation of acids to the phase of the formation of alcohols and the relationship of the latter with sporogenesis are analyzed. The main key genes (*ak, pta, buk, ptb, thl, crt, bcd, BdhAB, ctfAB, adc, rub, Spo0A, adhE, hdb*) that affect their course have been identified. The possibility of improving strains by genetic manipulations (inactivation of genes, entering of genes of other microorganisms, etc.) is shown. The effect of gene inactivation on solvent production is shown. An analysis of methods for increasing the accumulation of butanol showed the need to create effective recombinant producer strains for butanol supra synthesis using renewable raw materials.

Key words: butanol, producer strains, clostridia, solventogenesis.

Today, the microbiological conversion of biosphere renewable resources for the purpose of receiving of commercial products, in particular, the biofuel, is one of the recent problems of biotechnology [1]. Anaerobic bacteria of the *Clostridiaceae* family are known as producers of one of the most promising biofuels, the biobutanol. Currently, the microbiological synthesis of butanol during the classical acetone-butanol-ethanol (ABE) fermentation is economically unprofitable. The creation of commercial biobutanol technology requires high-yielding strains and an affordable, cheap and preferably renewable raw material — the plant biomass [2].

Industrial production of butanol based on microbiological synthesis was established in the early twentieth century and is related to the production of acetone (as a concomitant process) by *Clostridium acetobutylicum* with the production of acetone, butanol and ethanol on a 3:6:1 ratio and the using of corn flour as a substrate [3, 4]. In the classical ABE fermentation, the oil, propionic, lactic and acetic acids (acid formation stage) were produced by *C. acetobutylicum* at the initial stages, and eventually, the hydrogen index decreased and the production stage of butanol, acetone, and ethanol began (alcohol formation stage). The increasing demand for butanol and the sharp increase in petrochemical production have led to the fact that the biotechnological process for the butanol production became economically unprofitable and has been replaced by a more efficient chemical synthesis. In recent years, there has been renewed interest in butanol biotechnology not only as a raw material in the plastic, paint and varnish production and in printing and pharmaceutical applications, but also as an alternative fuel [5]. Today, the biofuels account for only 2% of all fuels used. Biofuel consumption volume in the fuel market is projected to reach 30% in the next 5–7 years [6].

The recent studies were related to the search for new productive strains that produce butanol and cheap non-food raw materials as a substrate [7, 8] and the optimization of butanol biotechnology. The following stages of butanol biotechnology optimization can be identified: 1 — primary selection of producer strains; 2 — determination of preferable

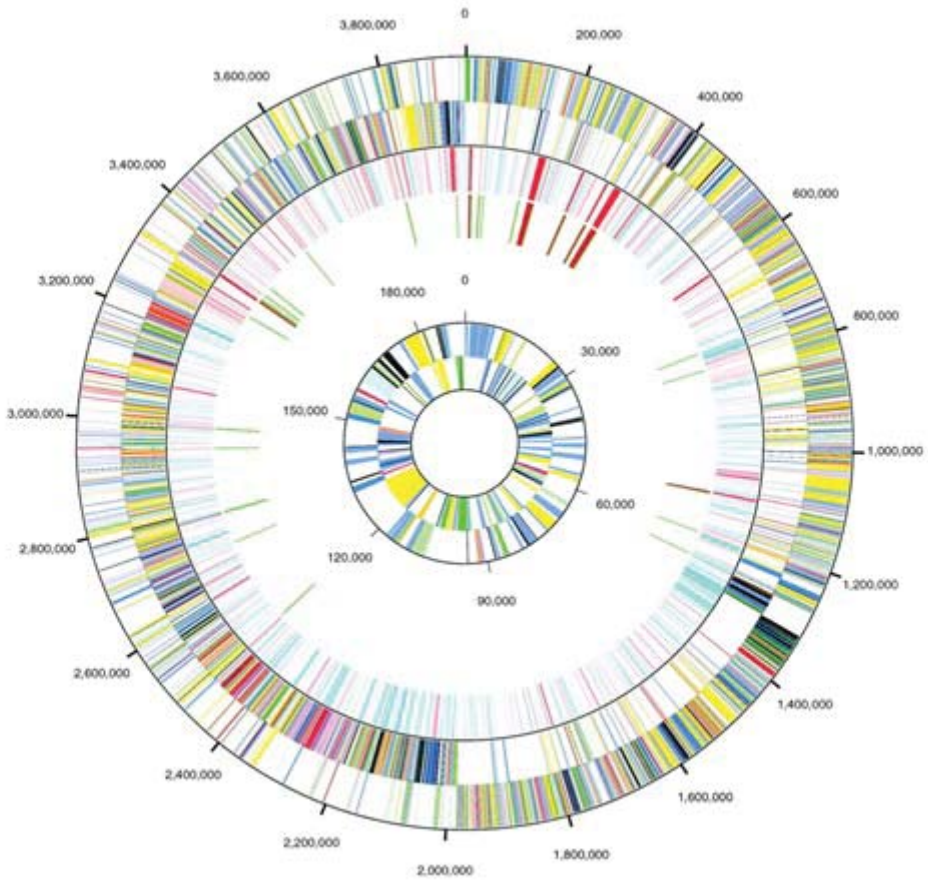

Fig 1. ***Clostridium acetobutylicum*** **ATCC 824 genome and megaplasmid [9]**

technological parameters (pH, temperatures, nutrient requirements) and preferable mode of nutrition and biomass accumulation, yield of the product in terms of the consumed substrate, rate of product formation; 3 — immobilization of producing cells; 4 — changes in genetic structure of a microorganism for increase in the accumulation of butanol.

Genetic manipulations with the *Clostridia* were made possible thanks to the determination of the nucleotide sequence/decoding of the complete genome of the *Clostridium acetobutylicum* ATCC 824 strain (Fig. 1).

Circular representation of the *C. acetobutylicum* genome and megaplasmid. The outer two rings indicate the positions of genes on the forward and reverse strands of the genome, respectively, color-coded by function. Moving inward, the third ring indicates the G1C content of each putative gene: turquoise (27%), gray (27 to 35%), pink-red (35%); the fourth ring indicates the positions of tRNA (green) and rRNA genes (dark red). The inner rings show the positions of genes on the forward and reverse strands of pSOL1, respectively, color-coded by function (the distance scale for the inner rings differs from the scale of the outer rings, as indicated). The functional color-coding is as follows: energy production and conversion, dark olive; cell division and chromosome partitioning, light blue; amino acid transport and metabolism, yellow; nucleic acid transport and metabolism, orange; carbohydrate transport and metabolism, gold; coenzyme metabolism, tan; lipid metabolism, salmon; translation, ribosome structure, and biogenesis, pink; transcription, olive drab; DNA replication, recombination, and repair, forest green; cell envelope biogenesis, outer membrane, red; cell motility and secretion, plum; posttranslational modification, protein turnover, and chaperones, purple; inorganic ion transport and metabolism, dark sea green; general function prediction only, dark blue; conserved protein, function unknown, medium blue; signal transduction mechanisms, light purple; predicted membrane protein, light green; hypothetical protein, black [9].

The genome size of *C. acetobutylicum* ATCC 824 is 4.13 Mbp, of which the chromosome consists of 3940880 bp and the megaplasmid pSOL1 is 192000 bp. The ratio

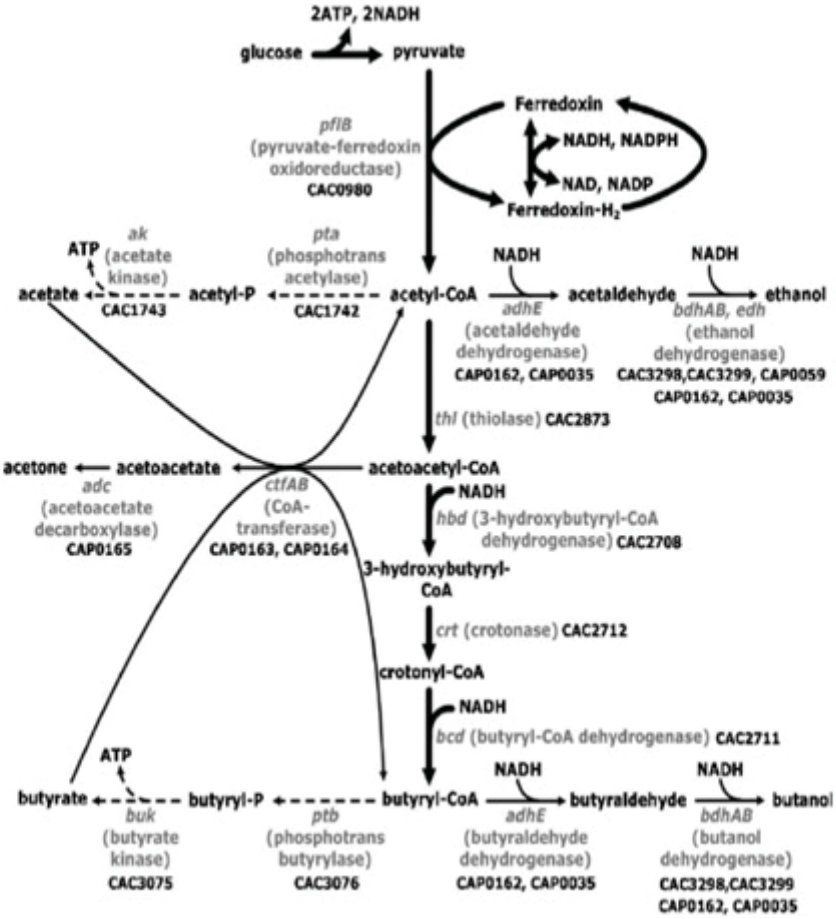

Fig. 2. **The metabolic pathway of *Clostridium acetobutylicum* [10]**

of G-C pairs is 30.8%. The genome consists of 3778 genes. The genetic information of the chromosome contains the major genes responsible for alcohol formation and encodes 178 polypeptides. It is these genes and the genes that encode the multifermental complex that is located on the cell surface (cellulosome) that is a unique metabolic profile of the *Clostridia* [9]. They demonstrate the role of horizontal gene transfer in the evolutionary development of these bacteria and determine the pathway for the synthesis of metabolites (Fig. 2).

The ABE process is discussed in detail in [11]. The genes responsible for the ABE process in *C. acetobutylicum* ATCC 824 may or may not be the part of certain groups (clusters). Two enzymes are involved in the formation of acetate-acetate kinase (*ak*) and phosphotransacetylase (*pta*), the genes of which are the part of acetate operon [12]. Butyrate kinase (*butK*) and phosphotransbutyrylase (*ptb*), which are the part of butyrate operon, are associated with the formation of butyrate. Both kits (sets) of these genes coexist in tandem on the chromosome and form operons in which *ptb* is located on the 5′-direction from *butK* and *pta* — from *ak*. The conversion of acetyl coenzyme A (*CoA*) involves the enzyme thiolase (*thl*), a gene of which is not the part of the functional units of organizations of genetic material in which there are several open reading frames (operons). The major genes responsible for converting 3-hydroxybutyryl-CoA to butyryl-CoA are the butyryl-CoA synthesis operon (BCS), namely crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*), the genes of two electron (*etfA, B*) transport flavoprotein subunits, β-hydroxybutyryl-CoA dehydrogenase (β*hbd*) [13].

Genes responsible for the formation of butanol dehydrogenase (*bdhAB*) are not clusters. Genes of acetaldehyde dehydrogenase (*aad*), acetyl-CoA acetate/butyrate CoA transferase (*ctfAB*), acetone

acetate decarboxylase (*adc*) make up the solventogenesis operon (*sol*) contained in the megaplasmid pSOL1 [14–16]. Genes of basic metabolism enzymes such as hydrogenase (*hydA*), flavodoxin (*flav*), rubredoxin (*rub*), the transcription factor responsible for the activation of sporulation (*Spo0A*) do not belong to clusters at all [17]. It should be noted that depending on the species and strain of bacteria of the genus *Clostridium*, the number of genes and their location in the clusters might vary [18–23]. Thus, the selective use of the positive effect of *Spo0A* on solvent formation is necessary to improve the productivity of the solvent. This requires *Spo0A* functioning as a gene for the activation of solventogenesis without the function of sporulation activation.

Sporulation is a major factor in solvent synthesis

The solvent synthesis process is closely related to the sporulation process (Fig. 3).

Some clostridia strains can convert acetone to isopropanol, producing a mixture of isopropanol, butanol and ethanol (IBE process), each of which can be used as a biofuel. The natural strain producer of isopropanol *C. beijerinckii* BGS1 accumulated 10.21 g/l of butanol and 3.41 g/l of isopropanol [25]. During normal vegetative growth, *C. acetobutylicum* cells asymmetrically divide by double division and exhibit typical bacillar morphology during glucose assimilation and acid accumulation [26]. During acid accumulation, the pH drops sharply and the medium becomes toxic to the cells. In response to changes in cell pH, the mechanisms of survival — sporulation and solventogenesis — are initiated [27].

Solventogenesis provides the cells with protection from low pH through the secondary consumption of acids and their conversion into solvents, which reduces the toxicity of the environment. Sporulation leads to high culture stability and provides a long-term survival mechanism until favourable conditions occur [28]. In the process of sporulation, various morphological changes of the cells occur [29]. The first morphological change that occurs in the cells is called the clostridial form. The cell acquires a cigar-shaped or swollen form with granulosa (vesicles of amylopectin) inside [30].

Previously, it was believed that cells with clostridial form produce solvents, but solventogenesis is started before they acquire it. Solventogenesis depends on three aggregate factors: the environment, the metabolic responses of a cell, and the activation of regulatory genes [31]. More than 256 genes that are differentially expressed during the transient phase are involved in solventogenesis [32].

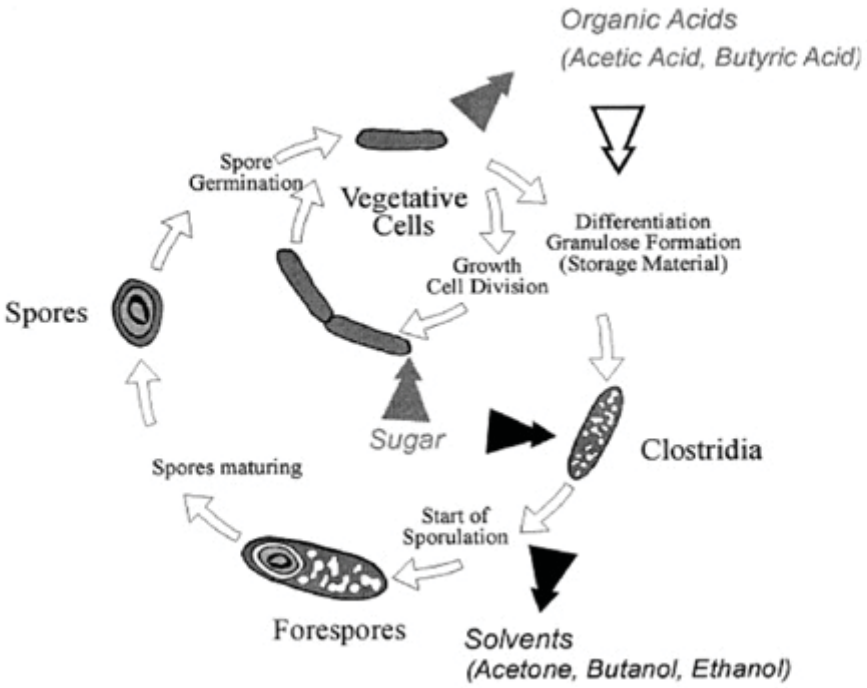

Fig 3. **Life cycle and sporulation of *Clostridia* [24]**

The transcription factor responsible for the activation of sporulation (*Spo0A*) also initiates the solvent synthesis in *C. acetobutylicum* by activating the transcription genes of acetoacetate decarboxylase (*adc*), alcohol dehydrogenase (*adhE*) and coA transferase (*ctfAB*). *Spo0A* is activated by phosphorylation, which is provided by the two-component signal transduction system. Phosphorylation is carried out by histidine kinase, which is encoded by the *ca_c0323, ca_c0903, ca_c2730, ca_c0437 and ca_c3319* genes in *C. acetobutylicum,* in *C. botulinum,* CBO1120 (CBO0336, CBO0340 and CBO2762) is responsible for it, and in *C. thermocellum* — *clo1313_0286, clo1313_2735* and *clo1313_1942*. Sigma factors that affect the transcription of sporulation genes in *C. acetobutylicum* are encoded by the *spoIIGA, sigE,* and *sigG* genes (Fig. 4).

The transcription factors encoded by sinR and abrB also regulate the onset of sporulation. The authors of [35] have suggested that abrB310 may also be a regulator during the transition between acidification and solvent phases.

For instance, in the case of the *C. beijerinckii* NCIMB 8052 mutant strain, the production of the solvents was controlled by the transcription factor *Spo0A*, which was the main regulator of stationary phase gene expression. The enzymes involved in solventogenesis have not been encoded in the plasmid, and therefore, are less sensitive to removal or loss [35].

Bacteria of the genus *Clostridium*, including *C. acetobutylicum*, are prone to degeneration (loss of increased solvent production) during cultivation on rich and easily digestible (carbon source) media.

The loss of a 210-tpn plasmid fragment containing the genes of the solvent synthesis enzymes leads to the emergence of *C. acetobutylicum* mutants that could not produce the solvent enzymes — degenerate, by definition, and could occur at the loss of 210 kbp of the plasmid where a few several genes are situated encoding several enzymes, solvent production [36, 37].

For *C. beijerinckii* NCIMB 8052 mutants, in addition to the abnormalities and degeneration caused by genetic alterations, the rapid cell growth in a rich medium with a high sugar concentration caused the inability to form solvents and spores. During the rapid growth of volatile fatty acids (acetic and oily) products that have been forming and accumulating in the medium quite intensively, simultaneously, the pH of the medium has also decreased. The rate of acid formation was so high that cells could not effectively induce solvent production [37–39].

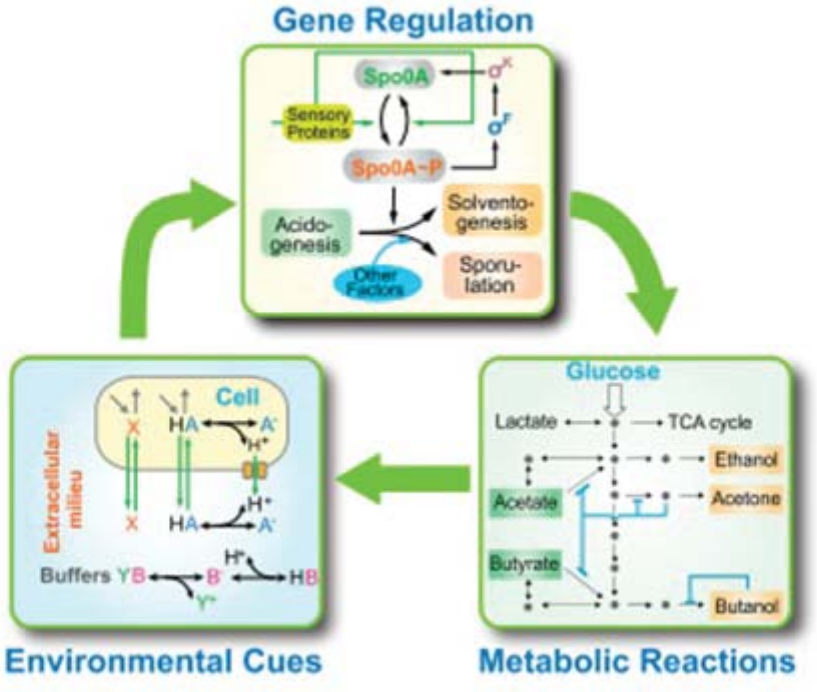

Fig. 4. **A system-level view of the acetone-butanol-ethanol (ABE) fermentation of *C. acetobutylicum* [34]**

Vectors for metabolic engineering

One of the major problems of genetic engineering of any microorganisms is the selection and development of vectors for the transfer of genetic information. The most promising vectors are plasmid-based — the extrachromosomal autonomous replication double-stranded ring DNA molecules. There are several types of plasmids: autonomous (not related to the bacterial chromosome) and integrated (built into the chromosome) plasmids.

F-plasmids carry information that ensures their own conjugative movement from one cell to another. R-plasmids carry antibiotic resistance genes. There are plasmids that have a specific set of genes that are responsible for the disposal of non-specific metabolites (degradation plasmids). There are plasmids that contain genes with unknown functions. If two or more plasmids cannot exist in the same cell, they are assigned to the same incompatibility group. Some plasmids carry a specific point of origin of replication (origin) and can replicate only in cells of a particular species, while others make this replication initiation site less specific and replicate in a variety of bacterial cells. Thus, the plasmids can be distinguished by the spectrum of hosts — wide or narrow.

For effective use in genetic engineering, the plasmids must have three characteristics.

1. Plasmids must be small in size because the transfer of exogenous DNA to *Escherichia coli* significantly reduces if plasmid lengths are greater than 15 kbp [40].

2. Each plasmid must have restriction sites at which insertion can occur [40].

3. A plasmid must have one or more selective markers to identify recipient cells carrying recombinant DNA [40].

In view of the above facts, pFNK1 vectors have been developed to increase the transformation efficiency of *C. acetobutylicum* ATCC 824 strain [41]. One of the first used was the integrative plasmid technology for metabolic pathway inhibition aimed for obtaining of acetate and butyrate through *C. acetobutylicum* ATCC 824. The copy number of plasmids commonly used for *C. acetobutylicum* was approximately 7–20 copies per cell [42–43].

Integration of the plasmid into the chromosome homologous region deactivated the *ack* and *pta* genes and formed a mutant with the deleted genes. Non-replicative integration plasmids (pAN1, pJC4, pJC7, pPUC-PTAK) that could be inserted into the *C. acetobutylicum* ATCC 824 genome were selected. Such plasmids required a host DNA fragment and an identified genetic marker. After the transfer, the plasmids can be anchored by integration in homologous regions. It were the integration plasmids that were introduced into the of *C. acetobutilicum* ATCC 824 chromosome [5]. Inactivation of *buk* and *pta* on the chromosome occurred by using the non-replicative integration plasmids containing the *buk* and *pta* genes. By inactivating the genes involved in the formation of the acid, it was possible to redirect the carbon stream to produce the solvent and increase its concentration.

Inactivation of the *buk* and *pta* genes allowed reducing energy costs for the production of acetate and butyrate. This resulted in a more than 10% increase in butanol production and 50% decrease in acetone production. Strains with changes in these genes accumulated more biomass and remained stable for 30 generations. The results of genetic engineering studies have demonstrated the possibility of using a gene inactivation method to control the metabolic pathway of glucose butyrate formation [44].

The *C. beijerinckii* BA 101 mutant (superproducer) was obtained from the original *C. beijerinckii* strain NCIMB 8052 by using the *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) chemical mutagen. Subsequently, the selection of the treated culture for resistance to 2-deoxyglucose was performed. This mutant produced twice as much solvent and utilized carbohydrates better. For the study of genetic mutations, the pJT 297 and pBUT 23 plasmids were used as compared to the original strain. These plasmids were used to study the activity of the fermentation genes of the *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* BA 101 strains at different cultivation periods. It is the activity of the *ptb* and *ctfAB* genes, which was determined by the mRNA accumulation level, which is the distinguishing difference between the strains. The activity of *ptb* and *ctfAB* genes in the *C. beijerinckii* BA 101 strain was 2-fold higher than in the *C. beijerinckii* NCIMB 8052 strain, and this coincided with the increased level of solvent production. Based on these results, it was concluded that genetic mutations occurred in these genes and they were essential in butanol synthesis increase [45].

