NATIONAL ACADEMY OF SCIENCES OF UKRAINE
Palladin Institute of Biochemistry

# BIOTECHNOLOGIA ACTA

***Vol. 14, No 6, 2021***

***BIMONTHLY***



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According to the resolution of the Presidium of the National Academy of Sciences of Ukraine from 27.05.2009 №1-05 / 2 as amended on 25.04.2013 number 463 Biotechnologia Acta has been included in High Attestation Certification Commission list of Ukraine for publishing dissertations on specialties "Biology" and "Technology".

Certificate of registration of print media КВ series №19650-9450ПР on 01.30.2013

Literary editor — H. **Shevchenko**; Computer-aided makeup — **O. Melezhyk**
Authorized for printing 30.12.2021, Format — 210×297. Paper 115 g/m$^2$. Gaqrn. SchoolBookC. Print — digital. Sheets 11.6. An edition of 100 copies. Order 6.6. Make-up page is done in Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. Print — **O. Moskalenko** FOP

NATIONAL ACADEMY OF SCIENCES OF UKRAINE
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# BIOTECHNOLOGIA ACTA

## Scientific journal

*Bimonthly*

*Vol. 14, No 6, 2021*

### *REVIEWS*



### *EXPERIMENTAL ARTICLES*

**НАЦІОНАЛЬНА АКАДЕМІЯ НАУК УКРАЇНИ**
**Інститут біохімії ім. О. В. Палладіна**

# BIOTECHNOLOGIA ACTA

## Науковий журнал

***Том 14, № 6, 2021***

### *ОГЛЯДИ*



### *ЕКСПЕРИМЕНТАЛЬНІ СТАТТІ*

UDC **577.112.7:612.115** **https://doi.org/10.15407/biotech14.06.005**

# MOLECULAR MECHANISMS OF INHIBITION OF INTRAVASCULAR, AND ACTIVATION OF EXTRAVASCULAR THROMBUS FORMATION

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Received 16.08.2021
Revised 30.10.2021
Accepted 30.21.2021

The hemostasis system is designed to maintain a balance between the processes of blood clotting, anticoagulation, and fibrinolysis, as well as to ensure constant effective blood circulation in the body and rapid cessation of bleeding in the event of their occurrence. The procoagulant potential of the hemostasis system is based on molecular mechanisms that lead to the formation of fibrin in the bloodstream, which is the framework of the thrombus, and to the aggregation of platelets — the basis of the thrombus body. The anticoagulant potential of blood plasma is provided by mechanisms aimed at inhibiting blood coagulation processes.

Thorough study of these mechanisms will open up numerous treatments for pathologies associated with both intravascular thrombosis and bleeding of various origins. The purpose of this review is to analyze ways to prevent intravascular thrombosis and stimulate extravascular thrombosis. The review describes and analyzes available data on thrombosis prevention, in particular, direct and indirect anticoagulants and antiplatelet, as well as methods of effective stimulation of thrombosis, which is necessary in case of vascular damage.

This analysis will determine the nodal points of the protein network of the hemostasis system, whose action by specific molecular effectors will control the process of thrombosis.

***Key words:*** anticoagulants, antiplatelets, activator, blood clotting, thrombosis.

The hemostasis system is designed to maintain a balance between the processes of blood clotting, anticoagulation and fibrinolysis, to ensure constant efficient blood circulation in the body and rapid cessation of bleeding in the event of their occurrence. To do this, the enzyme-cell complex of the hemostasis system must at any time, depending on the needs of the body, initiate either coagulation, anticoagulant mechanisms, or fibrinolysis. The basis for providing procoagulant and anticoagulant potentials of the hemostasis system are molecular mechanisms, which have been the subject of research for many years. Such studies are not only fundamental but also important in practice, as they allow influencing the pro- and anticoagulant potential of the hemostasis system, which opens the possibility of treating a number of serious diseases associated with intravascular thrombosis and bleeding of various origins.

Intravascular thrombosis occurs due to pathological activation of the blood coagulation system and is manifested in the formation of fibrin deposits, fibrin clots, and thrombi, which completely or partially block the lumen of the vessel, impeding the blood supply to vital tissues and organs. Intravascular thrombosis is the cause of diseases such as myocardial infarction, pulmonary embolism, ischemic stroke, deep vein thrombosis, and often accompanies a number of cardiovascular, metabolic and cancer diseases, surgical, gynecological and obstetric diseases. It is one of the main causes of patient mortality. Timely proper antithrombotic and sometimes

fibrinolytic therapy is required to correct such complications.