It was shown that the placement of a transposon between restriction sites *Sna*BI

and *Eco*RI resulted in decrease in the rate of growth of the culture, decrease in the tendency to degeneration and increase in viability. Mutants actively grew in environments with high sugar content, had long-term stability of solvent production and the ability to grow at 42 °C according to phenotype and, as a result, the target product accumulation increased 2-fold [46].

To improve the *C. cellulovorans* strain, a shuttle plasmid pYL001 with pMRL83151 was developed by moving the 1138 bp fragment containing the *Cce*7431/743 II restriction site and changes in ColEI and in the non-coding region. This plasmid enabled to incorporate genes that encode butanol synthesis (*adhE1, adhE2, bdhB, aorm*) [47].

Several metabolic pathways were also altered by integration plasmid technology [48].

As an alternative to plasmids, a λ phage vector was used. Typically, this vector is used to create genomic libraries that require work with DNA fragments larger than possible to clone in a plasmid (greater than 10 kbp). Such vector makes it possible to work with large genetic constructs and incorporate them into clostridial cells [49].

Genetically modified strains

Genetic manipulations with clostridia were aimed at the altering of selected strain genes to increase the accumulation of butanol, to increase the tolerance of the strain, to expand the range of raw material (substrate) for cultivation and industrial production of butanol not mixed for obtaining of other solvents and acids [50]. The first effective result of metabolic engineering was an increase in acetone production. Acetoacetate decarboxylase of the recombinant *C. acetobutylicum* strain with amplified *adc* (encodes acetoacetate decarboxylase) and *ctfAB* (encodes COA transferase) genes that was involved in the synthesis of acetone, became much more active in the process of cultivation then usually, which caused the early induction of acetone formation. As a result, the concentrations of acetone, butanol and ethanol increased by 95%, 37% and 90% respectively, compared to the parent strain [51].

Most of the works were focused on the inactivation of genes responsible for the accumulation of acetate, butyrate and acetone and enhancing the expression of solventogenesis genes. High production of butanol (16.7 g/l) was obtained by inactivation of the butyratkinase (*buk*) gene. Metabolic pathway analyses showed a 300% increase in the butanol formation flow in the recombinant strain. Cumulative silencing of phosphatacetyltransferase (*pta*) butyratkinase (*buk*) genes and *adhE1* overexpression (encodes mutant aldehyde/alcohol dehydrogenase) resulted in a 60% increase in butanol production (from 11.8 to 18.9 g/l) with a 145% increase in bioconversion butanol (0.71 vs. 0.29 mol/mol glucose) [52, 53].

Inhibition of acetyl-CoA acetate/butyrate coenzyme transferase (CoAT) ratio increases the ratio of butanol to acetone, but decreases the accumulation and titer of butanol [54, 55]. Various strategies were used to increase the accumulation of butanol by *C. acetobutylicum* strains by overexpression of 6-phosphofructokinase (*pfkA*) and pyruvate kinase (*pykA*) glycolytic pathways [56]. The engineered strain showed an increase in the intracellular level of nicotinamidudinucleotide (NADN) and adenosine triphosphate (ATP) and an increase in butanol tolerance in connection with increase in butanol concentration from 19.12 g/l to 28.02 g/l.

Genetic analyses has shown that minor alcohol dehydrogenase (*sadh*) was responsible for the conversion of acetone to isopropanol [57–59]. *Sadh* overexpression shows the change of ABE to IBE processes in solventogenic clostridial strains. Overexpression of *sadh* and *hydG* (encodes the electron transfer protein) led to an increase in the total amount of IBE production (27.9 g/l) [60–65].

The silencing of histidine kinase in the *C. acetobutylicum* ATSC 55025 asporogenic strain has increased its tolerance to butanol and its accumulation by 44% (18.2 g/l vs. 12.6 g/l) and its productivity by 90% (0.38 g/lh vs. 0.20 g/lh) [66, 67].

A butanol producing strain was also constructed based on *C. tyrobutyricum* with overexpression of *adhE2*, which was asporogenic and accumulated butanol (20 g/l) by cultivation with nutrition [68]. The *C. tyrobutyricum* strain with inactivated *ack* and *adhE2* overexpression accumulated 16 g/l butanol versus 10 g/l in the original strain by using mannitol as a substrate [69].

To increase the yield of butanol and ethanol from lignocellulosic raw material, a *C. cellulolyticum* producer strain was created with overexpression of solventogenesis genes and inhibited function of acidogenesis genes. Alcohol dehydrogenase (*adh*) and pyruvate decarboxylase (*pdc*) genes from *Zymomonas mobilis* were incorporated into *C. cellulolyticum* H10 strain to produce

cellulose ethanol. Malatdehydrogenase (*mdh*) and lactate dehydrogenase (*ldh*) genes were added to increase ethanol accumulation by the *C. cellulolyticum* H10 strain [70–72].

In [73], a strain of *C. cellulolyticum* was improved by integrating thiolase (*atoB*) with *E. coli* and a CoA-dependent butanol synthesis pathway (*adhE2, hbd, crt, bcd*) with *C. acetobutylicum*. However, the resulting strain produced only 0.12 g/l of butanol from cellulose after 20 days of cultivation.

In order to obtain butanol by using cellulose, the *C. cellulovorans* strain was enhanced by the integration of alcohol dehydrogenase (*adhE2*) with *C. acetobutylicum*. Thus obtained *C. cellulovorans* producing strain has been producing butanol (3.47 g/l) from cellulosic raw materials [47, 74–76].

In order to obtain butanol from the thermotolerant strain of *C. thermocellum*, a 2-keto acid strain was created for the biosynthesis of isobutanol, which produced 5.4 g/l of solvent during periodic fermentation with use of cellulose [77].

Synthetic gases may be the alternative substrates for butanol. To obtain butanol from gas, the pathway of butanol biosynthesis was transferred to *C. ljungdahlii* from *C. acetobutylicum*. Butanol accumulation of 0.15 g/l was obtained by using gas as a carbon source, which eventually decreased to 0.015 g/l [78].

By silencing the phosphatacetyltransferase and acetoaldehyde dehydrogenase and integrating the part of the genes from the butanol synthesis pathway (*thl, hbd, crt, bcd,* butyraldehyde dehydrogenase and NAD-dependent butanol dehydrogenase), the *Clostridium* sp. MTButOH1365 mutant strain was obtained from *Clostridium* sp. MT1962 into the chromosome. The resulting strain accumulated 22 g/l of butanol from the CO/H_2 gas mixture during periodic cultivation [79].

The complexity of regulation of clostridium genes has prompted the researchers to explore other organisms (for example, *E. coli*) as hosts for butanol producing genes [80]. Metabolic engineering of *E. coli* produced a strain that accumulated butanol at a concentration of 18.3 g/l. The formation of by-products such as acetate and butyrate can be avoided in the process of improving the activity of *adhE2* [81].

Despite some advances in producing strain genetic engineering researches, the use of genetically modified butanol producing strains in butanol accumulation increase remains imperfect and complex.

Recently, the attention of researchers has returned to butanol, as butanol as a fuel has significant advantages over traditional ethanol-based biofuels. Butanol is already starting to be used as a liquid biofuel for current vehicles without engine modification. However, there are some limitations in the process of microbiological synthesis and commercial butanol technology — low productivity of producing strains and inhibition of synthesis products in the cultivation process. This review provides examples of genetic modifications of butanol producing strains by overexpression or inactivation of the respective genes to enhance butanol accumulation. The gene of solventogenic clostridia, genes involved in the transition between acidification and solvent production, and the influence of sporulation on the accumulation of alcohols are described. The review literature can serve as a basis for further research and the creation of genetically modified butanol-accumulated strains.

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МЕТАБОЛІЧНА ІНЖЕНЕРІЯ СОЛВЕНТОГЕННИХ *Clostridia*

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Наведено дані літератури щодо організації геному, участі генів у продукуванні та регуляції синтезу розчинників і метаболічної інженерії спиртоутворювальних клостридій. Проаналізовано перехід від фази утворення кислот до фази утворення спиртів та зв'язок останньої зі спорогенезом. Визначено основні ключові гени (*ak, pta, buk, ptb, thl, crt, bcd, BdhAB, ctfAB, adc, rub, Spo0A, adhE, hdb*), які впливають на їх перебіг. Показано можливість удосконалення штамів шляхом генетичних перетворень (інактивації генів, уведення генів інших мікроорганізмів тощо). Установлено вплив інактивування генів на продукування розчинників. Аналіз методів підвищення накопичення бутанолу засвідчив необхідність створення ефективних рекомбінантних штамів-продуцентів для надсинтезу бутанолу в разі використання відновлювальної сировини.

Ключові слова: бутанол, штами-продуценти, клостридії, солвентогенез.

МЕТАБОЛИЧЕСКАЯ ИНЖЕНЕРИЯ СОЛВЕНТОГЕННЫХ *Clostridia*

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Приведены данные литературы относительно организации генома, участия генов в продуцировании и регуляции синтеза растворителей и метаболической инженерии спиртообразующих клостридий. Проанализирован переход от фазы образования кислот до фазы образования спиртов и связь последнего со спорогенезом. Определены основные ключевые гены (*ak, pta, buk, ptb, thl, crt, bcd, BdhAB, ctfAB, adc, rub, Spo0A, adhE, hdb*), которые влияют на их ход. Показана возможность усовершенствования штаммов путем генетических преобразований (инактивации генов, введения генов других микроорганизмов и др.). Установлено влияние инактивации генов на продуцирование растворителей. Анализ методов повышения накопления бутанола показал необходимость создания эффективных рекомбинантных штаммов-продуцентов для надсинтеза бутанола при использовании возобновляемого сырья.

Ключевые слова: бутанол, штаммы-продуценты, клостридии, солвентогенез.

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THE USE OF HERBAL REMEDIES IN THE TREATMENT OF HEPATOBILIARY DISEASES: TRENDS AND PROSPECTS

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Hepatobiliary system diseases represent an important medical and social problem due to increasing morbidity rates worldwide. Liver and biliary diseases are characterized by complex pathophysiology as well as by multi- and comorbidity. The treatment of such diseases necessitates multitarget drug development. The effectiveness of current drugs in the treatment of hepatobiliary disorders remains low and the incidence of side-effects are profound. This actualizes the search and development of highly effective hepatoprotectors with a low incidence of side effects. Medicinal plants potentially constitute a sourse of such preparations. The review summarizes the data concerning mechanisms of hepatoprotective and immunomodulatory effects of medicinal plants and their phytoconstituents. The prospects for the development and use of herbal remedies in the treatment of hepatobiliary diseases are outlined.

Key words: hepatobiliary diseases, medicinal plants, hepatoprotectors, immunomodulators.

Digestive system diseases (DSD) are among the most common pathologies of internal organs, found in 10 to 15% of population of the developed countries [1, 2]. Usually, the anatomically and functionally related systems and organs are involved in the pathological process of these diseases. For many years, the prevention and treatment of DSD have been one of the most important medical problems. The most frequent pathological conditions of the digestive tract are diseases of the hepatobiliary system. In particular, the diagnosis of hepatobiliary disorder is among the top 100 in the US and many European countries. As of 2018, hepatobiliary pathology accounts for more than a quarter of cases per year of all DSD in USA [3–5]. According to WHO, more than 2 billion people worldwide suffer from the pathology of the hepatobiliary system, 100 times the number of patients with HIV. Annually, 500 000 to 1 million patients with liver, gallbladder and biliary tract diseases are registered in the Commonwealth of Independent States. In Ukraine, the pathology of the hepatobiliary system is 60.32% of all DSD. The prevalence of that pathology increased by 20.1% in Ukraine in the latest decade [6, 7]. Diseases of the hepatobiliary system include a wide range of pathological conditions of the liver, gallbladder and biliary tract of infectious and non-infectious etiology. The liver diseases are classified into diseases related to malformations, hereditary diseases, and chronic diseases which account for the largest share. Chronic types of hepatitis are of viral, congenital or autoimmune nature, and take the leading place among chronic liver diseases. Pathological conditions of the biliary system include diseases caused by birth defects of the gall bladder, and diseases of the biliary tract. Those, in turn, are divided into functional disorders in the

forms of dyskinesias of hypotonic, hypokinetic and hyperkinetic types, and cholecystitis, cholangitis and cholelithiasis [8]. The cholelithiasis is the most costly hepatobiliary disease. Medically, it involves the formation of concretions in the gallbladder due to the abnormally high cholesterol or bilirubin (heme breakdown product) in bile [9]. Almost 20% of adult Europeans, and nearly as many Asians have gallstones. Cholelithiasis is mostly found in female patients. It is a chronic state, with prevalence increasing with age (reaching plateaus after the ages of 50 and 60 in women and men, respectively). Hence, highly prevalent cholelithiasis in the elderly people is considered to be one of the most serious medical problems of the contemporary aging human population. In addition, cholelithiasis is also a major cause of gallbladder carcinoma, which is fifth of the most common cancers and has an extremely high patient mortality rate. The main reasons for the increasing prevalence of cholelithiasis are the dominance of high-calorie diets combined with a general decrease in physical activity [10–13].

Diseases of the hepatobiliary system (HBD) can occur in acute and chronic forms, as well as be accompanied or cause a number of threatening conditions with characteristic symptoms and syndromes: jaundice, portal hypertension, hepatic coma, hepatic insufficiency, cirrhosis, general intoxication [14, 15].

The aforementioned suggests that functional disorders of the liver and biliary tract are one of the most important problems for healthcare professionals worldwide. Different groups of pharmaceuticals are used in the complex treatment of HBD, but special place among the medical preparations are those with a selective effect on the liver, hepatoprotectors. The mechanisms of direct protective action of most hepatoprotectors are not yet fully understood. However, they are known for their membrane-stabilizing, antitoxic, anti-inflammatory, choleretic, antiviral, antioxidant, immunomodulatory and other effects [16, 17]. Hepatoprotective preparations normalize metabolic processes and homeostasis in the liver, increase the resistance of hepatocytes to pathogenic effects, stimulate regenerative processes, restore the liver parenchyma and normalize its physiological functions. However, the existing hepatoprotective preparations for the treatment of HBD are still poorly effective, primarily due to the side effect caused by toxic chemicals [18, 19]. Hence, medicines with low or no side effects are needed, which incited research that is aimed at finding and developing effective hepatoprotective herbal remedies [20–24].

Polyherbalism in phytotherapy

Phytotherapy (PT) is a form of complementary and/or alternative medical practice [25]. It is usually implemented with the common treatment, not instead of it [26]. Today the share of phytopreparations in the world pharmaceutical market is over 40%. According to WHO, this proportion will increase to 60% of the total list of medicines over the next ten years. The fact that the Nobel Prize in Physiology and Medicine in 2015 was awarded to Tu Youyou, William C. Campbell and Satoshi Omura for the discovery of natural products for the treatment of tropical parasitic diseases is in favor of the progressive development of phytopharmacology [27, 28].

There is a plethora of crude drugs derived from medicinal plants which are used in the treatment of various human diseases and ailments. For a systemic study of crude plant-derived medications it is very important to classify them in proper system. There are several classifications of crude plant-derived drugs: alphabetical cassification (crude phytopreparations are arranged alphabetically either in Latin name or in English name); taxonomical (botanical) classification (phytopreparations are arranged in a group according to their division, class, order, family, genus and species); morphological classification (phytopreparations are arranged in a group according to the used part of plant, e.g. flower, root, etc.); pharmacological classification (phytopreparations are grouped according to their pharmacological action, e.g. anticancer, anti-inflammatory, antibacterial, etc.); chemical classification (phytopreparations are classify according to their content of active substances, e.g. alcaloids, volatile oils, etc.). Additionally, all phytopreparations are divided into three broad categories (Fig. 1): preparations based on dried raw materials (compositions of collected plants, briquettes); extraction preparations; preparations composed by separate fractions of raw materials (juices, oils). Extraction preparations, in turn, are divided into galenic, neogalenic, and preparations of individual biologically active compounds (BAC) of plant or more complex origin. Galenic formulations are preparations which have a complex chemical composition and are a product of treatment of herbal medicinal raw materials for preservation of BAC in the native state.

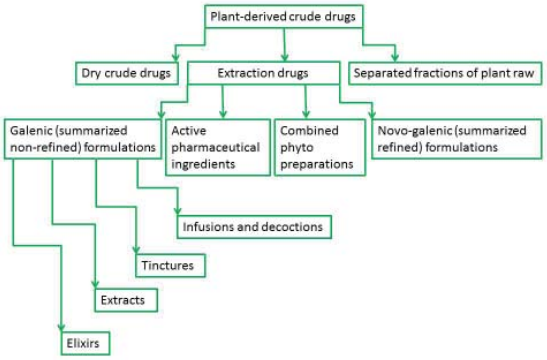

Fig. 1. **Classification of plant-derived crude drugs**

Neogalenic formulations are mixtures of active substances of plants, purified from ballast and related substances. Complex preparations, in addition to plant BAC, may include chemical constituents. Galenic preparations, in turn, are divided into infusions and decoctions, tinctures, extracts (liquid, thick and dry), and preparations from fresh raw materials. By the method of implementation of drugs in PT, the preparations can be divided into those intended for internal and external application. The liniments (balms), ointments, creams, and compresses are intended for external use.

Plant extracts are the base of all herbal preparations for internal use. The extracts separate the useful (medicinal) components from the fibrous, less useful part of the plant. Tinctures are highly concentrated, mostly alcohol-based extracts of fresh or dried plants. Most BAC of medicinal plants are soluble in alcohol, thus this way of separating them from the plant is the most effective. Elixirs are tinctures with the addition of sweeteners. Extraction can be also realized from an herbal tea, which is the most popular, simplest and least concentrated aqueous extract of medicinal plants. The use of herbal extracts in a tea preparation is ideal for chronic conditions, including HBD, when long-term exposure to BAC of medicinal plants in low concentration is desirable. Among tea preparations, there are decoctions and infusions. Infusions are mostly prepared from the above-surface plant parts (flowers, leaves), by treatment of plant material with boiling water and letting it steep for a varying period of time to obtain drugs with different BAC concentrations. Not only flowers and leaves, but also seeds, roots and bark are used to make decoctions. In that case, raw plant material is added to cold or boiling water and maintained at a temperature of 50–100 °C also for different time intervals [29–31].

An important element of the development of PT is the development of complex herbal products based on mixtures of medicinal plants. This is due to the increasing level of comorbidities (coexistence of two or more syndromes or diseases in one patient, pathogenetically interrelated or coincidental) and polymorbidities (presence in the individual of several diseases having synchronous course in different phases and stages, both related and unrelated genetically and in their pathogenesis) in current therapeutic practice [32–36]. The comorbidity is also characteristic of HBD [37–39]. The simultaneous presence of several pathological conditions, as well as complex pathophysiology of many diseases, including HBD, dictates the need for using BAC of medicinal plants with biological action of different nature. This requires an in-depth study of the biological effects of herbal mixtures, and the possible synergism and antagonism of herbal BAC in their composition. The multicomponent mixtures of medicinal plants are preferable because of the proven fact of synergistic and additive action of plant BAC in the certified and newly created polyherbal compositions. Recently, the traditional “one drug, one target, one disease” approach in the development of preparations and treatment strategies has become increasingly replaced by a new approach, the therapy combining the use of several active substances. This change in priorities is partly due to the limited therapeutic efficacy of mono-component treatment of poly-etiological diseases that have complex pathophysiology, such as cancer, neurodegenerative diseases, diabetes, most chronic diseases, including liver and gall bladder disorders, etc. Another reason is the development of drug resistance in case of mono-component therapy, as well as the side effects of synthetic monopreparations [40–42]. In addition, the development of analytical chemistry and molecular biology techniques has broadened our understanding of the therapeutic targets of many diseases and multicomponent therapeutic approaches. Phytotherapeutic medical systems also use multicomponent herbal remedies in many cases, as numerous studies have proven their superior efficacy compared to single medicinal plants [43, 44].

Herbal preparations in the pathogenetic treatment of hepatobiliary diseases

The use of herbal remedies in the treatment of HBD worldwide is considered as an alternative to existing pharmaceuticals because of the formers’ safety, availability, cost-effectiveness, and therapeutic efficacy [45]. IUCN has proposed the use of about

50000 to 80000 flowering plants for medicinal purposes, many of which are used in the treatment of liver disease, gallbladder and biliary tract ailments. According to the literature, about 35% patients suffering from chronic HBD prefer to use herbal remedies for treatment, and more than 60% use herbal remedies in combination with synthetic drugs [46, 47]. The main directions of phytotherapy for HBD are rehabilitation after acute diseases, treatment of exacerbated chronic diseases, prevention of possible relapses of liver and biliary diseases, restoration of disturbed metabolic processes (in steatosis and steatohepatitis of different etiology, post-cholecystectomy syndrome, etc.), reduction of side effects of chemotherapy, and restoration of reduced overall reactivity of the body due to adverse environmental factors.

According to numerous literature data and our own results, hepatoprotective properties have been found in plants of many families (Fig. 2), most often *Acanthaceae, Amaranthaceae, Asteraceae, Cyperaceae, Euphorbiaceae, Fabaceae, Primulaceae, Rosaceae, Rutaceae, Schisandraceae, Scrophulariaceae, Solanaceae, Utricaceae*, and many others [48–52]. Constituents of medicinal plants with hepatoprotective properties are mainly secondary metabolites produced by the plant against herbivores, phytopathogenic microorganisms, insects and competing plants [53].

The most well-known hepatoprotective plant constituents (Fig. 3) include numerous phenolic compounds, including flavonoids, terpenoids or terpenes, alkaloids and some others [53, 54].

Most publications on the hepatoprotective properties of plant constituents concern polyphenolic compounds. Plant polyphenols have various pharmacological effects on oxidative stress, lipid metabolism, insulin resistance and inflammation, which are the most important pathological processes in the etiology of liver disease [55]. This puts the polyphenols in the spotlight when looking for phytotherapeutic drugs to treat HBD. High content of polyphenols is present in many vegetables (soy, pepper) and fruits (pomegranate, guava, peach), tea and a large group of medicinal plants. Phytophenol compounds include several classes of substances: *flavonoids* (flavones, such as luteolin; flavonols, such as quercetin; flavanone, for example, hesperidin, and flavanonols, including taxifolin), *biflavonoids* or dimers of flavonoids (for instance, bilobetol), *isoflavones* (such as genistein), *chalcones* (e.g., phloretin), *phenolic acids* (among which there are two classes, benzoic and cinnamic acid derivatives). Hydroxybenzoic acids include gallic, ρ-hydroxybenzoic, protocatechuic, vanilla and syringic acids [56]. Hydroxycinnamic acids are more common than hydroxybenzoic and are composed mainly of ρ-coumaric, caffeic, ferulic and sinapinic acids [57], as well as esters of caffeic acid with chlorogenic acid, and 2-hydroxyhydrocaic acid (rosemary acid), capable of hydrolysis and condensed tannins, lignans and stilbenoids.

Antioxidant action is the most important hepatoprotective mechanism of phenolic compounds inherent in a wide range of medicinal plants. Plant phenolic compounds normalize the enzymatic activity of liver cells, maintain the balance of the oxidant-antioxidant system, as well as can selectively activate apoptosis of malignantly transformed cells. The biological activity of plant phenolic compounds depends on the method of their extraction and composition, the dose dependence of the effects of these drugs varies greatly depending on the type of compound and is still under investigation [58, 59].

The polyphenolic plant compounds with hepatoprotective action which attract the most attention are flavonoids, the largest

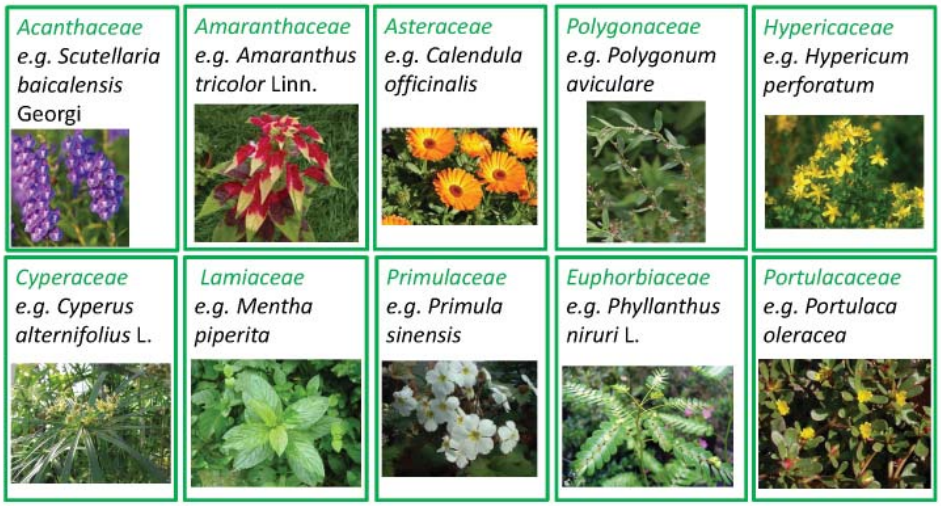

Fig. 2. **Commonly used medicinal plants with hepatoprotective properties**

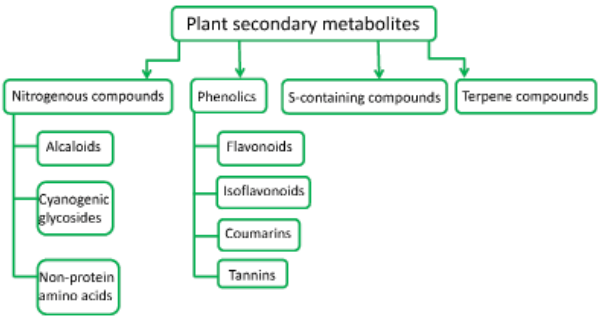

Fig. 3. **Plant secondary metabolites**

class of plant polyphenols. More than 6000 plant flavonoids have been described. Plant flavonoids are characterized by significant structural diversity, high multifaceted biological activity and low toxicity. Herbal flavonoids have a wide range of pharmacological properties: antioxidant, angioprotective, hepatoprotective, choleretic, diuretic, neurotropic, etc. It is medicinal plants containing flavonoid compounds that are considered to be the most promising source for the creation of herbal preparations. In view of this, numerous attempts have been made to systematize and classify plant materials containing flavonoids. One such classification is proposed by pharmacologists of the Samara State Medical University, according to which medicinal plants that accumulate flavonoids are divided into pharmacopoeial plants containing flavonoids as a leading group of BAC (immortelle, pigweed, etc.), pharmacopoeial essential oil plants containing flavonoids (yarrow, peppermint, etc.), pharmacopoeial plants containing bitter compounds and flavonoids (dandelion, plants of the genus *Leonurus*, etc.), pharmacopoeial plants containing saponins and flavonoids (licorice, horse chestnut, etc.), pharmacopoeial plants containing vitamins and flavonoids (calendula, tickweed, rosehips, etc.), pharmacopoeial plants containing simple phenols and flavonoids (sharp-leaf willow, etc.), pharmacopoeial plants that contain tanning substances and flavonoids (bird cherry, blueberries, etc.) and pharmacopoeial plants containing alkaloids and flavonoids (celandine, etc.) [60].

One of the most widely used and thoroughly researched herbal hepatoprotective flavonoid drugs are flavonoid lignans of *Silybum marianum*, known as silymarin and characterized by distinct antioxidant, cytoprotective and anticarcinogenic properties. In addition, the antiviral properties of silymarin have been reported in patients with viral hepatitis. Silymarin has also been shown to be effective in patients with non-alcoholic liver steatosis, where it has a potent antioxidant effect, stabilizes hepatocyte membranes, and restores mitochondrial function [61, 62]. The membrane-stabilizing effect of the thistle flavonoids is also due to the fact that silibinin, the basic BAC of silymarin, is able to interact directly with hepatocyte membranes [63].

Catechins, the flavonoid components of green tea, can stimulate the synthesis of antioxidant defense enzymes, such as glutathione transferase (GT) and superoxide dismutase (SOD), thereby realizing their hepatoprotective effect [64].

Luteolin, a flavone of weld, can enhance the activity of antioxidant defense enzymes and modulate the synthesis of xenobiotic metabolizing enzymes [65].

Genistein, a legume isoflavone, is capable of regulating NFκB-dependent signaling and thus influences the synthesis of many inflammatory mediators. The flavonone naringenin, found in many citrus fruits, as well as flavonol quercetin, are capable of the same effect [55]. Polyphenolic compounds such as resveratrol (stilbenoid), curcumin etc. can activate apoptosis of malignantly transformed cells, including liver carcinoma cells, possess antifibrotic properties, activate numerous signaling cascades involved in the regulation of lipidoximidoxide oxidase [66].

It is also traditional to use glycyrrhizin (saponin, contained in the roots of licorice) to treat pathologies of hepatobiliary system. This herbal remedy has been used for many years in disorders of liver function associated with obesity, as well as in the treatment of non-alcoholic liver steatosis. Numerous animal models have shown that glycyrrhizin is capable of reducing hepatic lipogenesis, has antioxidant activity, and restores insulin sensitivity [67, 68].

Terpenoids are divided into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes and politerpenes. Terpenoids are widespread in medicinal plants and are part of essential oils and resins. Terpenoids include saponins, steroid compounds, triterpene bitternesses, carotenoids, rubber and gutta-percha. The ether-bearing plants that accumulate terpenoids are representatives of *Lamiaceae, Cupressaceae, Pinaceae, Apiaceae, Brassicaceae, Myrtaceae, Asteraceae and Rosaceae*. Essential oils can accumulate in flowers, fruits, roots and wood of plants. Plant bitternesses are represented mainly by monoterpenoid iridoid

glycosides and accumulate in *Valerianaceae, Lamiaceae, Scrophulariaceae, Plantaginaceae*, etc. Sesquiterpene bitternesses are found in yarrow and wormwood. The most common plants containing terpenoids are anise, mountain arnica, birch, valerian, goldenrod, coriander, medicinal dandelion, oregano, peppermint, sage medicinal and some others [69]. Powerful hepatoprotective properties are observed in tetracyclic terpenic saponins such as dammarane (found in *Apiaceae*), derivatives of lanostane and cycloartan (in *Ranunculaceae*), cucurbitan (accumulates in *Cucurbitaceae*), as well as pentacyclic terpene glycosides, which are found in *Lamiaceae* and have the lowest level of hepatotoxicity [70].

Much attention has been paid in recent years to herbal terpene hepatoprotective preparations with andrographolide, a labdane diterpenoid, which is the main biologically active component of the medicinal plant *Andrographis paniculata* and known a "king of bitterness" for its exceptional taste. In addition to hepatoprotective properties, this phytoconstituent has powerful antioxidant properties, antidiabetic, antiviral, antibacterial, antimalarial and antiatherosclerotic effects. It is considered by experts in the field of pharmacology as a substrate for a number of preparations with anti-inflammatory properties [71, 72].

As for plant alkaloids, hepatoprotective activity has been reported for phylantine, a compound from *Phyllanthus niruri*, plant of the family Phyllanthaceae. It is capable of antioxidant activity and in animal model studies has demonstrated the ability to restore enzyme homeostasis [73]. One of the most famous barberis alkaloids, berberine, is also used in the treatment of HBD and has choleretic and antispasmodic effects. This alkaloid lowers the viscosity of bile and promotes bile excretion, reduces the tone of the gallbladder smooth muscle, and reduces the amplitude of its contractions [74, 75].

For the treatment of disorders of the biliary system, the most commonly used preparations are herbal choleretic drugs, which are divided into choleretics (enhancing the formation of bile by the liver), cholekinetics (stimulating the reduction of the gallbladder) and spasmolitics (increasing the excretion of bile by removing spasm of bile ducts) [76]. To date, more than 100 medicinal plants whose preparations can be used as choleretics have been described in the literature. Medicinal plants with choleretic properties can be divided into the following groups by the mechanism of action: 1) medicinal plants with choleretic properties (cumin, yarrow, calendula, mint, corn stalks, roots and stalks of dandelion and rose, etc.); 2) medicinal plants with anti-inflammatory action (plants of the genus *Hypericum*, buckthorn, chamomile, yarrow, nettle, etc.); 3) medicinal plants used against the biliary tract dyskinesia of hypertonic type (valerian, belladonna, chamomile, barberry, etc.); 4) medicinal plants used in dyskinesia of the biliary tract of hypotonic type (peppermint, thyme, tansy, etc.) [77].

It is quite difficult to classify medicinal plants that affect only the liver or only the bile ducts. The reason is that each plant affects several components of the hepatobiliary system. There are plants that affect mainly the liver parenchyma, others may affect mainly the excretion of bile, some have mainly antispasmodic effect. On the other hand, there are medicinal plants with choleretic and cholelitic action, which in addition have a bacteriostatic or bactericidal effect, thus they can be used as complementary preparations in the treatment of cholangitis and cholecystitis [78].

To conclude that review on the use of medicinal herbs in the pathogenetic treatment of HBD, it should be noted that phytotherapy in case of chronic liver and biliary tract diseases usually lasts for several months. Usually, complex herbal remedies are utilized containing multiple medicinal plants with synergistic and/or additive action, or several different medicinal plants are used sequentially [79]. The principal hepatoprotective mechanisms of herbal remedies are mainly to restore normal levels of liver enzymes in serum, including alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and enzymes of antioxidant protection, as well as total bilirubin and total protein levels. The choleretic influence of medicinal plants includes effects on both the formation and secretion of bile [80].

Prospective directions in the search for new herbal hepatoprotective agents are the creation of medicinal plants compositions that contain BAC complementing each other without causing toxic effects.

Immunomodulatory effects of medicinal plants

One of the most important mechanisms of hepatoprotective action of medicinal plant preparations is the influence of their constituents on various components of the inflammatory response. The ability to modulate immune reactivity is one of the most significant manifestations of the biological activity of herbal remedies. The

function of the immune system is closely linked to the general state of human health, so the pathogenesis of many diseases involves disorders of the immune reactivity. Local and systemic inflammation is an important component of the pathophysiology of diseases of the hepatobiliary organs [81–84] (Fig. 4). However, it is still rare to incorporate medicinal plants with immunomodulatory properties into polyherbal remedies in medical practice. It is more common in experimental studies [85–87]. In the modern society, there is a significant proportion of persons of working age with compromised immune reactivity, that is, with abnormally functioning effector mechanisms of both innate and adaptive immunity [88–90]. Immune reactivity disorders, associated with many diseases (including HBD), as well as the compromised immune system of otherwise healthy individuals require the development of safe and effective means of immunocorrection. Existing synthetic immunocorrectors (immunomodulators) have low therapeutic and prophylactic efficacy and cause a number of side effects, which, for instance, can adversely affect the functioning of some parts of the immune system. Thus, particular attention in recent years has been paid to the study of natural immunomodulators of microbial, animal and plant origin [91, 92].

Phytoconstituents of medicinal plants can exert immunosuppressive, immunostimulatory and homeostatic (normalizing) immunomodulatory effects. In addition, herbal remedies enhance the efficacy of the anti-infective chemotherapeutic agents in the treatment of infections [93, 94]. Due to immunostimulatory properties, herbal remedies have long been used as vaccine adjuvants [95]. In particular, the extracts of *Azadirachta indica* leaves and of ginseng root exhibit adjuvant activity in the composition of antitumor vaccines compared to those of the complete and incomplete Freund's adjuvant [96]. The most famous herbal adjuvants are saponins. Many of these biologically active substances are found in edible crops such as potatoes (α-solanine, α-chaconin) and tomatoes (α-tomatine). The ability of plant saponins to stimulate cellular immune responses is now considered in the prospect of developing so-called edible vaccines, a type of mucosal vaccines that are considered as an alternative to injected vaccines [97]. Vegetable proteins, such as carbohydrate-binding lectins, have adjuvant activity comparable to that of cholera toxin, which has been demonstrated in animal models using mucosal vaccines for intranasal administration [98]. The adjuvant action of the abovementioned herbal preparations is based on their ability to activate nonspecifically the functions of the immune system cells involved in the initiation of the immune response, such as macrophages, monocytes, and neutrophils. Genetically modified plant organisms are also used for the creation of antitumor vaccines. The extracts of such plants contain targeted tumor-associated and tumor-specific antigens, which are used not only to manufacture antitumor vaccine preparations, but also for the development of diagnostic test systems in oncology. Vaccines based on such extracts contain not only the transformed plant-targeted protein or DNA, but also the adjuvant biologically active substances synthesized by the plant [99, 100].

Echinacea is one of the herbs that have been used for a long time to restore compromised immune reactivity, including in childhood. The preparations of all existing species, *Echinacea angustifolia*, *Echinacea purpurea* and *Echinacea pallida*, are used to enhance suppressed immune reactivity. Echinacea preparations enhance the cytotoxic activity of macrophages and natural killer cells, activate the metabolism of neutrophils, stimulate the synthesis of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-12, etc. [101, 102]. Extracts of root and aerial plant parts of *Echinacea purpurea* can significantly enhance the antigen presenting activity of dendritic cells by activating

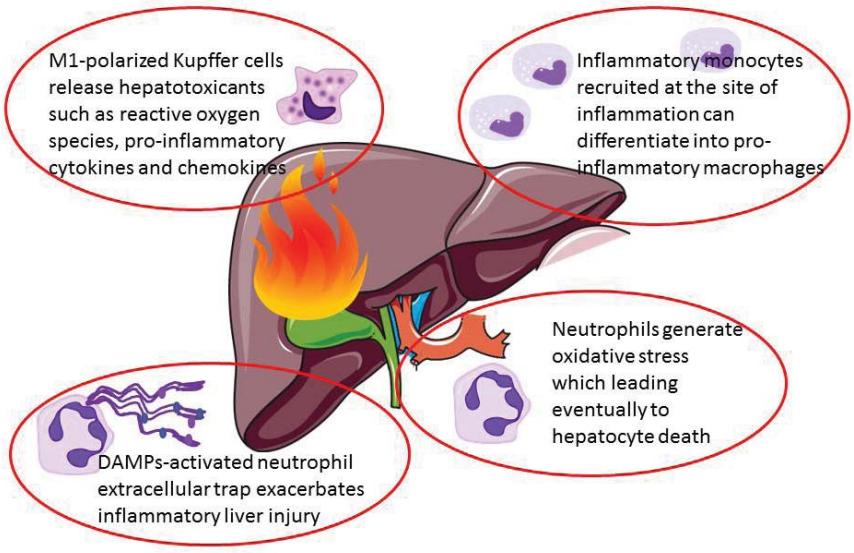

Fig. 4. **Innate immune cells in liver and gallbladder inflammation**

JNK, p38-MAPK and NF-κB-dependent signaling pathways [103]. Another example of the oldest used plants, whose preparations are characterized by a powerful immunostimulatory action, is garlic (*Allium sativum*). Garlic is called a "plant antibiotic" because of its antiseptic properties and anthelmintic effect. Garlic extracts contain more than 200 biologically active constituents, including about 33 sulfur-containing compounds, numerous enzymes, amino acids and minerals, for example, selenium. Aqueous extract of garlic has a dose-dependent stimulatory effect on the leukocyte oxidative metabolism and enhances the proliferative activity of T cells. Lectins, contained in raw garlic, affect the isotype switching of immunoglobulins, reducing IgE synthesis while enhancing IgG and IgM production. Garlic preparations (mainly aqueous fresh extracts and tinctures) are used to overcome stress-induced immunosuppression [104–106]. The immunostimulatory properties of ginseng (*Panax ginseng*) are also widely used. The saponins and glycosides from this plant have powerful adaptogenic properties. Ginseng extracts enhance the migration ability of leukocytes, including macrophages and T-cells, stimulate the synthesis of proinflammatory cytokines and plant factors such as IL-1, IL-2, TNF-α, granulocyte-macrophage colony-stimulating factor, etc., and enhance B-cell antibody generation and mitogen-induced proliferative activity of lymphocytes. Ginseng-based dietary supplements enhance vaccination efficacy by acting as mucosal adjuvants [107–109]. Powerful immunostimulatory properties are described for *Sambucus nigra*. Syrups and aqueous extracts of its berries, as well as teas based on it, stimulate the leukocyte migration to the foci of infection and increase the functional activity of myeloid cells in the acute phase of inflammation [112]. To enhance the suppressed immune reactivity, different forms of preparations can be used. They may contain extracts of different parts of one plant, selected individual BAC and their mixtures, as well as polyherbal phytopreparations, which include extracts or phytoconstituents of several plants. A wide range of commercial phytopreparations with immunostimulatory activity is introduced into the medical and veterinary practices, among which polyherbal compositions are predominant, with therapeutic and prophylactic action based on the synergistic action of BAC of various medicinal plants. Here are several examples of such drugs:

• ImmuPlus^{R} — polyherbal veterinary immunostimulatory drug comprising four medicinal plants: *Ocimum sanctum*, *Tinospora cordifolia*, *Emblica officinalis* and *Withania somnifera* [111].

• Echinacealiquid^{R} — polycomponent syrup, which includes extracts of three species of echinacea. Used to restore immune reactivity suppressed by prolonged infectious processes, etc. [112].

• Sambucol^{R} — a preparation, 38% of which is made up by a standardized elderberry extract, and the rest are polyphenolic compounds of other medicinal plants. It is used to enhance immune reactivity in patients with viral infections [113].

Many of phytopreparations have immunosuppressive activity, the action of which is aimed at controlling the inflammatory activation of the immune system. Often, a systemic inflammatory response syndrome (SIRS) may develop in the case of many diseases of inflammatory etiology, both infectious in nature (bacterial, viral, fungal diseases and infectious processes of mixed etiology), and aseptic inflammatory diseases (rheumatoid arthritis, diseases of the hepatobiliary system, metatabolic syndrome, sugar mellitus, gout) if the inflammatory process is generalized. SIRS is accompanied by cytokine storm which is the high level of synthesis of anti-inflammatory cytokines, possibly dangerous to the patient's life. The cytokine storm is particularly characteristic for viral infections caused by flu virus. The cytokine storm in this case is most often the cause of lethality [114]. Numerous studies have revealed the high efficiency of multicomponent herbal remedies in overcoming cytokine storms, especially in infectious diseases. The authors of these publications convincingly prove the synergistic effect of phytoconstituents of various medicinal plants in inhibiting the proinflammatory immune response and stimulating restorative, homeostatic immune responses [115]. As noted above, the vast majority of effective herbal immunomodulators are multicomponent drugs, and physicians that practice phytomedicine convincingly prove that the synergistic or additive effects of BAC of different parts of one plant or several in the composition of a complex preparation are fundamentally important for the drug's immunomodulatory activity and therapeutic efficacy. However, the evidence base for this assertion is still insufficient and requires an in-depth study of the immunomodulatory properties of complex herbal preparations with the ability to modulate immune reactivity to optimize their use in the complex treatment

of human pathology. The ancient recipes of multicomponent phytocompositions that have been used for a long time in Traditional Chinese Medicine or Ayurvedic practice have only recently been subjected to an analysis of their high efficiency mechanisms. In particular, the immunomodulatory effect of a mixture of black pepper (*Piper longum*) and ginger (*Zingiber officinalis*) is based on the ability of the piperine alkaloid contained in black pepper to increase the bioavailability of ginger phytoconstituents. The result of the high immunomodulatory efficacy of multicomponent herbal remedies can be more than a synergy (a more pronounced effect in the case of a combination of preparations in comparison with individual use). In some cases, the phytoconstituents of one of the components of the multicomponent preparation help to preserve the nativeness and biological activity of the other. For example, the antioxidant BAC contained in large quantities in valerian, garlic, or ginger extracts can help to preserve the integrity of the BAC of combined medicinal plants. Studies of ancient multicomponent phytopreparations have shown that their immunomodulatory activity is completely or substantially lost when they are fractionated or used in separate components. The mechanisms of high immunomodulatory activity of combinations of medicinal herbs such as bell pepper (*Piper methysticum*) and valerian (*Valeriana officinalis*), ginseng and ginkgo, are still under investigation [116, 117].

An example of a plant whose preparations have long been used in medical practice to control inflammation is *Glycyrrhiza glabra*. Phytochemical analysis of licorice preparations, carried out by numerous scientific groups, proved that the phytoconstituents responsible for the anti-inflammatory action of its preparations are saponins, flavonoids and pectins. Licorice preparations inhibit phospholipase A, enhance the synthesis of IL-10 and other anti-inflammatory cytokines, stimulate differentiation of regulatory T-cells, etc. [118, 119].

Curcuma longa L. also has potent anti-inflammatory activity. One of the mechanisms of action of turmeric BAC-based preparations is the inhibition of cyclooxygenase-2 and the stimulation of cytokine shift toward the predominance of Th2 type cytokines [120]. A herb with a pronounced anti-inflammatory effect is *Zingiber officinale*. More than 400 BAC have been found in ginger root, of which about 70% are carbohydrate compounds. Aqueous extracts of ginger root inhibit the lipoxygenase and cyclooxygenase activity of leukocytes, enhance the synthesis of anti-inflammatory and immunoregulatory cytokines, such as transforming growth factor-β (TGF-β) and IL-12. Dietary supplements which include ginger are effective in the complex treatment of peptic ulcer disease. It should also be noted that ginger preparations are capable of controlling Th2 inflammation which is characteristic to an allergic pathology [121]. The anti-inflammatory properties of *Nigella sativa*, which has only recently been adapted to cultivation in Ukraine, have been used for more than 2000 years. The anti-inflammatory immunomodulatory action of preparations and supplements based on this plant is realized due to the presence of polyunsaturated fatty acids in its composition, which inhibit the induced oxidative metabolism of mononuclear phagocytes, reduce the synthesis of eicosanoids and enhance production of anti-inflammatory and immunoregulatory Th2-profile cytokines [122]. Anti-inflammatory immunomodulatory activity is characteristic of herbal remedies based on cinnamon and aloe vera, hibiscus and calendula, chamomile, plants of the genus *Hypericum* and many other medicinal plants.

The anti-inflammatory immunomodulatory action is realized in phytoconstituents of medicinal plants due to numerous different mechanisms. For example, alkaloids of *Corydalis turtschaninovii* Besser are able to inhibit the phosphorylation of ERK and p38, resulting in the inhibition of NFκB-dependent signaling pathways and the reduction of the synthesis of proinflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-6, IL-1β, etc. The essential oils of many medicinal plants are able to interfere with MAPK-dependent signaling cascades, thus inhibiting the synthesis of proinflammatory cytokines, prostaglandins, and reactive oxygen species by leukocytes, and adversely affect the migration of myeloid and lymphoid cells. Flavonoid compounds of medicinal plants also inhibit NFκB signaling pathways, activate PPAR transcription factors involved in the synthesis of anti-inflammatory mediators, prevent the formation of synapses between cells of the immune system through inhibition of leukocyte synthesis of molecules of intercellular adhesion. Thus, activation of transcription factors, such as NF-κB, ERK, and STAT3 involved in the proinflammatory immune response, is inhibited by plant stilbene and terpenoids [123].