The hemorrhages are no less dangerous for human life and health, if the extravascular thrombosis which is designed to stop the bleeding is not effective enough. Bleeding that cannot be stopped in time is the cause of death from various injuries, catastrophes, combat injuries, in surgery, obstetrics and in patients with hemophilia.

That is why this review is devoted to the analysis of available and promising ways to prevent intravascular thrombosis and stimulate extravascular thrombosis in case of vascular damage.

*Intravascular pathological thrombosis and basic antithrombotic agents*

Intravascular pathological thrombosis is one of the leading causes of death in the world. It can occur in cardiovascular diseases, surgery, injuries and burns, cancer and metabolic diseases, sepsis, immobilization, infectious and inflammatory processes, including COVID-19 [1], etc.

Intravascular thrombosis occurs due to the following pathogenic factors (Virchow's triad): 1) damage to endothelial cells with exposure to thrombogenic subendothelial structures and factors; 2) hyperactivation of the blood coagulation system, which leads to the appearance of active thrombin in the bloodstream, pathological activation of platelets, decreased fibrinolytic potential; 3) decrease in blood flow velocity [2].

Understanding the mechanisms associated with hyperactivation of the blood coagulation system and pathological platelet activation is the basis for the development and improvement of antithrombotic agents (Fig. 1) [3, 4]. Fig. 1 presents a generalized scheme of the hemostasis system, which combines the system of blood clotting, which leads to the formation of a three-dimensional network of fibrin, the framework of the thrombus; platelet link, the activation of which leads to the formation of platelet "body" of the thrombus; the protein C system, which regulates the activity of the blood coagulation system; the fibrinolysis system, which provides hydrolysis of polymeric fibrin.

The following symbols are used in the legend of Fig. 1: Fg — fibrinogen; Fn — fibrin; fXIIIa — activated factor XIII; Plat — platelets; $Plat_a$ — activated platelets; fIXa — activated factor IX; fXIa — activated factor XI; fXIIa — activated factor XII; KI — kallikrein; TF — tissue factor; fXa — activated factor X; fVIIa — activated factor VII; PCa — activated protein C; Coll — collagen; Adr — adrenaline; TxA — thromboxane A; ADP — adenosine diphosphate; Pg — plasminogen; Pm — plasmin; tPA — tissue plasminogen activator; uPA — urokinase; FDPs — fibrin degradation products; DD — D-dimer; $E_1$ — high molecular weight E-fragment; $E_2$, $E_3$ — hydrolyzed E-fragment.

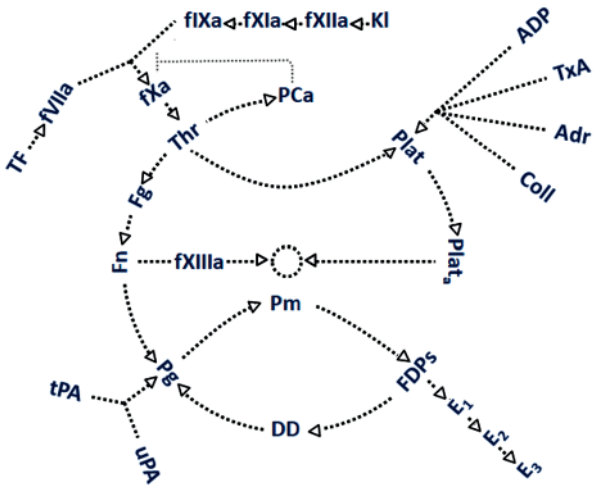


*Fig. 1.* **Generalized scheme of the hemostasis system, combining blood clotting, platelet, protein C and fibrinolysis systems**

When the blood coagulation factor X is activated as a result of a cascade of successive enzymatic reactions through either the external or internal pathway, it then activates prothrombin to thrombin by limited proteolysis (Fig. 1) [5–8].

Thrombin converts fibrinogen, which circulates freely in the blood, to fibrin, capable of spontaneous polymerization. Fibrin polymerizes with the formation of oligomers and polymers of different lengths, which then laterally associate, forming fibrils, branching and eventually forming a three-dimensional fibrin network, which serves as the framework of the thrombus [9].

The body of the thrombus, which fills the fibrin framework, is formed by aggregated platelets. Inactive non-nuclear round cells are activated by direct thrombin action and by the vascular endothelial damage. Active platelets change shape and aggregate due to multipotent binding to fibrinogen molecules and fibrin of the blood clot.