The majority of studies on the molecular basis of the effect of phytopreparations on immune reactivity relates

to the immunomodulatory action of plant polyphenolic compounds in their composition. Polyphenols are well-known, pharmacologically active compounds with immunomodulatory activity [124]. This category includes flavonoids, phenolic acids and stilbenoids, which are universally formed in plants and exist as free aglycones (non-carbohydrate glycoside fragments) or in the esterification state of glucose and other carbohydrates (glycosides) [125]. Absorbed polyphenols are stable in the conditions of gastrointestinal digestion and interact, first and foremost, with the immune system of the intestinal mucosa, initiating both local and systemic immunomodulatory effects [126].

Decades of research into polyphenols have led to several conclusions regarding their effects on immune system function. Each type of polyphenol binds to one or more immune system cell receptors and thus triggers intracellular signaling pathways that ultimately regulate the host immune response. Phytopreparations and dietary supplements which contain plant polyphenols can modulate the immune response by affecting epigenetic mechanisms such as regulatory DNA methylation, histone modification, and microRNA-mediated posttranscriptional repression that alters the expression of genes encoding key immune factors [127].

Immune system cells express a number of polyphenolic receptors. For example, epigallocatechingallate (EGCG), which is found in large quantities in different varieties of tea, in apples, plums, etc., can interact with three different cellular receptors: the 67 kDa laminin receptor (67LR), associaited with 70 kDa protein chain (ZAP-70) and with RIG-I cytosolic pattern recognition sensor [128, 129]. Of these, 67LR is expressed by neutrophils, monocytes / macrophages [130, 131], mast cells and T cells [132, 133] and regulates the adhesion and inflammatory responses of these cells. RIG-I downstream signaling pathways trigger interferon synthesis [134]. Toll-like receptor (TLR) 4, antigenic T-cell receptor (TCR) $\alpha\beta$, and IgM- (sIgM-) B-cell receptor are receptors for baicalin (a flavone glycoside, contained in large amount in *Scutellaria* plants) on T and B cells. By regulating these receptors, baikalin may affect innate and adaptive immunity responses [135]. In studies with laboratory animals, daidzein and a few other phytoestrogens have been shown to modulate immune cell function by interacting with estrogen membrane and intracellular receptors [136].

Polyphenolic compounds can suppress the dendritic cell (DC) function under conditions of their inflammatory activation. In particular, daidzein (isoflavone contained in soybeans, cereals, and certain medicinal plants), silibinin (flavono-lignan of milk thistle), fisetin (flavonol), epigenin and baikalin can inhibit functional maturation of DC, stimulated by bacterial lipopolysaccharide (LPS): decreasing the expression of histocompatibility molecules of class II and costimulatory molecules [137, 138]. Inhibition of the proinflammatory activation of DC is also characteristic of curcumin and some other phytophenols [139]. The inhibitory effect of phytophenols on the proinflammatory activation of DC is inhibition of the adaptive Th1 immune response. Plant polyphenols, such as daidzein, enotelin B (polyphenol isolated from fireweed and other medicinal plants), activate the functions of $\gamma\delta$T cells and natural killer cells, enhancing IFN-γ synthesis and increasing the expression level of intercellular CD69 molecules (CD25, CD69) [140, 141]. Alcoholic extracts of plants of the milkweed family with high content of biflavonoids enhance the production of antibodies by B cells *in vitro* and *in vivo* [142]. A similar effect was reported for quercetin and its derivatives.

The differentiation of naive T cells and their production of cytokines can be enhanced by numerous phytophenolic compounds. In particular, plant flavones, catechins and flavonones inhibit the synthesis of cytokines involved in the activation of isotype switching of B lymphocytes to IgE synthesis and thus have the ability to suppress allergic inflammatory responses [143, 144]. Contact hypersensitivity reactions are inhibited by the same mechanism by phytoflavones of *Artemisia vestita*, ginkgo, and many other medicinal plants. It should be noted that phytophenols have a general ability to induce a shift of the cytokine profile in serum and other biological fluids from the Th1 profile to the Th2 profile, which is characterized by the activation of humoral immunity reactions, inhibition of inflammatory reactions and activation of reparative processes [145, 146]. Due to this immunomodulatory action, phytophenols activate isotype switching of B cells to IgG and IgM synthesis while inhibiting the synthesis of immediate-type allergic reagins, IgE and IgA. Changes in the local and systemic cytokine profile due to the action of plant polyphenols are characterized by a decrease in IL-1β, IL-6 and TNF-α levels, which are a triad of major cytokines that initiate, support and enhance

inflammation, as well as the synthesis of IL-17, one of the major mediators of autoimmune inflammation. As a rule, the synthesis of IL-4 is enhanced, which is involved in the regulation of antibody production by B-cells [147–149]. It should be noted that phytophenols also have a bimodal dose-dependent modulatory effect on the synthesis of some cytokines. For example, the synthesis of IL-2 that regulates T-cell proliferation can both be enhanced and inhibited by the action of plant phenolic compounds. A peculiarity of the stimulatory effect of phytophenols on the synthesis of this cytokine is the simultaneous enhancement of differentiation of helper type 2 T cells, which inhibit the inflammatory responses of adaptive immunity. The bimodal modulatory effect of phytophenols has also been reported in relation to the synthesis of IL-12, a cytokine whose main source is macrophages, and with a function to stimulate Th1-immune responses. The synthesis of this cytokine by non-sensitized intact macrophages is slightly enhanced in the presence of phytophenols. However, treatment of activated LPS macrophages by phytophenol compounds causes inhibition of their production of IL-12 [150, 151].

The inhibition of inflammation by phytophenols is also achieved by activating differentiation of T-regulatory cells and enhancing their synthesis of immunoregulatory cytokines [152].

Plant polyphenols have the most pronounced modulatory effect on the function of mono- and polymorphonuclear phagocytes (macrophages and neutrophils, respectively). Phytophenols mainly affect MAPK-dependent and NFκB-dependent signaling of these cells. The consequence of this effect is a shift of phagocyte metabolism toward an anti-inflammatory phenotype with synthesis of immunoregulatory cytokines, a decrease in the production of inflammatory metabolites (reactive oxygen and nitrogen forms, eicosanoids, phagolysosome components and enzymes in the cytoplasmic granules of neutrophils). The restorative and reparative processes in tissues are enhanced. That metabolic polarization of phagocytes also has another consequence: inhibitory effect on the inflammatory responses of adaptive immunity effectors, Th1 helper cells [153–155]. These effects are characteristic for phytophenols of plants of the genus *Acanthaceae, Euphorbiacea, Clusiaceae*, and some others.

Perspectives of using phytopreparations in treatment of diseases of hepatobiliary system

The dominant paradigm in the development of medicinal preparations is the concept of constructing optimally selective ligands to influence individual therapeutic targets. However, advances in systemic biology have convincingly shown that selective compounds exhibit less clinical efficacy than multifunctional preparations. Hence, a new approach to the development of medicines occurred, and a one-drug-one-disease treatment strategy is increasingly replaced by the use of combination therapy with several active substances. Such a change in priorities is partly due to the limited therapeutic efficacy of mono-component treatment in the treatment of polyetiological diseases with complex pathophysiology, including HBD. Another reason is the formation of drug resistance to the factors of single-component therapy, as well as the side effects of synthetic monopreparations [41, 42]. In addition, the development of analytical chemistry and molecular biology techniques has broadened our understanding of the therapeutic targets of many diseases and multicomponent therapeutic approaches. Advances in these fields of science form the basis for the following paradigm in drug development: network pharmacology, an interdisciplinary science based on pharmacology, network biology, systems biology, bioinformatics, and other related scientific disciplines. Network pharmacology is aimed at understanding the network interactions between a living organism and the preparations that affect its normal and abnormal functions. This scientific approach aims to use the pharmacological mechanism of action of a medicinal product in a biological network with well-defined therapeutic targets and to enhance the therapeutic efficacy of the drug [156–158].

The scientific principles of network pharmacology are also used in PT, in particular, to create an evidence base on the efficacy of Traditional Chinese Medicine [159, 160]. Phytotherapeutic medical systems in many cases use multicomponent herbal remedies, because numerous studies have proved their higher efficacy compared to the use of individual medicinal plants due to the multi-purpose, synergistic and additive effects of phytoconstituents [43, 44]. Synergy, by definition, is the interaction of two or more agents to produce a combined effect that exceeds the sum of the individual effects of the individual components [161]. Spinella et al. (2002) proposed the classification of synergies into two categories: pharmacodynamic and pharmacokinetic

[162]. In the first case, two or more agents act on the same receptor structures or biological targets, which increases the effect compared to the action of the individual components. Pharmacokinetic synergy happens if the components of a complex preparation interact during the pharmacokinetic processes: absorption, distribution, metabolism of elimination etc. Unlike a synergistic effect, which is the sum of the action of two or more components that exceed their effect on self-administration, an additive effect is a set of effects of components of a combined preparation that do not interact and do not affect the effects of each other [163, 164]. Literature data indicate the synergistic effect of phytochemical components of multicomponent phytoteas [165], synergistic and additive effect of combined extracts of medicinal plants and their essential oils with antibacterial and antiviral effects [166, 167], synergistic anti-inflammatory effect of combined phytochemicals [167, 168]. The future is undoubtedly in the use of therapeutic agents based on medicinal plants for the treatment of HBD by multicomponent herbal remedies. Particular attention should be paid to the introduction of medicinal plants with immunomodulatory properties into the composition of such preparations for directed effect on the immune system, which is one of the integral physiological systems with protective and regulatory effects. An important question is the principle of the arrangement of medicinal plants in the composition of multicomponent herbal preparations. To date, the choice of components of complex phytopreparations is based mainly on knowledge of the biological action of individual medicinal plants. At the same time, a new scientific direction in phytomedicine is being developed, known as phytomix (metabolome analysis for each of the components of the complex phytopreparation, taken separately and in combination with other components, along with the analysis of the correlative relationship between the phytome composition and the desired effect on the biological target) [169, 170]. Phytomix allows evolving from the empirical approach to polyherbal compositions to a scientifically sound creation of complex phytopreparations. To date, several mechanisms of synergistic action of complex phytopreparations have been deciphered: activation or inhibition of signaling by the same receptors; regulation of enzymes and transporters involved in liver and intestinal metabolism to influence the bioavailability of plant BAC; complex influence on factors of formation of drug resistance of target cells; neutralization of side effects of some BAC by the action of others, etc. [171]. Investigation of the synergistic effects of polyherbal compositions will not only facilitate the creation of new complex phytopreparations, but will also reveal the negative synergism between BAC from different medicinal plants and thereby achieve their maximum therapeutic efficacy.

Thus, herbal remedies have been and remain effective, safe and, therefore, promising drugs for the treatment of diseases of the hepatobiliary system. Most promising approach for the development phytoremedies for the treatment of liver and biliary ailments is the use polyherbal formulations combining hepatoprotective and immunomodulatory potentials. Nevertheless, it is necessary to point that current use of herbal medicines in the complementary and altrnative treatment of hepatobiliary disorders is mostly rooted in experience and observation. Metodological approaches of modern evidence-based phytotherapy are needed to increase and proof of efficacy and safety of hepatoprotective and immunomodulatory phytoremedies.

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ЗАСТОСУВАННЯ ФІТОПРЕПАРАТІВ У ЛІКУВАННІ ПАТОЛОГІЇ ГЕПАТОБІЛІАРНОЇ СИСТЕМИ: ТЕНДЕНЦІЇ ТА ПЕРСПЕКТИВИ

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Захворювання органів гепатобіліарної системи становлять важливу медико-соціальну проблему у зв'язку з дедалі зростаючими показниками морбідності в усьому світі. Хвороби печінки та біліарної системи характеризуються комплексною патофізіологією і можуть супроводжуватися розвитком супровідних патологічних станів. Їх лікування потребує застосування багатоцільових препаратів, біологічна дія яких спрямована на різні терапевтичні мішені. Ефективність лікарських засобів для терапії розладів гепатобіліарної системи на сьогодні є низькою, а частота побічних ефектів — достатньо високою. Це актуалізує пошук і розроблення високоефективних гепатопротекторів з низькою частотою побічних ефектів. Джерелом таких препаратів можуть бути лікарські рослини. В огляді узагальнено дані стосовно механізмів гепатопротекторної та імуномодуляторної дії лікарських рослин та їхніх фітоконституєнтів. Окреслено перспективи розробки та застосування фітопрепаратів у лікуванні захворювань органів гепатобіліарної системи.

Ключові слова: захворювання органів гепатобіліарної системи, лікарські рослини, гепатопротектори, імуномодулятори.

ПРИМЕНЕНИЕ ФИТОПРЕПАРАТОВ В ЛЕЧЕНИИ ПАТОЛОГИИ ГЕПАТОБИЛИАРНОЙ СИСТЕМЫ: ТЕНДЕНЦИИ И ПЕРСПЕКТИВЫ

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Заболевания органов гепатобилиарной системы представляют важную медико-социальную проблему в связи с возрастающими показателями морбидности во всем мире. Болезни печени и билиарной системы характеризуются комплексной патофизиологией и могут сопровождаться развитием сопутствующих патологических состояний. Лечение таких заболеваний требует применения многоцелевых препаратов, биологическое действие которых направлено на различные терапевтические мишени. Эффективность существующих на сегодняшний день лекарственных средств для терапии расстройств гепатобилиарной системы остается низкой, а частота побочных эффектов — достаточно высокой. Это актуализирует поиск и разработку высокоэффективных гепатопротекторов с низкой частотой побочных эффектов. Источником таких препаратов могут быть лекарственные растения. В обзоре обобщены данные, касающиеся механизмов гепатопротекторного и иммуномодулирующего действия лекарственных растений и их фитоконституэнтов. Очерчены перспективы разработки и применения фитопрепаратов в лечении заболеваний органов гепатобилиарной системы.

Ключевые слова: заболевания органов гепатобилиарной системы, лекарственные растения, гепатопротекторы, иммуномодуляторы.

EXPERIMENTAL ARTICLES

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PRODUCTION OF MAGNETICALLY CONTROLLED BIOSORBENTS BASED ON FUNGI *Agaricus bisporus* AND *Lentinula edodes*

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The aim of the study was to produce magnetically controlled biosorbent based on fungi of champignon and shiitake, to determine the proportion of the magnetically controlled phase of the biomass of fungi when the magnetic fluid (MF) was added to the substrate and to explore the efficiency of extraction Fe^{3+} ions by shredded biomass of the fungus. The object of the study was mushrooms champignon *Agaricus bisporus* and shiitake *Lentinula edodes* grown in the laboratory. An effective and cheap way to remove waste biosorbent from the working environment is a high-gradient magnetic separation (HGMS), which takes place in high-speed mode. The separation of the magnetically controlled phase of fungi biomass *A. bisporus* and *L. edodes* was carried out by HGMS methods. It was investigated that when using the biomass of champignon grown on MF, the properties of the sorbent were significantly improved, the full saturation was 6 times faster in comparison with the biosorbent based on the biomass of the fungus grown without MF.

Key words: biogenic magnetic nanoparticles, magnetite, magnetically controlled biosorbent, champignon *Agaricus bisporus*, shiitake *Lentinula edodes*.

The interest in the biosynthesis of iron-containing biogenic magnetic nanoparticles (BMNs) is associated with their ferrimagnetic properties. BMNs are the subject of intense research when they were first discovered in magnetotactic bacteria (MTB) [1]. BMNs are found in organisms that belong to all three domains: prokaryotes, archaea and eukaryotes [1–11]. BMNs has been experimentally detected in algae and protozoa [12], worms [7], chitons [9], snails [13], ant and butterflies [14–16], honey bees [5, 15], termites [6], lobsters [17], tritons [18], migratory and non-migratory fish [8, 19–24], turtles [10, 25], birds [26–29], bats [30], dolphins and whales [31], pig [32], humans [33–35], plants [36, 37] and mushrooms [37–39].

It was found that the mechanism of biomineralization of BMN is the same for all living organisms [40–42].

All species are potential producers of BMNs among investigated representatives of the divisions of fungi the Ascomycetes (Ascomycota) and Basidiomycetes (Basidiomycota), which decoded the genomes of more than 50% in the database GenBank NCBI as it was proved by the methods of comparative genomics. At the same time, experimental studies of BMNs in fungi samples *A. bisporus* and *L. edodes* by means of methods atomic force microscopy (AFM) and magnetic force microscopy (MFM) showed that the BMNs in the fungi form chains that are localized on the walls of hyphae of samples of fungi. In recent decades, the search for sorbents of biological origin has become one of the most promising areas of problem solving for combating pollution with heavy metals in the environment. Heavy metals are elements of pollution from transport and many enterprises

in various industries. These metals entering into the human body cause poisoning and lead to serious disruption of metabolic processes and vital body functions [43].

And it is known [43–45] that the fruiting bodies of macromycete fungi (*Boletus edulis* (penny bun), *Ganoderma lucidum* (lingzhi mushroom), *Calvatia excipuliformis* (handkea excipuliformis), *Paxillus involutus* (brown roll-rimy), *Tricholoma terreum* (grey knight), *Armillaria mellea* (honey fungus) are natural and safe sorbents of heavy metal ions, dyes and pesticides.

Chitin is the only polysaccharide that contains nitrogen atoms and has uniquely high sorption properties. Fungi were chosen as the main active agent for the production of magnetically controlled biosorbent, because of the high content of chitin in the cell wall [43, 44].

Fungi can accumulate high concentrations of heavy metals [46–50]. Zinc (Zn), copper (Cu), manganese (Mn), lead (Pb), chromium (Cr), mercury (Hg), cadmium (Cd), nickel (Ni) and iron (Fe) can be accumulated in the largest quantities in fruiting bodies and the mycelium of many species of fungi [43, 44, 51].

However, the problem of removing metal-saturated biosorbent from the working solution remains relevant. The known method — filtering through a filter-paper, is quite long and inefficient [52, 53]. Therefore it is important to find a more efficient way to extract metal-saturated biosorbent from the working solution. Such a cheap and effective method is high-gradient magnetic separation (HGMS) [54], which operates in a high-speed mode.

Therefore, the aim of the work is to obtain a magnetically controlled biosorbent based on champignon and shiitake, the fraction of the magnetically controlled phase of the biomass of the studied fungi when added to the substrate magnetic fluid (MF) and study the efficiency of the extraction of Fe^{3+} ions by the biomass of the fungus *A. bisporus*.

Materials and Methods

Agaricus bisporus and *Lentinula edodes* were grown according to the standard method [55, 56].

Champignons and shiitake, were grown on medium with the addition of MF (magnetite — iron oxide Fe_3O_4), using a concentration MF of 0.1 mg/ml, which is close to the content of magnetite in soils [57–59] and 1 mg/ml, to study the characteristics of sorbents from the biomass of fungi.

Preparation of fungus biomass for high gradient magnetic separation includes the following steps: drying of a fresh fungus in a oven at t = 60 °C to a constant mass, grinding dry biomass using an electric mill for 1–5 min, sifting the biomass of fungi through a sieve with a cell diameter of 0.5 mm.

Suspensions were prepared for HGMS by mixing dried and crushed biomass of *A. bisporus* and *L. edodes* fungi with water, so that the ratio of the mass of the biosorbent to the water mass was 1: 200 (1 g per 200 ml of water). It is optimal concentration of mushroom/water, because its increase causes the grinding of a ferromagnetic matrix.

The suspensions (200 ml) that are based on fungus biomass of fungi *A. bisporus* and *L. edodes* were separated by high-gradient ferromagnetic matrix. The value of the magnetic field flux density is 3500 G. The diameter of the coils is 41.0 cm, the size of the magnet tips is 20.0×15.0 cm and size of the cuvette is 3.5×5.0 cm. External magnetic field is homogeneous, since the size of the cuvette with a ferromagnetic matrix of low carbon steel (according to the Ukrainian standards 380–2005 and 1050–90, composition: C — 0.25%, Si — 0.35%, Mn — 0.8%, S — 0.06%, P — 0.08%) is much less than the size of the pole tips of the electromagnet (dimensions are shown in Fig. 1).

The working fluid laminar flows through a high-gradient magnetic separator. The particles retained on the ferromagnetic matrix in the filter are washed with a small amount of distilled water.

The experimental setup for separating the magnetic phase of fungi from the nonmagnetic is shown in Fig. 1 [60].

Dry and crushed, using a laboratory mill, the biomass of the fungus *A. bisporus* grown on substrates with addition of MF of different concentrations, was tested for sorption capacity with respect to Fe^{3+} ions.

The process of biosorption was carried out with mechanical stirring 180 rpm, sorption duration 30 min.

Concentration of Fe^{3+} ions in solution — 50 mg/l.

Biosorbent concentration — 2 g/l.

Sampling time — 5 min, 10 min, 20 min, 30 min.

After sampling, the blue ribbon filter was used to determine the residual amount of Fe^{3+} ions.

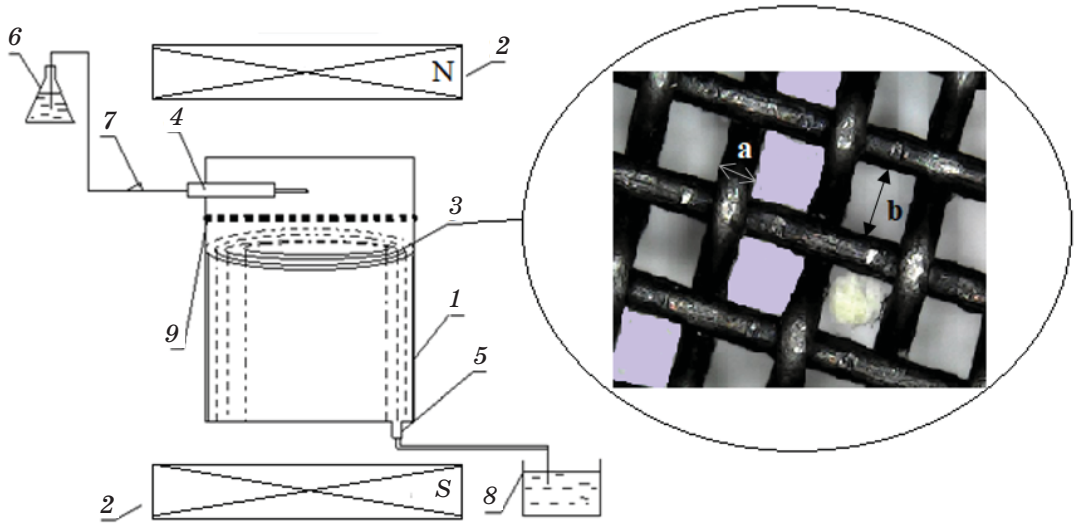

Fig. 1. **The scheme of an experimental installation for HGMS:**
1 — cuvette; *2* — magnetic system; *3* — a ferromagnetic matrix of low carbon steel (a is wire diameter — 0.58mm, b is cell size — 0.5×0.5 mm); *4* — an inlet pipe; *5* — outlet pipe; *6* — container for the working fluid; *7* — the speed control fluid; *8* — container for resetting the nonmagnetic phase; *9* — perforated plate for distributing the flow of liquid

Table 1. **Cluster size before and after HGMS, percentage of separated parts of dry crushed biomass of shiitake (*L. edodes*), penny bun (*B. edulis*) and champignons (*A. bisporus*) depending on grinding, time**

Fungi	Characteristic	The time of grinding dry mushroom, min				
		1 min	2 min	3 min	4 min	5 min
L. edodes	Size of the clusters before HGMS, μm	1.74±1.03	1.7±1.19	1.68±0.78	1.52±1.03	1.49±0.85
	Size of the clusters after HGMS, μm	3.6±1.2	3.93±0.51	4.1±1.05	4.4±0.45	4.3±0.78
	% magnetic phase	3.3	3.7	4.38	4.37	4.34
B. edulis	Size of the clusters before HGMS, μm	1.83±1.19	1.76±1.14	1.5±0.32	1.36±0.24	1.3±0.47
	Size of the clusters after HGMS, μm	1.93±1.01	2.1±1.07	2.38±1.51	2.27±0.61	2.25±0.68
	% magnetic phase	1.62	1.94	2.23	2.1	2.15
A. bisporus	Size of the clusters before HGMS, μm	2.44±1.1	2.23±0.93	1.73±0.56	1.66±1.21	1.58±0.85
	Size of the clusters after HGMS, μm	2.96±0.63	3.61±0.78	3.18±1.03	3.58±1.05	3.97±0.68
	% magnetic phase	1.01	1.01	1.05	1.44	1.17

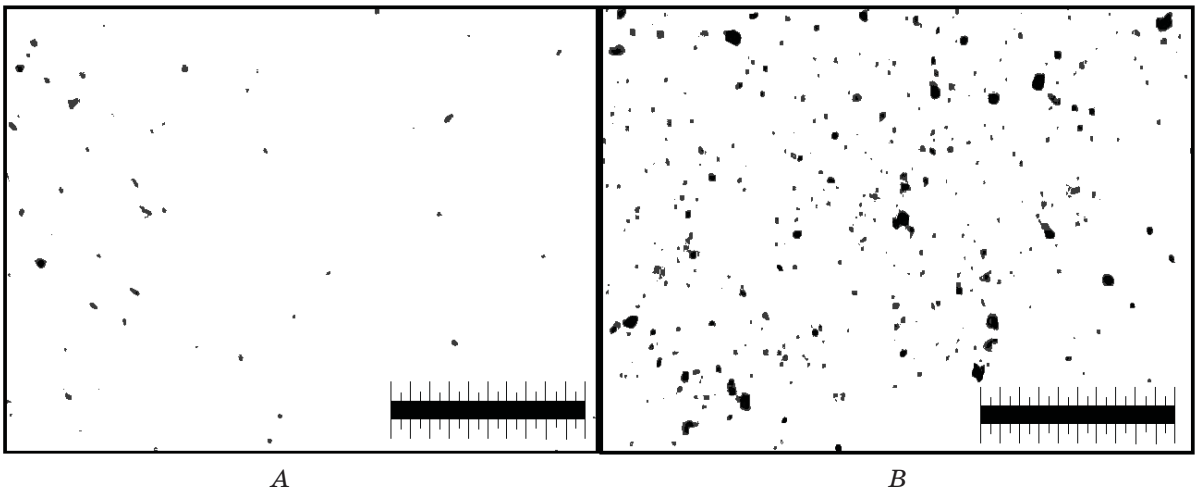

Fig. 2. **Optical microscopy of dry biomass champignons before (*A*) and after separation (*B*) (scale bar 100 μm)**

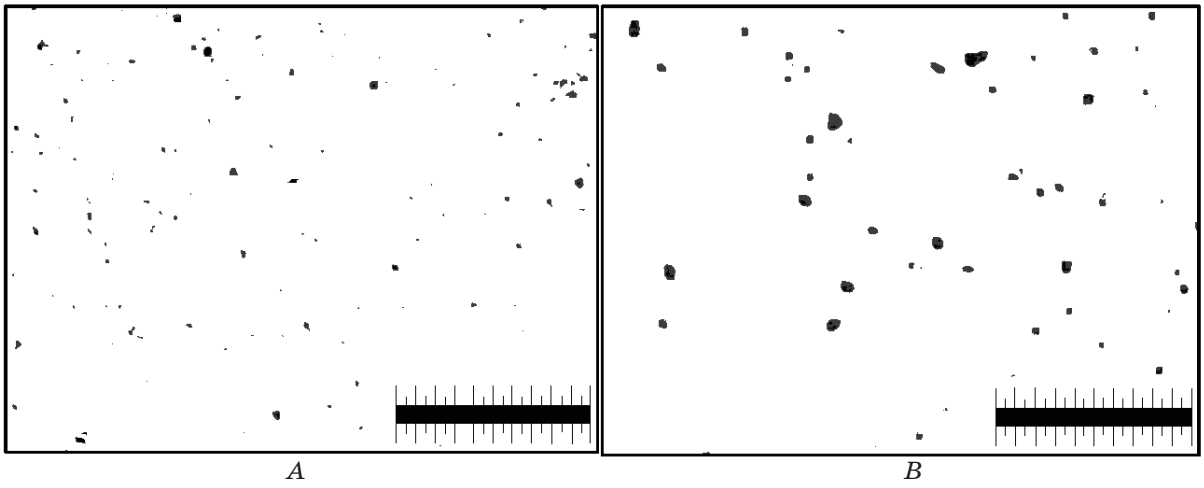

Fig. 3. **Optical microscopy of dry biomass shiitake before (*A*) and after separation (*B*) (scale bar 100 μm)**

Results and Discussion

The separation of the magnetized magnetic phase of the biomass of fungi was carried out with the installation for the HGMS. The average size of clusters was calculated using "Gwyddion" software. The results are shown in Table 1.

The results (Fig. 2, 3) show that after the HGMS the size of clusters and% of separated magnetically controlled phase increased significantly. The optimal time of grinding the dry biomass of the fungus is 3–4 min, so as further grinding does not reduce the size of the clusters and does not increase the number of magnetically controlled phase.

It is investigated (Table 2) that after HGMS the size of clusters of fungi biomass, grown on substrates with the addition of concentrated magnetite, increased almost 4 times, compared to the size before separation for shiitake and 3.3 times for champignons. This can be explained by the coagulation of clusters, containing magnetic particles, in the external magnetic field of the separator. Shiitake contains more magnetically controlled phase (3.3–4.8%) compared to champignon (1.05–1.8%).

Biosorbent based on the biomass of mushrooms was prepared as follows to

Table 2. **Comparison of the results of HGMS of fungi *A. bisporus* and *L. edodes* grown on the substrate with the addition of MF of different concentrations**

Fungi	Characteristic	Control	MF 0.1 mg/ml	MF 1 mg/ml
L. edodes	Size of the clusters before HGMS, μm	1.52±0.05	1.56±0.08	1.61±0.2
	Size of the clusters after HGMS, μm	4.4±1.1 (189%)*	5.1±1.1 (227%)*	5.8±1.4 (260%)*
	% magnetic phase	3.3%	3.6%	4.8%
A. bisporus	Size of the clusters before HGMS, μm	1.73±0.1	1.52±0.56	1.59±0.05
	Size of the clusters after HGMS, μm	3.18±1.1 (83%)*	4.1±1.3 (170%)*	4.9±0.9 (208%)*
	% magnetic phase	1.05%	1.5%	1.8%

* $P < 0.05$ compared with the size of clusters before HGMS.

Table 3. **The efficiency of sorption of Fe^{3+} ions**

Sorption time, min	Efficiency of remote ions Fe^{3+} on the basis of biomass *A. bisporus*, %	Efficiency of remote ions Fe^{3+} on the basis of biomass *A. bisporus* (MF 0.1 mg/ml), %	Efficiency of remote ions Fe^{3+} on the basis of biomass *A. bisporus* (MF 1 mg/ml), %
5	43±2	91.5±0.5	94±0.5
10	67±2	93.5±0.5	95±0.5
20	80±2	94.0±0.5	96±0.5
30	90±2	94.5±0.5	99.5±0.5

determine the sorption capacity: fresh biomass of the fungus *A. bisporus* were dried to constant weight in a drying cabinet at $t = 60$ °C to a constant mass; grinding dry biomass using an electric mill for 1 min, sifting the biomass of fungi through a sieve with a cell diameter of 0.5 mm. Carried out the sorption of iron ions Fe^{3+} and carried out the determination of residual amount of iron in the solution after mixing. The results of the experiments are presented in Table 3 and Fig. 4.

Thus, dry biosorbent based on mushroom biomass champignons has a high sorption capacity with respect to Fe^{3+} ions since the efficiency of extraction of iron (III) ions at 30 min of sorption in all samples is more than 90%. It is proved that the full saturation is 6 times faster, that is, 5 min, compared with 30 min for biosorbent based on the biomass of the fungus grown without MF.

Conclusions

The HGMS divided the magnetically controlled phase of fungi biomass *A. bisporus* and *L. edodes*. It was investigated that after HGMS the particle size for *A. bisporus* increased by 1.8–3 times, for *L. edodes* by 2.8–

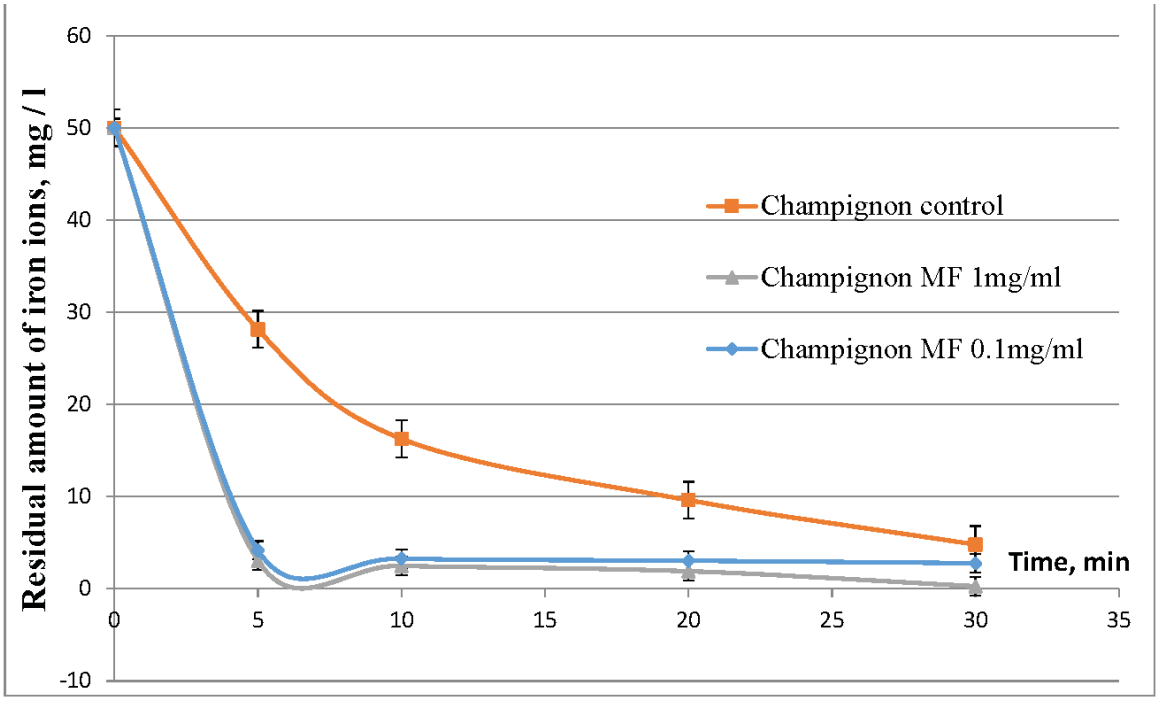

Fig. 4. **Effect of germanium coordination compounds on activity of *P. tardum* α-L-rhamnosidase**

3.6 times. Shiitake contains more magnetically controlled phase (3.3–4.8%) compared to champignon (1.05–1.8%).

The sorption properties of the fungus *A. bisporus* were investigated, when using the biomass of champignons grown on MF, the properties of the sorbent are significantly improved, the full saturation is 6 times faster, that is, 5 min, compared with 30 min for the biosorbent based on the biomass of the fungus grown on a conventional substrate.

The work was done according to the Ukrainian State theme № 2107 f, "Research of biosorbents with natural ferromagnetic properties for water purification".

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ОТРИМАННЯ МАГНІТОКЕРОВАНОГО БІОСОРБЕНТУ НА ОСНОВІ ГРИБІВ *Agaricus bisporus* ТА *Lentinula edodes*

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Метою роботи було отримати магнітокерований біосорбент на основі грибів печериці та шиїтаке, визначити частку магнітокерованої фази біомаси грибів за додавання до субстрату магнітної рідини (МР) і дослідити ефективність вилучення іонів Fe^{3+} подрібненою біомасою гриба печериці. Об'єктом дослідження були гриби печериці *Agaricus bisporus* і шиїтаке *Lentinula edodes*, вирощені в лабораторії. Ефективним та дешевим способом вилучення відпрацьованого біосорбенту з робочого середовища є високоградієнтна магнітна сепарація (ВГСМ), яка проходить у швидкісному режимі. Методами ВГМС здійснено відділення магнітокерованої фази біомаси грибів *A. bisporus* та *L. edodes*. Установлено, що в разі використання біомаси печериці, вирощеної на МР, значно поліпшуються властивості сорбенту, повне насичення відбувається в 6 разів швидше порівняно з біосорбентом на основі біомаси гриба, вирощеного без МР.

Ключові слова: біогенні магнітні наночастинки, магнетит, магнітокерований біосорбент, печериця *Agaricus bisporus*, шиїтаке *Lentinula edodes*.

ПОЛУЧЕНИЕ МАГНИТОУПРАВЛЯЕМОГО БИОСОРБЕНТА НА ОСНОВЕ ГРИБОВ *Agaricus bisporus* И *Lentinula edodes*

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Целью исследования было получить магнитоуправляемый биосорбент на основе грибов шампиньона и шиитаке, определить долю магнитоуправляемой фазы биомассы грибов при добавлении к субстрату магнитной жидкости (МЖ) и исследовать эффективность извлечения ионов Fe^{3+} измельченной биомассой гриба шампиньона. Объектом исследования были грибы шампиньоны *Agaricus bisporus* и шиитаке *Lentinula edodes*, выращенные в лаборатории. Эффективным и дешевым способом удаления отработанного биосорбента из рабочей среды является высокоградиентная магнитная сепарация (ВГСМ), которая проходит в скоростном режиме. Методами ВГМС осуществлено отделение магнитоуправляемой фазы биомассы грибов *A. bisporus* и *L. edodes*. Установлено, что при использовании биомассы шампиньона, выращенной на МЖ, значительно улучшаются свойства сорбента, полное насыщение происходит в 6 раз быстрее по сравнению с биосорбентом на основе биомассы гриба, выращенного без МЖ.

Ключевые слова: биогенные магнитные наночастицы, магнетит, магнитоуправляемый биосорбент, шампиньон *Agaricus bisporus*, шиитаке *Lentinula edodes*.

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ANTIMICROBIAL, ANTIBIOFILM-FORMING AND SOME BIOCHEMICAL PROPERTIES OF *Potentilla erecta* RHIZOME EXTRACT

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The purpose of the work was to study the antimicrobial, antibiofilm-forming, antioxidant and some biochemical properties of alcoholic extracts of *Potentilla erecta* L. rhizome. The plants for the study were gathered around the village of Luta, Velyky Berezny rayon, Transcarpathia. From the *Potentilla erecta* L. rhizome, ethyl and methyl alchogol extracts were produced. The aim of the study was their antioxidant activity (by DPPH method), total tannin and flavonoids (by spectrophotometric method), and antimicrobial activity (by diffusion-into-agar method). The clinical isolates were isolated with the use of differentially diagnostic nutrient media. The antibiofilm activities of the extracts were tested in standard 96-well microtitration plates.

Ethyl and methyl extracts of *Potentilla erecta* L. rhizome were shown to reveal high antioxidant activity. Antimicrobial activity of the extracts against *Staphylococcus* genus bacteria and *Candida* genus fungi was established. The study proved high capacity of ethanol extract for bacterial biofilm destruction.

Thus, the study showed the antimicrobial, antioxidant and antibiofilm-forming activity of tormentil ethyl extract against the isolates from the mouth cavities of patients suffering from parodentium inflammatory diseases, which fact contributes to the application prospects of this extract as an active base for mouth cavity hygiene preparations.

Key words: antimicrobial effect, antibiofilm formation, plant extracts, antioxidant activity, flavonoids, tannins.

Studies aimed at the search of natural substances with antimicrobial activity, including those derived from plants. This trend is connected with the diversity of biologically active compounds that have a broad spectrum of pharmacological activity and exhibit antioxidant, anti-inflammatory and even anticancer properties [1]. Substances of plant origin are widely used both in conventional and folk medicine, as well as in food, pharmaceutical and beauty industries. Studies aimed at the search of substances that, apart from their antimicrobial activity, can destroy bacterial biofilm are also of significant importance nowadays. The microorganisms of the biofilm are known to possess a higher level of resistance to antimicrobial preparations, and as such they serve an additional factor of pathogenicity [2, 3]. This problem is especially vital for mouth cavity diseases, where the prevailing majority of agents of inflammatory diseases are part of the biofilm, which complicates treatment of persisting diseases [4]. In our previous works, we showed the high percentage of antibiotic-resistant microorganism strains

within microbial associations of mouth cavity affected by chronic inflammatory process [5, 6]. In that case, it was *Staphylococcus* spp. genus bacteria and *Staphylococcus* spp. + *Candida* spp.; *Staphylococcus* spp. + *Enterobacteriaceae* spp. microorganism associations that were the dominating associates during an inflammatory process, on the background of the most complicated clinical course [7]. In [8] it was shown that the microorganisms being part of the biofilm were characterized by a higher level of resistance to antimicrobial preparations. This is why, the search of the substances with antimicrobial and antibiofilm-forming activities presented a particular interest. In our previous works we also showed the antimicrobial activity of essential oils and cowberry extract against clinical microorganism isolates [9, 10].

The *Potentilla* genus is a member of the *Rosaceae* family, *Rosoideae* subfamily, which is mainly distributed in temperate, arctic and Alpine zones of the Northern hemisphere. Extracts of the aerial and/or underground parts have been applied in traditional medicine for the treatment of inflammations, wounds, certain forms of cancer, infections due to bacteria, fungi and viruses, diarrhoea, diabetes mellitus and other ailments [11].

The substances extracted from rhizomes of *Potentilla* genus plants are known to possess antimicrobial properties, but no data on the effect of the extracts upon antibiotic-resistant clinical isolates and their antibiofilm-forming properties have been available so far.

The purpose of the work was to study the antimicrobial, antibiofilm, antioxidant and some biochemical properties of alcoholic extracts of *Potentilla erecta* L. rhizome.

Materials and Methods

The plant materials were collected in the vicinity of the village of Luta, Velyky Berezny rayon, Trancarpathia, dried at the temperature of 30–35 °C in shadow, then ground and placed in tightly closed containers.

Extracts manufacturing techniques. We made ethyl and methyl extracts of *Potentilla erecta* L. rhizome. A 10 g batch of dry plant material was pulverized to powdery mass. In an Erlenmeyer flask, 10 g of plant material was blended with 200 ml of or 96° ethyl or methyl alcohol (Sigma, Germany). The opening was closed with a food wrap to avoid evaporation. Following a 30-minute-long incubation in the ultrasonic bath (Kraintek) at 35 °C, the blend was filtered through Whatman No. 1 filter paper. The clear solution was placed in an evaporative device (16–17/32″×34-59/64″G5B, Coated Dry Ice Condenser Rotary Evaporator) to obtain pure alcoholic extract at 50 °C, 82 rpm. Then, extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove or ethyl or methyl. As a result, the following pure extracts were obtained: ethyl extract of 0.50 g; methyl extract of — 1.07 g. For the purpose of study, 0.50 g of extract was chosen.

Antimicrobial assay. As test cultures, the following bacteria and yeasts from the American Type Culture Collection were used: *Candida albicans* ATCC 885-653; *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; *Enterococcus faecalis* ATCC 29212; *Streptococcus pyogenes* ATCC 19615; reference *S. aureus* CCM 4223 biofilm-forming strain. We also used clinical strains of bacteria and yeasts (*S. aureus, E. coli, S. pyogenes*, *E. faecalis, C. albicans*) isolated from the oral cavities of patients suffering from inflammatory periodontium and pharynx. We chose the clinical strains with multiple resistance at least to two classes of antibiotics. As a positive control were used: gentamicin (10 mg/disk) for Gram-negative bacteria, ampicilin (10 mg/disk) for Gram-positive bacteria, nystatin (100 UI) for *Candida*. As negative control were used DMSO.

The microorganisms from the oral cavities of patients with chronic periodontium inflammatory processes were isolated on the basis of the Dental Polyclinic, Uzhhorod National University; the extracts were manufactured and their antioxidative activity and contents of tannins and flavonoids were determined on the basis of the Department of Pharmacognosy and Botany, University of Veterinary Medicine and Pharmacy in Košice, Slovakia; the antimicrobial activity of plant extracts was studied at the Microbiological Laboratory of the Department of Genetics, Plant Physiology and Microbiology, Uzhhorod National University, and Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice.

Antimicrobial activity of *Potentilla erecta* L. rhizome extracts was determined using agar diffusion test [12]. The bacterium inocula 100 µl in the physiological solution were adjusted to the equivalent of 0.5 McFarland standard, and evenly spread on the surface of Muller-Hinton agar (incubated at 37±2 °C for 24 hours); yeasts — on SDA agar (incubated at 35±2 °C for 48 hours). The extracts 20 µl

were introduced into wells 6 mm in diameter. The diameters of the inhibition zones were measured in millimetres including the diameter of the well. Each antimicrobial assay was performed at least three times.

Determination of antibiofilm activity. The antibiofilm activity of the EO were tested in standard 96-well microtitration plates (Greiner-BioOne, Austria) using a modified staining method according to O' Toole [13].

With the purpose of study of the antibiofilm-forming activity, a 18-hour culture of the reference *S. aureus CCM 4223* biofilm-forming strain grown at 37 °C was used. Into the wells, 180 µl of bacterial suspension, Mc Farland in broth (TSB, Himedia, India) were introduced. The *Potentilla erecta* L. rhizome extracts dissolved to the concentrations of 1%, 5% and 10% in dimetylsulfoxide (DMSO; Sigma-Aldrich, USA) was introduced into the wells in the amount of 20 µl. Following the addition of the bacterial suspension, the concentration of plant extracts in the broth equaled to 0.1%, 0.05% and 0.01%, respectively. The wells with only 180 µl of broth and 20 µl of 10% DMSO served as the control.

Following a 24-hour-long incubation in the thermostat at 37°, the supernatant was withdrawn and washed 3 to 5 times with distilled water. Following a 30-minute-long incubation, it was dyed with 200 µl of 0.1% solution of crystal violet; then the dye was withdrawn, and the supernatant washed 3 to 5 times with distilled water. Into every well, 200 µl of 30% acetic acid were added and incubated for 10 min. The optical density was measured on the Synergy HT (Biotek, USA) spectrophotometer at 550 nm.

More than 50% reduction in absorbance of CV was considered as significant inhibition. Statistical Analysis Values mentioned are the mean with standard deviations, obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Antioxidant activity. Detection of free radical scavenging activity of the samples was measured with 2.2-diphenyl-1-picrylhydrazyl (DPPH) [14]. A sample of 0.1 ml was mixed with 1.9 ml of DPPH solution in methanol (0.06 mmol l^{-1}). The absorbance of the reaction mixture was detected with a spectrophotometer Beckman Coulter DU 530. Following incubation in dark for 30 min, the absorbance of each solution was measured at 515 nm (A). The antioxidant activity was expressed as percentage (%) of the scavenging activity. The percentage of DPPH radical scavenging activity was calculated by using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs (control)} - \text{Abs(sample)}}{\text{Abs (control)}} \times 100,$$

where Abs (control): Absorbance of DPPH radical + methanol; Abs (sample): Absorbance of DPPH radical + extract.

Determination of Total Tannins (TT). The content of tannins was determined using Folin-Ciocalteus method [15]. The absorbance was measured as the absorbance at 750 nm (A), with the use of water as the compensation liquid. The percentage of tannins expressed as pyrogallol was calculated based on the following expression:

$$\text{Tannins (\%)} = \frac{3.125 \times \text{A}}{0.316 \times m},$$

where m — mass of the sample to be examined, in grams; A — absorbance.

The absorbance of the reaction mixture was detected with a spectrophotometer Beckman Coulter DU 530v.

Determination of *Total Flavonoids (TF)*. The flavonoid content was determined by a colorimetric assay as described by aluminium chloride colorimetric method [15]. The absorbance of the test solution was measured at 425 nm with a spectrophotometer Beckman Coulter DU 530.

$$X = \frac{A \times 1.25}{m},$$

where A — absorbance at 425 nm; m — mass of the herbal drug to be examined in grams.

For the results of experiment, we used statistical software Microsoft Office-Excel (2013) with the calculation of averages, error, and standard deviation.

Results and Discussion

The studies have shown that the highest antimicrobial effect of the extracts was registered against *Staphylococcus* genus, *Enterococcus faecalis* bacteria and *Candida* genus microscopic fungi. It was established that the extracts possessed a distinguished antibacterial effect upon MRSA *S. aureus*. Their effect upon *E. coli* was significantly lower. No antibacterial effect of the extracts upon *Streptococcus pyogenes* has been ascertained. The antimicrobial activity of methyl and ethyl extracts would not differ

statistically significantly against bacterial isolates, though ethyl extracts showed a more distinguished antimycotic activity.

The study of the biochemical and antioxidant properties of the extracts has shown a high antioxidant level of tormentil rhizome ethyl and methyl extracts (Table 2).

The results of the present study suggested that the ethanol extract from *P. erecta* rhizome is characterized by high concentrations of tannins and flavonoids (Table 2). The study of the antibiofilm-forming ability of the extracts showed a high antibiofilm-forming effect of ethyl extracts from *Potentilla erecta* L. (Fig. 1). Thus, 0.1% ethyl extracts reduced the biofilm-forming activity of *S. aureus* CCM 4223 by 91.72% as compared with the control (ethyl alcohol). The reduction of extract concentration insignificantly affected the antibiofilm-forming properties of the extract. Say, 0.05% extract caused reduction of the antibiofilm-forming properties of staphylococci by 86.2%, and 0.01% extract — by 83.4%.

The study of antibiofilm-forming properties of methyl extract from *Potentilla erecta* L. rhizome showed that the use of 0.1% extract caused a 71.3% biofilm destruction; the use of 0.05% extract resulted in a 66.6% reduction of biofilm formation; the application of 0.01% extract led to a 50% reduction (Fig. 2).

Thereby, high antibiofilm-forming activity of ethyl and methyl extracts was recorded, however the antibiofilm-forming activity of ethyl extract was more expressive and did not reduce significantly with the reduction of extract concentrations.

The antimicrobial properties of tormentil have been shown in the works by other scholars. Say, tormentil rhizome extract was shown to have an effect against Gram-positive microorganisms that provoke food infections. The extract was shown to display an inhibiting effect against Gram-positive bacteria such as *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, as well as against yeast such as *Candida lipolitica* KKP 322 and Hansenula anomala R 26. The extract did

Table 1. **Antimicrobial activities of the *Potentilla erecta rhizome* extract against typical and clinic opportunistic infectious agents, mm ($n = 3$, $x \pm SD$)**

Test culture	Ethyl extract	Methyl extract
S. aureus ATCC 25923	17.67±0.58*	18.17±0.29*
S. aureus CCM 4223 (biofilm formation)	16.5±0.50*	17.67±0.58*
S. aureus MRSA (clinic), isolate from mouth cavity	16.0±0.50*	17.50±0.50*
Streptococcus pyogenes ATCC 19615	7.33±0.58*	7.50±0.80*
Streptococcus pyogenes (isolate from mouth cavity)	-	-
Escherichia coli ATCC 25922	11.17±0.29*	11.33±0.58*
Escherichia coli (isolate from mouth cavity)	8.17±0.29*	8.67±0.58*
Enterococcus faecalis ATCC 29212	15.67±0.58*	15.00±0.50*
*Enterococcus faecalis (*isolate from mouth cavity)	14.67±0.33*	14.67±0.33*
Candida albicans ATCC 885-653	20.33±0.58*	17.5±0.29*
*Candida albicans (*isolate from mouth cavity)	17.67±0.58*	12.33±0.58*

An extraction solvent (ethanol or methanol) were used as the control:
control of ethanol — no inhibition; control of methanol — no inhibition;
* the data were statistically significant as compared with the control ($P < 0.05$).

Table 2. **Level of tannins, flavonoids and antioxidant activity in ethyl and methyl extracts of P*otentilla erecta* L. rhizome**

Ethyl extract		Methyl extract	
Absorbance (nm)	%	Absorbance (nm)	%
tannins			
0.81*	8.04*	0.78*	7.74*
flavonoids			
0,112*	0.114*	0.11*	0.14*
antioxidant activity			
0.06*	88.44*	0.05*	91.08*

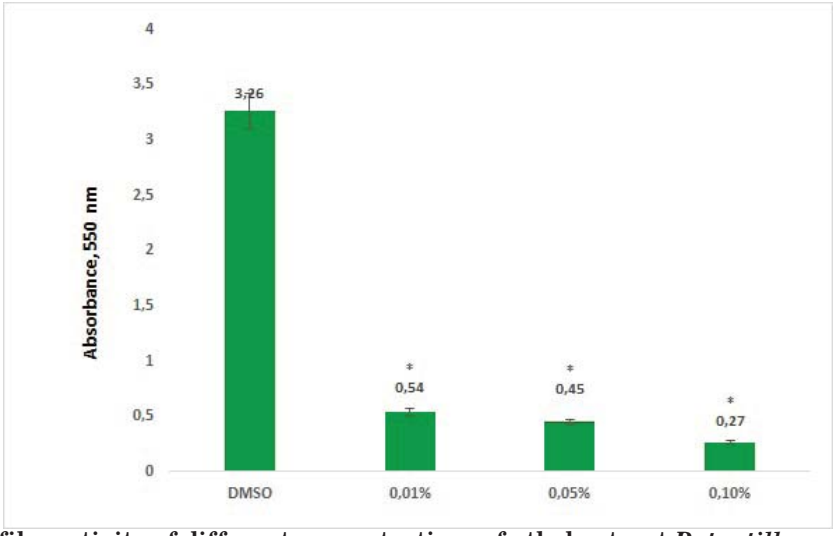

Fig. 1. **Antibiofilm activity of different concentrations of ethyl extract *Potentilla erecta* L. rhizome on biofilm-forming *S. aureus***
* the data were statistically significant as compared with the control ($P < 0.05$)

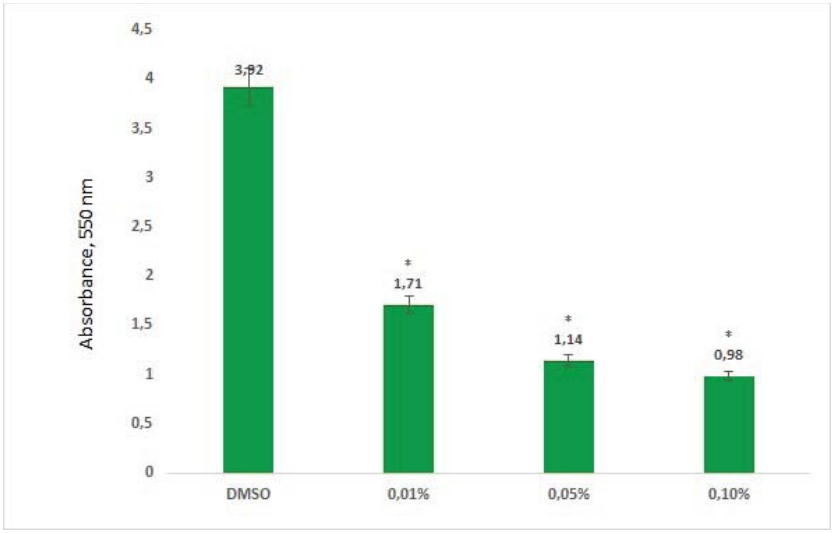

Fig. 2. **Antibiofilm activity of different concentrations of methyl extract *Potentilla erecta* L. rhizome on biofilm-forming *S. aureus***
* the data were statistically significant as compared with the control ($P < 0.05$)

inhibit the growth of Gram-negative bacteria [16]. Another work showed tha antibacterial and antimycotic activity of aqueous extracts.

Most of the biological effects of *Potentilla* species can be explained by the high amount of condensed and hydrolysable tannins present in the aerial and the underground parts, e.g. the antiviral and antimicrobial activities, immunomodulating effects, hepatoprotective and anti-inflammatory effects. Tannins have been known to be important constituents of *Potentilla* species and their extracts, respectively, and the cause for the astringent effects. Therefore thorough phytochemical studies on *Potentilla* species starting especially in the 1960s were primarily focussed on tannins [19].

Thus, our studies have demonstrated the antimicrobial activity of ethyl and methyl extracts of *Potentilla erecta* L. rhizome against *Staphylococcus* genus bacteria and *Candida* genus microscopic fungi. These trends were shown both on typical and clinical strains, the latter being isolated from the mouth cavities of patients suffering from chronic mouth cavity diseases and characterized by a high resistance to antibiotics. Ethyl extract of *Potentilla erecta* L. was shown to display a high antibiofilm-forming activity. A significant antioxidant activity of the reviewed extracts was also demonstrated. The obtained results indicated to good prospects for further research in order to create tormentil-based preparations as mouth cavity care and hygienic products, as far as they —

as contrasted with chemical preparations — may be used for a long period of time as part of mouth cavity care and preventive products; they as a rule have no side effects but possess an anastaltic effect and antioxidant properties. *Potentilla erecta* L. is a specially valuable plant product, for it has long since been used in folk pharmaceutics and medicine of concrete localities.

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АНТИМІКРОБНІ, АНТИБІОПЛІВКОУТВОРЮВАЛЬНІ ТА ДЕЯКІ БІОХІМІЧНІ ВЛАСТИВОСТІ ЕКСТРАКТУ КОРЕНЕВИЩА *Potentilla erecta*

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Метою роботи було дослідити антимікробні, антибіоплівкоутворювальні, антиоксидантні та деякі біохімічні властивості спиртових екстрактів кореневища *Potentilla erecta* L. Згідно з метою досліджень визначали антиоксидантну активність (DPPH методом), загальні таніни та флавоноїди (спектрометрично), антимікробну активність (дискодифузійним методом). Клінічні ізоляти виділяли з використанням диференційно діагностичних середовищ. Антибіоплівкоутворювальну здатність визначали у стандартних 96-лункових планшетах.

Показано високу антиоксидантну активність етилового та метилового екстрактів кореневища *Potentilla erecta* L. Встановлено антимікробну активність екстрактів стосовно бактерій роду *Staphylococcus* і мікроскопічних грибів роду *Candida* та високу здатність етилового екстракту до деструкції бактеріальної біоплівки.

Отже, дослідження показали антимікробну, антиоксидантну та антибіоплівкоутворювальну активність етилового екстракту калгану щодо ізолятів ротової порожнини людей із запальними захворюваннями пародонта, що зумовлює перспективність використання цього екстракту як активної основи препаратів для гігієни ротової порожнини.

Ключові слова: антимікробний ефект, утворення антибіоплівки, рослинні екстракти, антиоксидантна активність, флавоноїди, дубильні речовини.

АНТИМИКРОБНЫЕ, АНТИБИОПЛЕНКООБРАЗУЮЩИЕ И НЕКОТОРЫЕ БИОХИМИЧЕСКИЕ СВОЙСТВА ЭКСТРАКТА КОРНЕВИЩА *Potentilla erecta*

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Целью работы было исследовать антимикробные, антибиопленкообразующие, антиоксидантные и некоторые биохимические свойства спиртовых экстрактов корневища *Potentilla erecta* L. Согласно цели исследования определяли антиоксидантную активность (DPPH методом), общие таннины и флавоноиды (спектрометрически), антимикробную активность (дискодиффузионным методом). Клинические изоляты были выделены с использованием дифференциально диагностических сред. Антибиопленкообразующую способность определяли в стандартных 96-луночных планшетах.

Показана высокая антиоксидантная активность этилового и метилового экстрактов корневища *Potentilla erecta* L. Установлена антимикробная активность экстрактов относительно бактерий рода *Staphylococcus* и микроскопических грибов рода *Candida* высокая способность этилового экстракта к деструкции бактериальной биопленки.

Итак, исследования показали антимикробную, антиоксидантную и антибиопленкообразующую активность этилового экстракта калгана в отношении изолятов ротовой полости людей с воспалительными заболеваниями пародонта, что обусловливает перспективность использования данного экстракта в качестве активной основы препаратов для гигиены полости рта.

Ключевые слова: антимикробный эффект, образование антибиопленки, растительные экстракты, антиоксидантная активность, флавоноиды, дубильные вещества.

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USING OF *Lemna minor* FOR POLLUTED WATER TREATMENT FROM BIOGENIC ELEMENTS

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The aim of this work was to establish the possibility of *Lemna minor* usage to improve the efficiency of wastewater treatment from nitrogen and phosphorus compounds.

Due to their pollution resistanse, *Lemna minor* is often used for wastewater remediation. It is capable of absorption and transformation of various compounds, promotes the deposition of suspended matter, saturates water with oxygen, intensifies the purification processes. Due to the rapid growth rate, duckweed absorbs a large amount of pollutants, thereby purifying water from them. However, there is insufficient data and information on the efficiency of nitrogen and phosphorous compounds extraction by *Lemna minor* wastewater. That is why the research direction was to determine the efficiency of wastewater treatment from biogenic nitrogen and phosphorus compounds by higher aquatic plants of *Lemna minor*.

The necessary conditions for cultivation of higher aquatic plants of *Lemna minor* in autumn and winter were established. The efficiency of using duckweed of different mass for purification of waste water from biogenic compounds of nitrogen and phosphorus was investigated. The usage of duckweed together with other higher aquatic plants to investigate wastewater from biogenic nitrogen and phosphorus compounds was investigated.

It was established that the efficiency of sewage treatment in the autumn-winter period should adjust the mode of illumination and temperature regime.

Key words: wastewater, biological treatment, phosphates, nitrates, *Lemna minor*.

Biogenic compounds can enter the surface waters both naturally (leaching from the topsoil, atmospheric precipitation, various processes in the reservoir itself), and as a result of human activity through wastewater discharge from industrial, household, agricultural and livestock complexes [1–5]. Exceedance of maximum permissible concentrations of biogenic compounds in wastewater lead to surface water's flowering and eutrophication, increase of the content of biogenic and organic compounds, oxygen decrease, appearance of anaerobic zones in the bottom layers, increase of water turbidity, color change, contamination with microorganisms, including pathogens [6, 7]. With the accumulation of excess organic matter in the bottom sludge, the processes of formation of methane, hydrogen, hydrogen sulfide, ammonia take place. This leads to formation of gas bubbles, which, when dissolved in the water, have a toxic and damaging effect on the flora and fauna, which significantly damages indicators of drinking water when using the reservoir as a source of water supply [8, 9]. Therefore, the problems of the effective of nitrogen and phosphorus compounds removal from wastewater before their discharge into natural reservoirs and the improvement of existing biological wastewater treatment technologies are important [10].

The aim of the work was to study promising methods of wastewater biological treatment from biogenic compounds using higher aquatic plants *Lemna minor* to improve the efficiency

of wastewater treatment with nitrogen and phosphorus compounds, reduce the construction and operation costs of wastewater treatment plants, ensure the quality of treated water in accordance with existing discharge standards in natural ponds.

The objective was to determine the optimal conditions and to establish the efficiency of wastewater treatment from biogenic compounds of nitrogen and phosphorus by higher aquatic plants *Lemna minor*. That is why the experimental part was based the experiments using this type of plant. Today biological ponds are considered as an economic and promising method of biological treatment and purification of household, some non-toxic industrial sewage and atmospheric waters [11–12]. Also, biological ponds do not require the use of chemical reagents, and are characterized by ease of maintenance [11, 13, 14].

The principle of wastewater treatment in biological ponds is the same as the natural self-purification processes that can be observed in aquatic and near-water natural ecosystems. Higher aquatic have the abilities to absorb biogenic and organic compounds, accumulate heavy metals and biodegradable organic substances. Higher aquatic plants are capable of absorbing nitrogen, phosphorus, potassium, calcium, magnesium, manganese, sulfur, cadmium, copper, lead, zinc, phenols, petroleum products, synthetic surface active substances, etc. [15].

Higher aquatic plants are also noted for their properties of oxidizing (due to oxygen income to the reservoir during photosynthesis) and detoxification (due to the ability of plants to transform toxic substances into non-toxic ones). Biological ponds are often used to reduce the values of BOD and COD and further precipitate suspended matter due to the filtration capacity of higher aquatic plants [16].

Lemna minor is a species of free-floating higher aquatic plants belonging to one of the smallest flowering plants in the world. The duckweed biomass buildup increases with stagnant water and high concentrations of biogenic compounds of nitrogen and phosphorus [17–19]. Duckweed is widespread in slow-moving freshwater reservoirs: ponds, swamps, lakes, streams. Due to rapid growth rate, duckweed absorbs a large amount of contaminants, thereby purifying water from them [20]. Phosphate and nitrate are removed in biosorption process by root and leaf cells followed by assimilation [3].

Materials and Methods

A model solution with the KNO_3 salt was prepared to determine the efficiency of nitrate extraction with *Lemna minor*. The 0.2 g sample of KNO_3 salt was measured on laboratory scales. Then sample was dried in a drying oven at 110 °C. After drying, salt was moved to a desiccator for cooling. The 0.08 g sample of dried KNO_3 salt was measured on laboratory scales. Then, weighting was transferred to a 1000 ml volumetric flask and filled with distilled water to the mark. The opening of the measuring flask was covered and its content was mixed thoroughly. The solution thus prepared contained a concentration of nitrates — 50 mg/dm^3.

The 0.2 g sample of KH_2PO_4 salt was measured on laboratory scales. Then sample was dried in a drying oven at 110 °C. After drying, salt was moved to a desiccator for cooling. The 0.02 g sample of dried KH_2PO_4 salt was measured on laboratory scales. Then, weighting was transferred to a 1000 ml volumetric flask and filled with distilled water to the mark. The opening of the measuring flask was covered and its content was mixed thoroughly. The solution thus prepared contained a concentration of phosphates — 10 mg/dm^3. The model solution with a concentration of nitrates of 50 mg/dm^3 and phosphates of 10 mg/dm^3 was prepared to simulate household wastewater.

Experimental installations in the form of model biological ponds with size of 12×9×2 cm, 216 ml volume, made of plastic were used for the research. In total, 32 models of such installations were used in experimental studies. For the experiment, the model biological ponds was filled with a suitable model solution, biological plant material and left for the required period of time on the windowsill with direct sunlight.

All experiments were divided into two series: the first, the study of the biological treatment effectiveness with higher aqueous plants in a model solution from nitrates; the second, the study of the biological treatment effectiveness in a model solution from phosphates.

In the first experiment series, the determination of the purification efficiency of the model solution containing NO_3-concentration of 50 mg/dm^3 was carried out. In all installations, the volume of the model solution was 100 ml. All experiments were performed under the same conditions. The duration of the experiment was 7 days (144 hours). 8 model installations were used.

Higher aquatic plants of *Lemna minor* were the biological agent. Two types of duckweed sample weightings were used: m_1 =11 g and m_2 = 22 g. Sample weightings were used to determine the effect of the duckweed biomass amount on the process of phosphorus and nitrogen removal. The experimental ponds were filled with model solution and the biological agent was distributed as follows: installations 1, 2, 3, 4 were loaded with higher aquatic plants *Lemna minor* 11 g each, and installations 5, 6, 7, 8 — 22 g each. The samples were analyzed on the second, fifth, and seventh days of the experiment. On the first day of the experiment, an analysis of the NO_3-ions concentration was performed for water samples from installations 1 and 5; on the third day — from installations 2 and 6; on the fifth day — from installations 3 and 7; on the seventh day — from installations 4, 8. The ion meter I160 MI and the electrode measuring to it ELIS -121 NO_3^- were used to determine the concentration of NO_3^- ions in the solutions.

The experimental data were processed using a Microsoft Excel software. Data were considered significant at $P < 0.05$.

After biological treatment, the concentrations of nitrates and phosphates in treated water should be 10 mg/dm^3 and 2 mg/dm^3, respectively, before being discharged into natural waterbody [4, 20].

Results and Discussion

The change in the concentration of NO_3^- ions in the model solution depending on the duration of the treatment is presented at Fig. 1. The efficiency of the treatment was determined based on on the obtained concentrations (Fig. 2).

Therefore, from the obtained results, it could be argued that the purification of contaminated water from nitrogen compounds occured differently, depending on the mass of duckweed involved.

Decrease of nitrate concentration in the model solutions was better in Experiment 2, where a larger sample of *Lemna minor* duckweed was used. This is evidenced by the calculated efficiency of purification, where the purification effect in the experiment with a biomass density of 0.1 g/cm^2 (m_1) was 83.1% against 91.8% in the experiment with a biomass density of 0.2 g/cm^2 (m_2).

Fig. 3 shows the change in the concentration of phosphates depending on the duration of the purification process.

The efficiency of the model solution treatment from phosphates depending on the duration was determined from the obtained results (Fig. 4.).

The decrease in phosphate concentration in model solutions was better in Experiment 4, where a larger sample of *Lemna minor* duckweed was used. This is evidenced by the calculated efficiency of purification, where the treatment effect in the experiment with a biomass density of 0.1 g/cm^2 (m_1) was 72% against 80% in the experiment with a biomass density of 0.2 g/cm^2 (m_2).

As a result of the experiments performed and the calculations made, we could conclude the treatment efficiency of biogenic compounds of nitrogen and phosphorus in model solutions at a concentration of 50 mg/dm^3 and 10 mg/dm^3, respectively.

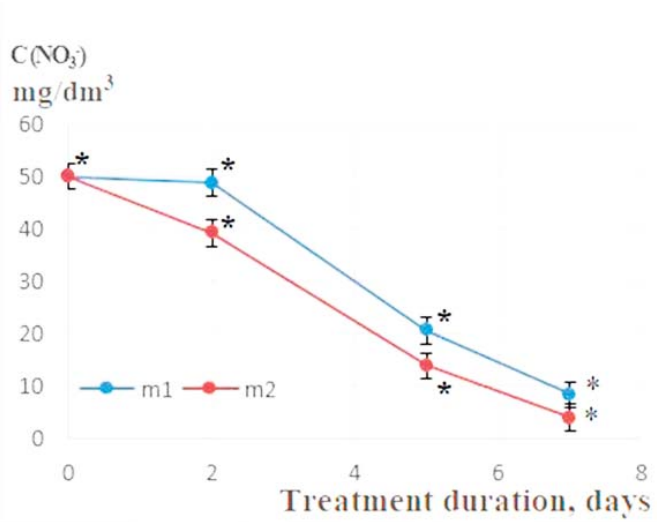

Fig. 1. **The change in the concentration of nitrates in the model solution from the duration of the process and the mass of duckweed in the installation: m_1 —11 g; m_2 — 22 g**

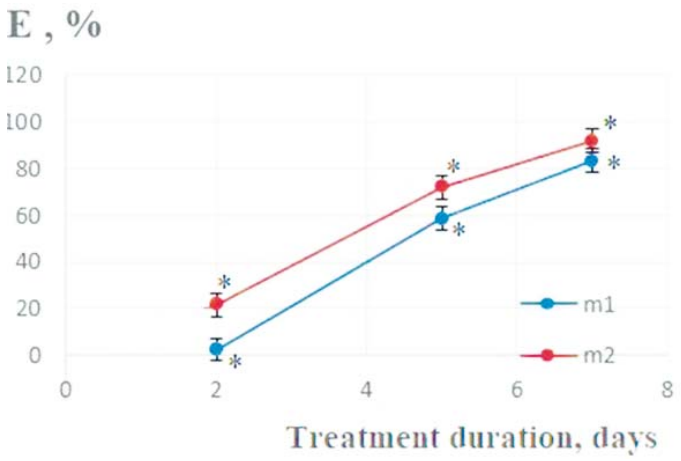

Fig. 2. **The change in the nitrate treatment effect in the model solution from the duration of the process and the mass of duckweed in the installation:** m_1 — 11 g; m_2 — 22 g

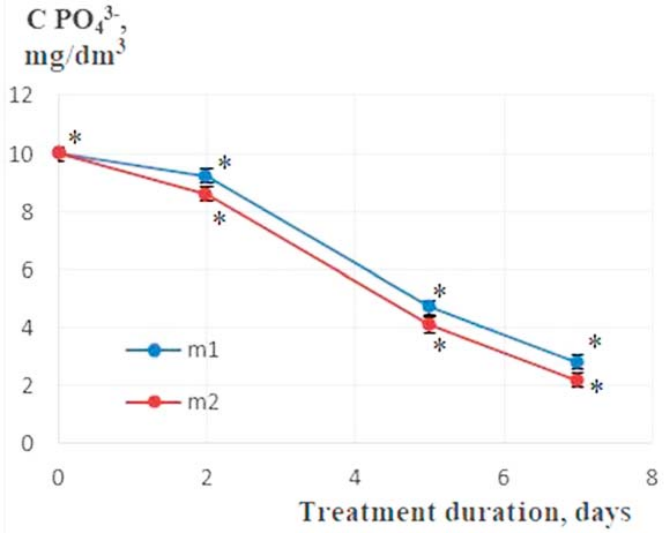

Fig. 3. **The change in the concentration of phosphates in the model solution from the duration of the process and the mass of duckweed in the installation:** m_1 — 11 g; m_2 — 22 g

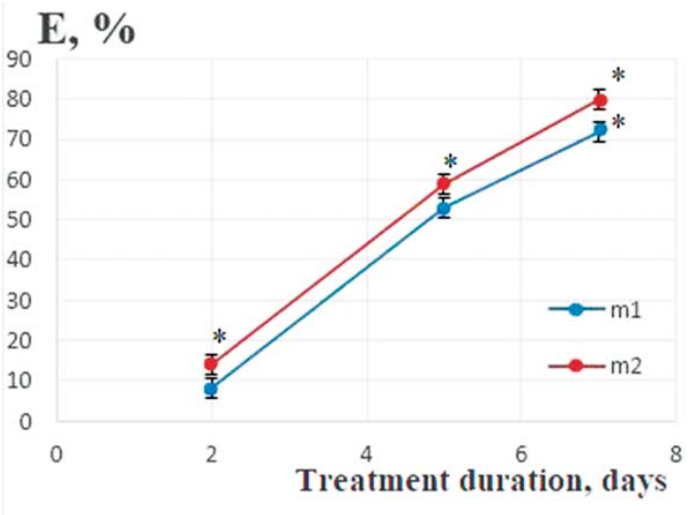

Fig. 4. **The change in the phosphate treatment effect in the model solution from the duration of the process and the mass of duckweed in the installation:** m_1 — 11 g; m_2 — 22 g

It has been found that with the increase of *Lemna minor* biomass, the treatment efficiency of nitrogen and phosphorus compounds in model solutions does not decrease, but rather increases. Even with the high density of the plants, there is enough light for photosynthesis and no death of duckweed is observed in the lower layer. The duckweed planted in a single layer shows good results, and the water in the model solutions treated by this method can be diverted to the nitrate content of the reservoir and require a slight purification of the phosphate content. The efficiency of such purification by nitrogen is 83% and phosphorus — 72%, and the concentrations of NO_3^- and PO_4^{3-} ions after purification are 8.45 mg/dm^3 and 2.8 mg/dm^3, respectively.

Using a doubled amount of *Lemna minor* duckweed, the purification efficiency of the model solutions from both phosphorus and nitrogen compounds increases to 80% and 91.8%, and the concentration of NO_3^- ions after purification under these conditions is 4.12 mg/dm^3 and phosphorus 2.2 mg/dm^3, respectively. Such concentration values do not exceed the maximum permissible concentrations norms for discharge of treated water into natural reservoirs.

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ВИКОРИСТАННЯ *Lemna minor* ДЛЯ ОЧИЩЕННЯ ЗАБРУДНЕНОЇ ВОДИ ВІД БІОГЕННИХ ЕЛЕМЕНТІВ

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Метою роботи було встановити можливість використання *Lemna minor* для підвищення ефективності очищення стічних вод від сполук азоту та фосфору.

Ряску *Lemna minor* завдяки її стійкості часто використовують для ремедіації стічних вод. Вона здатна до засвоєння та трансформації різних сполук, сприяє осадженню завислих речовин, насичує воду киснем, інтенсифікує процеси очищення. Через швидкі темпи росту ряска поглинає велику кількість забруднювальних речовин, очищуючи від них воду. Однак немає достатньої кількості даних та інформації стосовно ефективності вилучення ряскою *Lemna minor* сполук азоту та фосфору зі стічних вод. З огляду на це в роботі було визначено напрям досліджень щодо встановлення ефективності очищення стічних вод від біогенних сполук азоту та фосфору вищими водними рослинами *Lemna minor*.

Встановлено необхідні умови культивування вищих водних рослин *Lemna minor* в осінньо-зимовий період; досліджено ефективність використання ряски різної маси для очищення стічних вод від біогенних сполук азоту та фосфору, застосування ряски разом з іншими вищими водними рослинами для очищення стічних вод від біогенних сполук азоту та фосфору.

З'ясовано, що для ефективності очищення стічних вод в осінньо-зимовий період необхідно регулювати режим освітленості і температурний режим.

Ключові слова: стічні води, біологічне очищення, фосфати, нітрати, *Lemna minor*.

ИСПОЛЬЗОВАНИЕ *Lemna minor* ДЛЯ ОЧИЩЕНИЯ ЗАГРЯЗНЕННОЙ ВОДЫ ОТ БИОГЕННЫХ ЭЛЕМЕНТОВ

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Целью работы было установить возможность использования *Lemna minor* для повышения эффективности очистки сточных вод от соединений азота и фосфора.

Ряску *Lemna minor* благодаря ее устойчивости часто используют для ремедиации сточных вод. Она способна к усвоению и трансформации различных соединений, способствует осаждению взвешенных веществ, насыщает воду кислородом, интенсифицирует процессы очистки. За счет быстрых темпов роста ряска поглощает большое количество загрязняющих веществ, тем самым очищая от них воду. Однако нет достаточного количества данных и информации об эффективности извлечения ряской *Lemna minor* соединений азота и фосфора из сточных вод. Именно поэтому в работе было определено направление исследований по установлению эффективности очистки сточных вод от биогенных соединений азота и фосфора водными растениями *Lemna minor*.

Установлены необходимые условия культивирования высших водных растений *Lemna minor* в осенне-зимний период; исследована эффективность применения ряски различной массы для очистки сточных вод от биогенных соединений азота и фосфора, использование ряски вместе с другими высшими водными растениями для очистки сточных вод от биогенных соединений азота и фосфора.

Установлено, что для эффективности очистки сточных вод в осенне-зимний период необходимо регулировать режим освещенности и температурный режим.

Ключевые слова: сточные воды, биологическая очистка, фосфаты, нитраты, *Lemna minor*.

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MATHEMATICAL MODEL FOR DESCRIBING THE POST-CRYOPRESERVATION VIABILITY OF FRUIT AND BERRY CUTTINGS

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A mathematical model that simplifies the determination of optimal parameters ensuring the maximum viability of frozen-thawed fruit and berry cuttings was developed. Values of the minimum amount of intracellular water η1min, which minimizes the plasmolysis probability, and η2min, which minimizes the probability of intracellular ice formation, were determined with due account for the bioobject heterogeneity.

Free water amounts Δη, forming ice crystals inside the cell during cryopreservation of different of fruit and berry varieties, were calculated. The optimal conditions for cutting dehydration (temperature Ti and incubation time t2, minimum amount of intracellular water ηmin) ensuring the maximum viability after drying and low-temperature adaptation to cryopreservation were selected. The individual features of the viability of frozen-thawed cuttings of different species were quantitatively reflected in the free water index Δη. The maximum viability of frozen-thawed birch and blackcurrant cuttings was achieved, when intracellular water was in the bound, vitrified state $\Delta\eta = 0$. The calculated $\Delta\eta > 0$ for cuttings of different varieties of apple- and pear-trees as well as of raspberry-bushes leads to a decrease in the viability, and it is impossible to obtain viable plum, apricot or grape specimens after low-temperature cryopreservation with no bound water ηc at all.

Key words: mathematical model, cryopreservation, fruit and berry cuttings, viability.

In the gardening practice, it is conventional to use vegetative propagation methods, since segregation of multiple traits in the offspring occurs upon propagation by seeds [1, 2]. Therefore, in order to preserve valuable economic and biological features, propagation by cuttings is common. Ex situ preservation, in banks of plant genetic resources, is the most reliable way of long-term storage of plant samples [1, 3–7]. Creation of cryobanks makes it possible to preserve the natural diversity of plants, where genetically modified organisms are actively used.

Cryopreservation of apical meristems of shoots is the best way to preserve their gene pool for vegetatively propagating plants [8–10]. This method requires specific conditions of in vitro culturing of meristem cells, that is, sterile conditions, nutrient media, controlled temperature, lighting and humidity [11]. Therefore, there are difficulties while creating such cryobanks. There are only reports on regeneration of plants of about 16 species from meristems that had been in liquid nitrogen [1, 10, 12–14].

Cryopreservation difficulties for plant cells are associated with their relatively large volumes. Ninety percent of this volume is occupied by the central vacuole containing free water, which is not bound in hydration shells of macromolecules, and can freeze to form ice crystals. These crystals damage biological membranes causing the death of plant cells and organs [15]. Free water must be removed by preliminary partial dehydration of cells and reduction in freezing rates [12, 15–17]. Removing an excessively large portion of free water results in the protoplast compression,

which leads to irreversible changes and plasmolysis.

To ensure the integrity of cooled and frozen-thawed cells, many researchers use drying of fruit and berry specimens from 50–56% to 20–30% [1, 4, 18, 19]. It was noted that different frozen-thawed varieties and species of crops had various viability ranging from 0 to 100% [1, 4, 8, 9, 18–22]. At the same time, there are no data on the effect of water content in fruit and berry cuttings of different species on the probability of their germination; hence, it is necessary to study the effects of different factors on dehydration of cells of miscellaneous varieties of fruit and berry crops. We believe that this is one of the reasons for low reproducibility of cryopreservation results on fruit and berry cuttings of different varieties.

Our objective was to develop a mathematical model for optimizing the cryopreservation parameters for different fruit and berry varieties.

Materials and Methods

Plant cuttings were cryopreserved in the Laboratory of Preservation of the NCPGRU of the Plant Production Institute named after V. Ya. Yuriev of NAAS (Kharkiv) [18, 23, 24]. Cuttings of the following species and varieties were taken as the study object: blackcurrant (*Ribes nigrum* L.) — Dachnitsa, Kytaivska, Sofiivska, Yuvilei, Raduzhna, Titiniia, Dar Pavlovoi, Katyusha, Nadiia, Sofiia, Alta, Shedevr, Darunok Mliieva, Lentyay, Uvertyura, Mif, Halynka, Lybid, Ben Tiran, Biriulevska, Nimfa, Yuvilaina Kopania, Krasa Lvova, Biloruska Solodka, Slavuta, Vira, Chorna Krupnoplidna, Ametyst, Ozherelye; redcurrant (*Ribes rabrum*) — Kytaivska, Joker, Sviatkova; gooseberry (*Ribes uva-crispa*) — Krasen, Malakhit, Kolobok; raspberry (*Rubus idaeus* L.) — Novost Kuzmina, Skromnitsa, Struyka; grape (*Vitis labrusca* L.) — Lydiya, Rkatseteli, Aligote, Kober 5BB, Traminer Rozovyy; cherry (*Prunus cerasus* L.) — Stepnaya, Amulet, Pamiat Artemenka, Optymistka, Nochka; duke (cherry-sweet cherry hybrid) — Alpha; sweet cherry (*Prunus avium*) — Chitinskaya Chornaya, Donchanka, Lehenda Mliieva, Dar Mliieva; plum (*Prunus domestica* L.), reine-claude group — Altana, Pamiat Materi, Oposhnyanka; Hungarian plum group — Voloshka, Oda; apricot (*Prunus armeniaca* L.) — Moldavskyi Olimpiiets, Krymskyi Medunets; apple (*Malus domestica* L.) — Belyy Naliv, Amulet; Edera; Teremok; Radost; Katya; pear (*Pyrus communis* L.), summer group — Velyka Litnia, Uliublena Klapa, autumn group — Horodyshchenska, Osinnia Vdala, winter group — Bere Kyivska, Zelena Mliiska. Birch cuttings (*Betula pubescens*) were as a control for the selected cryopreservation methods.

Cuttings were cut from one-year shoots and divided into 10 individual specimens, with a length of 5–12 cm and a diameter of 0.5–1.0 cm. Cuttings had 2–5 vegetative buds. Before the specimens were dried, their viability and initial water content were determined.

The effects of low-temperature sublimation of intracellular water and plasmolysis were evaluated from the cutting viability. The control viability of cuttings was determined after each stage of drying and cooling. For this purpose, cuttings were placed in an exsiccator with distilled water at 5 °C for 14 days for their hydration, and then cultured in vitro (in glasses with water at 20–25 °C). Swelling and development of buds indicated that the specimens under investigation were viable. The percentage of viability was estimated as the ratio of the number of cuttings with evolved buds in vitro to the total number of buds in a specimen.

The water content in native cuttings varied within 50–20% at — 2 ± 2 °C. The water content was determined by weighing samples and calculating by the following formulae:

$$\eta_i = \frac{m_0 - m_k}{m_0} \times 100\%, \quad (1)$$

$$\eta_s = \frac{m_s}{m_0} \times \eta_0 \times 100\%, \quad (1a),$$

where: η_i — water content of a sample (%): i = 0 native sample; i = 1 after drying; i = 2 after cryopreservation; i = 3 after rehydration; m_0 — initial weight of a native sample (g); m_k — final weight of a sample after dehydration until constant weight (g); η_s — water content in a sample at a drying stage (%); m_s — sample weight at a drying stage (g).

To preserve the residual moisture, cuttings were waxed or paraffinned at both ends prior to cooling. Prior to freezing, they were exposed at 4 °C for 10–15 days and at –5 °C for 2 weeks to several months.

Fruit and berry cuttings were cooled on a step-by-step basis at a rate of 0.01–0.1 °C/h to –5÷–30 °C with the increment of 5 °C and exposed at these temperatures for 1, 3 or 7 days, respectively, in 2-liter household thermoses placed in a refrigerator. Specimens

were cooled from −20 and −30 °C to −196 °C by direct immersion into liquid nitrogen at a rate of 600–800 °C/min. Specimens were stored in liquid nitrogen for a period from 1 day to 1 year. They were thawed at the rate of 70 °C/min by direct placing in a room at 20 °C [25].

The data were statistically processed on a computer using conventional formulae in the standard program "Stadia" to compose regression equations.

Results and Discussion

The main criterion of the viability of a frozen-thawed bio-object is its ability for further development. Therefore, an in vitro culture is the simplest and most reliable way to quantify the viability of plant cuttings. Determination of the cutting viability is maximally reliable with the minimal usage of a bioobject, if the polynomial regression dependence (Fig. 1) is applied to experimental data of cultured specimens in combination with the dependence derived from Verhulst's formula

$$V_{k1}(t_1) = \frac{V_0 K e^{\mu_1 t_1}}{K - V_0 + V_0 e^{\mu_1 t_1}}, \qquad (2)$$

where: $V_k(t_1)$ — change in the viability of native cuttings related to the culturing time $V_k(t_1) = n/n_0$, a relative value expressed in relative units; n — the number of viable cuttings at a culturing stage $t_1 > 0$; n_0 — the total number of cuttings at the start of culturing $t_1 = 0$; V_0 — initial viability of cuttings at $t_1 = 0$, $V_0 < 1$; K — the maximum theoretical value of the cutting viability $K = 1$; μ_1 — specific rate of decrease in the cutting viability in culture reflecting individual characteristics of a bioobject's resistance and determined by simulation modeling (Fig. 1) or in the inverse coordinate system, day^{-1}; t_1 — culturing time, days.

Cuttings were dehydrated stepwise at sub-zero temperatures of −5÷−10 °C with concurrent empirical determination of the minimum moisture η_{min}, at which a high viability was observed according to the results of culturing. The cutting viability after removal of excess moisture was evaluated using Moser's formula

$$V_\eta(\eta) = \eta_0 + \frac{\eta^\alpha}{K_\eta + \eta^\alpha}, \qquad (3)$$

where η — water content in a cutting, η_0 — the empirically obtained correction coefficient, α and K_η — Moser's constants that reflect individual features of the bioobject viability, when its water content changes (determined by simulation modeling from experimental values, Fig. 2).

The post-dehydration viability was evaluated by the results of culturing

$$V_\eta(t_1\eta_{min}) = V_k(t_{1max}) \times V_\eta(\eta_{min}), \qquad (4)$$

where η_{min} — the minimum water content in cuttings, which ensures their maximum viability $V_\eta(\eta_{min})$ (Fig. 2).

To bind the residual free intracellular water, temperature adaptation of cuttings was carried out at T = −20÷−30 °C. The cutting viability in the temperature range close to the

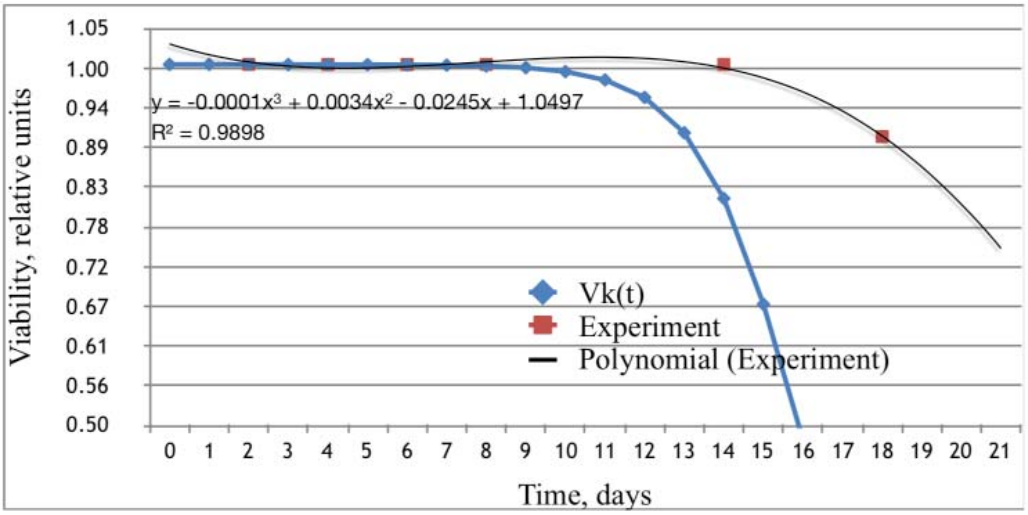

Fig. 1. **Changes in the viability of warty birch cuttings related to the *in vitro* culturing time**
Specific rate of decrease in the cutting viability — $\mu_1 = -0.78$; water content — $\eta = 38\%$

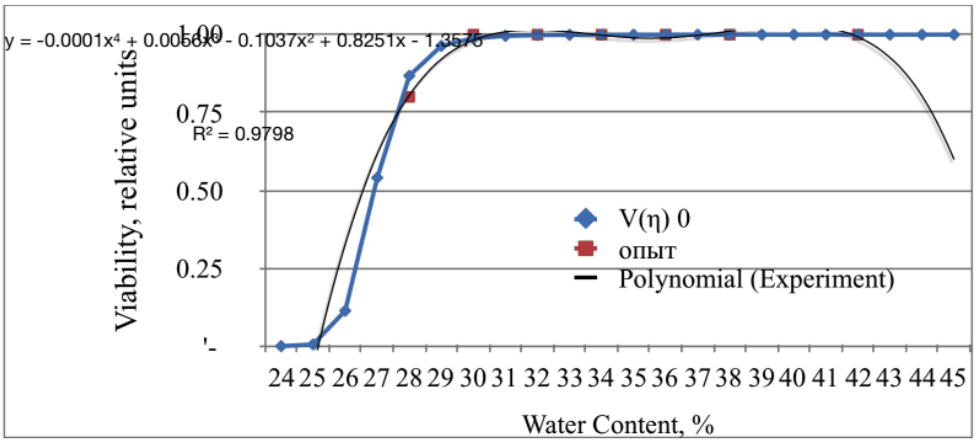

Fig. 2. **Changes in the viability of warty birch cuttings related to the *in vitro* culturing time after drying to a specified water content $\eta_{min} = 30\%$**
Moser's constants $K_s = 36770$, $\alpha = 7.70$, and the correction coefficients $\eta_0 = 24$

temperature of intracellular ice formation was determined experimentally and described by Verhulst's formula

$$V_T(t_2) = \frac{V_\eta(\eta_{\min}) \times K e^{\mu_2 t_2}}{K - V_\eta(\eta_{\min}) + V_\eta(\eta_{\min}) \times e^{\mu_2 t_2}}, \quad (5)$$

where: $V_T(t_2)$ — change in the cutting viability related to time at a specified temperature $V_T(t_2) = n/n_0$, a relative value expressed in relative units; n — the number of viable cuttings $t_2 > 0$; n_0 — the total number of cuttings in a sample $t_2 = 0$; V_η (η_{min}) — post-drying viability of cuttings at $t_2 = 0$, $K = 1$; μ_2 — specific rate of decrease in the cutting viability upon temperature adaptation reflecting individual characteristics of a bioobject's resistance and determined by simulation modeling (Fig. 3) or in the inverse coordinate system, day^{-1}; t_2 — exposure time, days.

The optimal exposure time t_2 at a temperature close to the intracellular crystallization point T^i and η_{2min} were determined experimentally from the obtained dependence ϕ (3) (Fig. 4).

The post-temperature adaptation viability were evaluated by the results of culturing

$$V_T(t_1, t_2, \eta_{2\min}, T^i) = V_\eta(t_1, \eta_{2\min}) \times V_T(t_2, T^i). \quad (6)$$

The viability of frozen-thawed cuttings V_d was estimated by the experimental results as the ratio of the number of viable cuttings n to the total number of cuttings in a sample no: $V_d = n/n_0$ (Fig. 5). This value depends on the initial viability $V_T(t_2, T^i)$ and the probability of intracellular crystallization P^i_k

$$V_d = V_T(t, T^i) \times (1 - P_k^i). \quad (7)$$

The probability of intracellular crystallization can be calculated as follows:

$$\eta_c = \eta_{\min} \frac{V_d}{V_T}, \quad (8)$$

where η_c — the fraction of bound (vitrified) water, and $\Delta\eta = \eta_{min} - \eta_c$ — the fraction of free water forming crystals at a temperature below T^i.

The amount of bound water can be calculated as follows:

$$P_k^i = \frac{\eta_{\min} - \eta_c}{\eta_{\min}}. \quad (9)$$

The post-dehydration and post-temperature adaptation viability of frozen-thawed cuttings was estimated by the results of culturing

$$V_d(t_1, t_2, \eta_k, T, \eta_c) = V_T(t_1, t_2, \eta_k, T) \times V_d(\eta_c). \quad (10)$$

The results show that birch cuttings retain their initial viability after different drying regimes, step-wise cooling to −20÷−30 °C, provided they are cooled at the rate of 0.1 °C/h, long-term storage at — 196 °C and subsequent warming at the rate of 70 °C/min. At the same time, we found (Fig. 2) that the water content in specimens should not be below 32% upon their drying. Below this critical value, the viability of specimens decreases dramatically as a result of excessive dehydration of cells (plasmolysis effect). At −30÷−196 °C, this value can be reduced to 14%. The calculations show (Fig. 5) that all intracellular water is in a bound state. This ensures a high viability of frozen-thawed birch cuttings.

To determine the optimum parameters ensuring the maximum viability of frozen-thawed cuttings of different varieties of

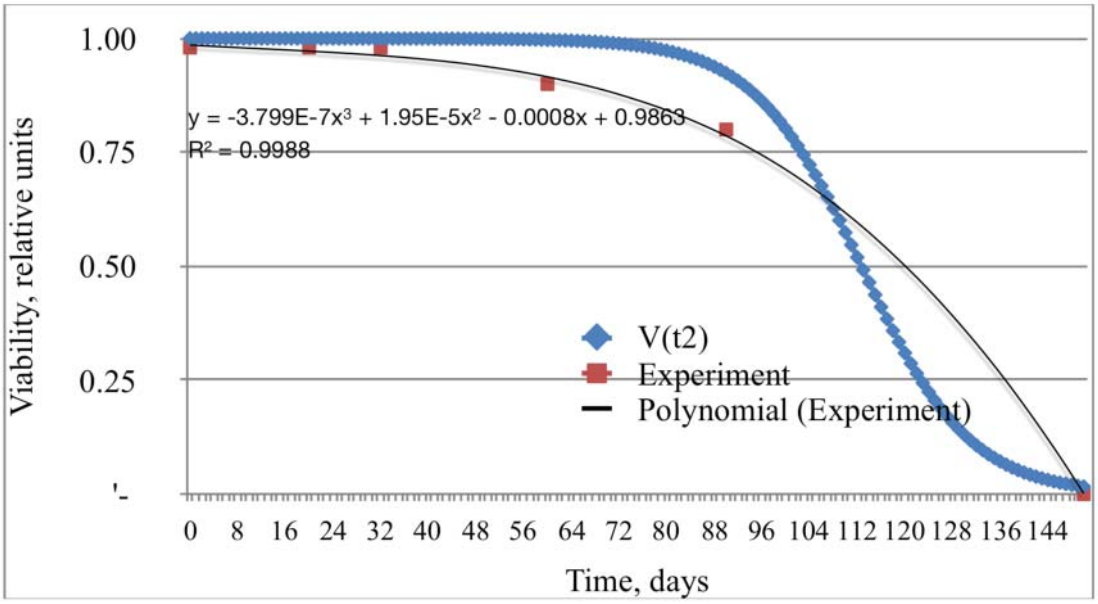

Fig. 3. **Changes in the viability of warty birch cuttings related to the exposure time at a temperature close to the temperature of intracellular crystal formation**
The initial viability of cuttings at a specified temperature — $V(t_2) = 0.99$;
specific rate of decrease in the cutting viability upon temperature adaptation $\mu_2 = -0.11$

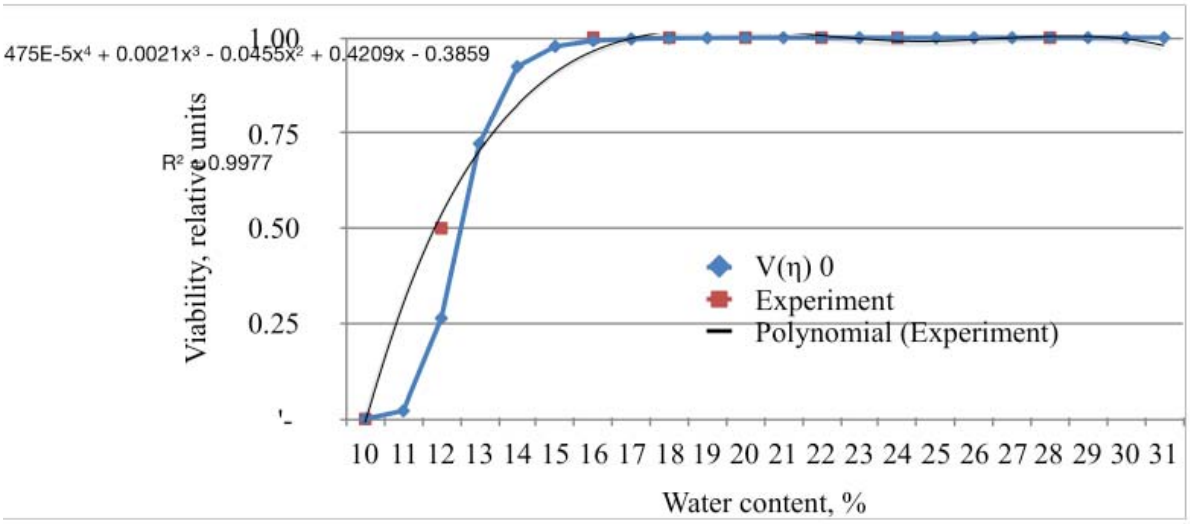

Fig. 4. **Changes in the viability of warty birch cuttings related to the in vitro culturing time after temperature adaptation to a specified water content $\eta_{min} = 15\%$**
Exposure time at −20 °C $t_2 = 30$ days.
Moser's constants $K_s = 36770$, $\alpha = 7.70$, and the correction coefficient $\eta_0 = 10$

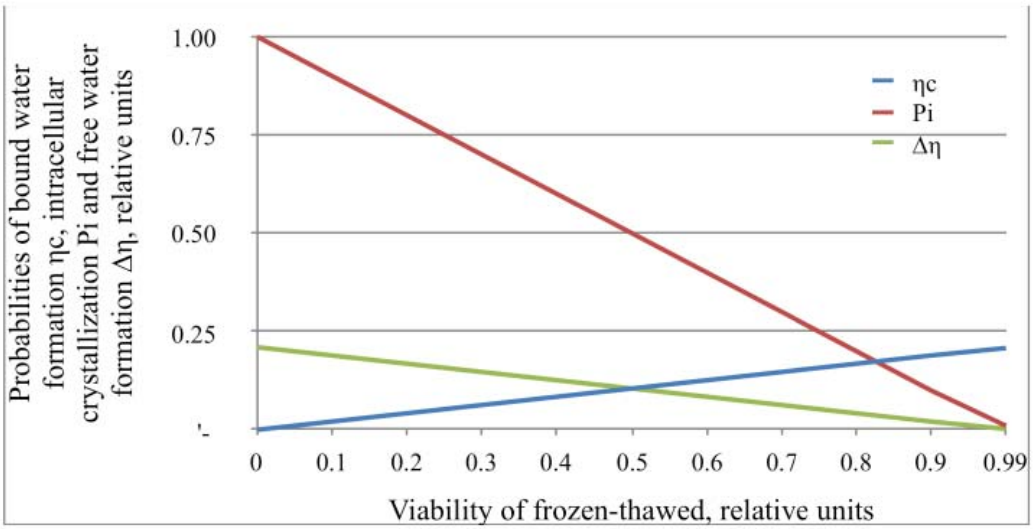

Fig. 5. **Dependence of the viability of frozen-thawed warty birch cuttings related to the *in vitro* culturing time after temperature adaptation to a specified water content $\eta_{min} = 20\%$, $t_2 = 30$ days**

Table 1. **The viability *V* and water content η of cuttings of different berry varieties evaluated at the cryopreservation, stages**

Species	Variety	Cutting viability after cooling to					
		−10 °C		−30 °C		−196 °C	
		Experiment	η_{1min}, %	Experiment	η_{2min}, %	Experiment	Δη,%
Black-currant	Dachnitsa	98.0±2[a]	38	94.0 ±4[a]	30	92.0±5.8[a]	1
	Yuvilei	80.0±7.1[b]	37	62.0±8.6[b]	31	54.0±13.6 [b]	4
	Sofiivska	94.0±4	39	60.0±14.1[b]	30	6.0±2[c]	27
	Kytaivska	86.0±4	38	12.0±3.7[c]	32	0.0±0 [d]	32
Red-currant	Kytaivska	92±3.7[a]	40	84.0±6.8[a]	30	54.0±7.5[a]	11
	Joker	68.0±3.7 [b]	42	6.0±2.4[b]	33	4.0±2.4[b]	11
	Sviatkova	66.0±4.0	38	0.0±0[c]	30	0.0±0	-
Goose-berry	Krasen	84.0±8.1	35	70.0±7.1	25	30.0±7.1	14
	Malakhit	82.0±8.0[a]	36	68.0±7.3 [a]	26	48.0±15.9[a]	8
	Kolobok	40.0 ±3.2[b]	35	8.0±3.7 [b]	25	0.0±0[b]	25
Rasp-berry	Novost Kuzmina	96±2.4	37	82.0±8.0	21	70.0±7.1[a]	3
	Struyka	94±2.4[a]	37	58.0±12.8[a]	22	0.0±0[b]	22
	Skromnitsa	68.0±3.7[b]	37	4.0±2.4[b]	20	0.0±0	20
Grape	Lidiya	94.0±2.4	40	84.0±2.4[a]	28	0.0±0	28
	Rkatseteli	94.0±4.0	41	22.0±5.8[b]	27	0.0±0	27
	Aligote	92.0±3.7	40	20.0±7.1[b]	26	0.0±0	26
	Kober 5BB	90.0±3.2	42	18.0±3.7 [b]	27	0.0±0	27
	Traminer Rozovyy	86.0±4.2	40	0.0±0[c]	27	0.0±0	-

η_{1min} — the minimum amount of intracellular water minimizing the probability of plasmolysis and η_{2min} — the minimum amount of intracellular water minimizing the probability of intracellular ice formation, Δη — the amount of free intracellular water, %.

fruit and berry crops (Tables 1, 2 and 3), we carried out experiments in a similar fashion to those on birch cuttings. In the experiments, we investigated cuttings with the initial viability of ≥ 99%. To improve the result reproducibility, we used the proposed mathematical model ϕ (2–10) for evaluation of the viability at 3 stages of cryopreservation.

The technological parameters for each variety of blackcurrant, redcurrant, gooseberry, raspberry and grape were optimized via step-by-step cooling to –10 °C followed by temperature adaptation at –30 °C and freezing at –196 °C. The maximum viability was achieved by exposure at –5 and –10 °C for 14–60 days, stepwise cooling of specimens at a rate of 0.1–0.5 °C/h from –10 to –20 and –30 °C, 3–7-day exposure, direct immersion in liquid nitrogen, storage for 1 to 30 days, and warming at a rate of 70–100 °C/min. The exposure length at –5 and –10 °C was determined by monitoring the water content in specimens set in accordance with the maximum permissible value established for each plant variety.

Analysis of the viability of frozen-thawed cuttings of berry crops showed that within one species the average values for different varieties varied by up to 92% in blackcurrant, 54% in redcurrant,

Table 2. **The viability *V* and water content η of cuttings of different drupaceous varieties evaluated at the cryopreservation stages**

Species	Variety	Cutting viability after cooling to					
		–10 °C		–30 °C		–196 °C	
		$M \pm m$, %	η_{1min}, %	$M \pm m$, %	η_{2min},%	$M \pm m$, %	$\Delta\eta$,%
Cherry	Stepnaya	96.0±2.4	34	78.0±8.6	33	64.0±12.9	6
	Amulet	94.0±4.5	35	90.0±8.6[a]	32	68.0±8.6	8
	Optymistka	92.0±3.7	34	62.0±10.2[b]	33	48.0±10.7	7
	Pamiat Artemenka	96.0±2.4	35	94.0±2.4[a]	34	34.0±2.4[a]	22
	Nochka	94.0±2.4	35	64.0±9.3[b]	35	8.0±3.7 [b]	31
Sweet cherry	Alpha*	98.0±2.0	35	76.0±9.3	33	60.0±13.0	7
	Donchanka	90.0±9.7	35	72.0±10.8[a]	35	54.0±10.8[a]	9
	Lehenda Mliieva	94.0±2.4[a]	36	28.0±8.6[b]	31	0.0±0.0[b]	31
	Chitinskaya Chornaya	58.0±12.0[b]	37	12.0±3.7	30	0.0±0.0	30
Plum	Oposhnyanka	92.0±3.7	40	28.0±8.6	28	0.0±0.0	28
	Pamiat Materi	90.0±3.2	41	22.0±8.6	20	0.0±0.0	20
	Voloshka	86.0±4.0	40	12.0±3.7	19	0.0±0.0	19
	Oda	78.0±7.3	42	10.0±3.2	18	0.0±0.0	18
	Altana	80.0±7.1	40	8.0±3.7	18	0.0±0.0	18
Apricot	Krymskyi Medunets	54.0±10.8	35	12.0±3.7	21	0.0±0.0	21
	Moldavskyi Olimpiiets	40.0±7.1	40	8.0±3.7	28	0.0±0.0	28

Footnote, see Table 1.

Table 3. **The viability V and water content η of cuttings of different apple and pear varieties evaluated at the cryopreservation stages**

Species	Variety	Cutting viability after cooling to					
		−10 °C		−30 ° C		−196 ° C	
		$M\pm m$, %	η_{1min},%	$M\pm m$, %	η_{2min}, %	$M\pm m$, %	Δη, %
Apple	Radost	92.0±3.7	40	52.0±3.7	19	36.0±5.1^{a}	6
	Teremok	90.0±3.2^{a}	42	48.0±3.7	22	22.0±8.0	12
	Belyy Naliv	70.0±4.5^{b}	41	52.0±3.7^{a}	19	8.0±3.7^{b}	16
	Katya	46.0±7.5^{c}	40	6.0±2.4^{b}	21	0.0±0.0^{c}	21
	Sprint	40.0±7.1	42	0.0±0.0^{c}	29	0.0±0.0	-
	Amylet	38.0±7.3	43	0.0±0.0	28	0.0±0.0	-
	Edera	26.0±8.4	40	0.0±0.0	23	0.0±0.0	-
Pear	Velyka Litnia	94.0±4.0	41	80.0±7.1	28	74.0±8.7^{a}	2
	Uliublena Klapa	90.0±3.2	40	82.0±5.8	28	28.0±7.3^{b}	18
	Osinnia Vdala	94.0±2.4	40	92.0±2.0^{a}	25	86.0±4.0^{a}	2
	Zelena Mliiska	88.0±3.7	40	72.0±8.6^{b}	21	8.0±3.7^{c}	19
	Bere Kiyvska	86.0±4.0^{a}	43	4.0±2.4^{c}	22	0.0±0.0^{d}	22
	Horodyshchenska	46.0±7.5^{b}	40	6.0±2.4	28	0.0±0.0	28

Footnote, see Table 1.

48% in gooseberry, and 70% in raspberry (Table 1). That is, the species difference of berry cuttings has approximately twice as much impact on the viability of frozen-thawed specimens as the variety one does. This is attributed to heterogeneity of the bioobject, namely, to the amount of free intracellular water Δη.

A similar dependence was observed for different varieties of drupaceous plants (Table 2). There are many-fold differences in the viability of frozen-thawed cuttings, depending on the variety. The exposure length at −10 and −30 °C was determined by monitoring the water content in specimens set in accordance with the maximum permissible value for each species of cuttings: 30–37% for cherry and sweet cherry and 35–45% for plum and apricot.

The cause of discrepancies in the viability is associated with intracellular crystallyzation, which is confirmed by numerical values of free intracellular water Δη calculated for these specimens. Significant differences for different varieties are likely to depend on sugar concentrations in the intracellular environment.

Similarly, the water contents in specimens were optimized in accordance with the maximum permissible value of 40% and 19–28% at −10 and −30 °C, respectively, obtained for apple and pear. The results show (Table 3) that there are many-fold differences in the viability of frozen-thawed cuttings and that the viability depends on the free intracellular water amount Δη estimated for each variety.

Analysis of the cryopreservation efficiency for different varieties of fruit and berry crops shows that within one species the average values vary for different varieties: by up to 90% in blackcurrant, 24% in redcurrant, 79% in cherry, 54% in apple, and 82% in pear. Such significant differences in the values obtained for cuttings of different species and varieties are accounted for influence of individual characteristics of a bioobject reflected in the amounts of free, unbound water.

Our review of the literature shows that many factors influence the results of the solution of cryobiological challenges. Experimental studies of mechanisms of all these phenomena are extremely difficult. Meanwhile, the problems of analysis and optimization of these processes must be solved. For these purposes, experimentally statistical methods are successfully applied for building descriptive mathematical models of objects and investigating relationships between the response of a system to changes in the parameters of interest.

No feedback during research is also an important obstacle to the use of existing methods of multi-factor optimization, which is manifested in the possibility of constructing graphical presentations depicting relationships between parameters of interest, both in the course of experiments and analysis of data. Thus, opportunities to trace causality mechanisms, which are a basis for constructing analytical models, are lost. At the same time, an essential shortcoming of conventional methods of research optimization in cryobiology is their specificity, that is, the suitability for certain areas of research is a solution to biological or physical problems. To study a cryobiological object, there is a need for an analytical mathematical model to increase reproducibility and comparability of data obtained. This allows us to accelerate the process of solving cryobiological problems associated with large usage of a bioobject and hereto related problems both of ethical and of economic nature.

The probability of generating fully functional offspring from frozen-thawed cuttings of fruit and berry crops is affected by their initial state and the effectiveness of a cryopreservation method. Basing on the study conducted, we propose a mathematical model (expressions 2–10) for evaluation of the viability of cuttings of different varieties at several stages of cryopreservation. Analysis of the post-cryopreservation viability of fruit and berry cuttings showed that the results depended on the variety as well as on cryopreservation and culturing methods. The model presented makes it possible to compile regulations for monitoring and optimization of cryopreservation of cuttings of different species and varieties of fruit and berry crops.

Thus,

1. The mathematical model providing the possibility of quantification of optimal cryopreservation parameters for different varieties of fruit and berry crops was developed.

2. The minimum values of the intracellular water amounts η_{1min} and η_{2min} minimizing the probabilities of plasmolysis and intracellular ice formation, respectively, were determined with due account for the heterogeneity of a bioobject.

3. The values of free and bound water quantitatively determining the viability of cuttings were estimated with due account for the individual characteristics of different fruit and berry varieties.

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МАТЕМАТИЧНА МОДЕЛЬ ОПИСУ ЖИТТЄЗДАТНОСТІ ЖИВЦІВ ПЛОДОВО-ЯГІДНИХ КУЛЬТУР ПІСЛЯ КРІОКОНСЕРВУВАННЯ

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Розроблено математичну модель, що спрощує процес визначення оптимальних параметрів, які забезпечують максимальну життєздатність деконсервованих живців плодово-ягідних культур. Визначено величини мінімальної кількості внутрішньоклітинної води — η_{1min}, що мінімізують ймовірність плазмолізу і — η_{2min} утворення внутрішньоклітинного льоду з урахуванням гетерогенності біооб'єкта.

Розраховано величини вільної води — $\Delta\eta$, що утворюють кристали льоду всередині клітини за кріоконсервування різних сортотипів плодово-ягідних культур. Визначено оптимальні умови зневоднення живців (температура T^i і час витримки t_2, мінімальна кількість внутрішньоклітинної води — η_{min}), що забезпечують максимальну життєздатність під час їх сушіння й низькотемпературної адаптації до кріоконсервування. Індивідуальні особливості життєздатності деконсервованих живців різних порід кількісно відображено в показниках вільної води $\Delta\eta$. Максимальної життєздатності деконсервованих живців берези і чорної смородини досягнено за умови, коли внутрішньоклітинна вода знаходиться у зв'язаному, вітрифікованому стані $\Delta\eta = 0$. Розраховане $\Delta\eta > 0$ для живців різних сортів яблук, груш, малини призводить до зниження життєздатності, а повна відсутність зв'язаної води — η_c для сливи, абрикоса, винограду унеможливлює отримання життєздатних зразків після низькотемпературного кріоконсервування.

Ключові слова: математична модель, кріоконсервування, живці плодово-ягідних культур, життєздатність.

МАТЕМАТИЧЕСКАЯ МОДЕЛЬ ОПИСАНИЯ ЖИЗНЕСПОСОБНОСТИ ЧЕРЕНКОВ ПЛОДОВО-ЯГОДНЫХ КУЛЬТУР ПОСЛЕ КРИОКОНСЕРВИРОВАНИЯ

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Разработана математическая модель, упрощающая процесс определения оптимальных параметров, обеспечивающих максимальную жизнеспособность деконсервированных черенков плодово-ягодных культур. Определены величины минимального количества внутриклеточной воды — η_{1min}, минимизирующие вероятность плазмолиза и — η_{2min} образования внутриклеточного льда с учетом гетерогенности биообъекта.

Рассчитаны величины свободной воды — $\Delta\eta$, образующие кристаллы льда внутри клетки при криоконсервировании различных сортотипов плодово-ягодных культур. Определены оптимальные условия обезвоживания черенков (температура T^i и время выдержки t_2, минимальное количество внутриклеточной воды — η_{min}), обеспечивающие максимальную жизнеспособность при их сушке и низкотемпературной адаптации к криоконсервированию. Индивидуальные особенности жизнеспособности деконсервировванных черенков различных пород количественно отражены в показателях свободной воды $\Delta\eta$. Максимальная жизнеспособность деконсервированных черенков березы и черной смородины достигнута при условии, когда внутриклеточная вода находится в связанном, витрифицированном состоянии $\Delta\eta = 0$. Рассчитанное $\Delta\eta > 0$ для черенков различных сортов яблок, груш, малины приводит к снижению жизнеспособности, а полное отсутствие связанной воды — η_c для сливы, абрикоса, винограда не дает возможности получить жизнеспособные образцы после низкотемпературного криоконсервирования.

Ключевые слова: математическая модель, криоконсервирование, черенки плодово-ягодных культур, жизнеспособность.