The main known antithrombotic agents are anticoagulants, which prevent the formation and polymerization of fibrin, and antiplatelet agents, which prevent excessive activation and aggregation of platelets.

*Anticoagulants*

Anticoagulants are substances that inhibit the rate of fibrin formation in the bloodstream, and therefore thrombosis.

According to the mechanism of action, there are direct- and indirect-acting anticoagulants.

*Direct-acting anticoagulants* immediately affect coagulation factors. According to the mechanism of action, they are divided into those that depend on or do not depend on antithrombin III.

Antithrombin-dependent anticoagulants are unfractionated high molecular weight heparin (UFH); low molecular weight fractionated heparins (LMWH), such as enoxaparin sodium, nadroparin calcium, bemiparin sodium, dalteparin sodium, tinzaparin sodium, etc., factor Xa inhibitors (fondaparinux sodium, hydraparinux sodium), and heparinoids.

Antithrombin-independent anticoagulants are preparations of antithrombin III and hirudin; preparations that directly inhibit thrombin, such as dabigatran etexilate, ximelagatran, melagatran, argatroban, bivalirudin and others; drugs that directly inhibit factor Xa (rivaroxaban, apixaban, edoxaban, betrixaban, otamixaban) and preparations that inhibit factors Va and VIIIa (human protein C, drotrecogin alpha) [10, 11].

*Indirect anticoagulants* include substances that inhibit the formation of coagulation factors, in particular prothrombin, in the liver and thus reduce the coagulation potential. They are also called vitamin K antagonists. These are coumarin derivatives (mono- and dicoumarins: warfarin, marcumar, syncumar, acenocoumarol, dicoumarin, tromexane) and indandione derivatives (phenindione, phenylin, dipaxin, omefin) [11].

*Indirect anticoagulants*

Indirect anticoagulants differ in that they do not immediately affect any of the links in the cascade of the blood coagulation system. They act only indirectly, inhibiting the synthesis, namely the carboxylation of vitamin K-dependent factors of the blood coagulation cascade, mainly prothrombin [12], the precursor of thrombin. The main preparation in this group is an affordable drug warfarin. Today, warfarin therapy is widely used in patients at risk of intravascular thrombosis.

Warfarin blocks the formation of vitamin-K-dependent coagulation factors in the liver, namely the factors II, VII, IX and X. The concentration of these components in the blood is reduced and the coagulation process is slowed down. Regular intake of warfarin lessens the risk of thrombosis, but does not eliminate the risk of bleeding, requires certain dietary restrictions and constant laboratory monitoring of the hemostasis system, MHO, to adjust the dosage, which is difficult to constantly perform in an outpatient setting [13, 14].

*Direct coagulants*

*Heparins*

The most common direct-acting anticoagulants are high molecular weight unfractionated heparin (UFH) and low molecular weight fractionated heparins (LMWH) [11–15].

Unfractionated heparin is a sulfated glycosaminoglycan (mucopolysaccharide) of mixed polysaccharide nature. It consists of polymers derived from D-glycosamine and L-iduronic or D-glucuronic acid [16, 17], with a molecular weight of from 3,000 to 30,000 Da, in commercial medicinal heparin preparations mainly from 12,000 to 16,000 Da [18].

Heparin has a negative charge in the solution, which promotes its interaction with proteins involved in blood clotting. In combination with antithrombin III, UFH primarily inhibits activity of thrombin and factor Xa. Binding of antithrombin III to heparin leads to conformational rearrangements in the molecule, which provide greater affinity of antithrombin III to thrombin and other coagulation factors and, consequently, increase the inhibitory properties of antithrombin by 1000 to 5000 times [19].

The heparin molecule contains a large number of reducing agents, and therefore has the ability to bind to free plasma proteins and blood cells. Heparin is also moderately related to fibrinogen and prothrombin, which also contributes to the inhibition of plasma procoagulant activity. In addition, UFH acts on other factors of hemocoagulation, in particular on factors IX, XI, Va and XII. Thus, the action of heparin extends to virtually all parts of the blood clotting system [20, 21]. However, the main mechanism of action of UFH on the coagulation system is still the inhibition of coagulation factors Xa and thrombin.

Smaller heparin molecules are able to inhibit factor Xa activity well and do not potentiate the effect of antithrombin in inhibiting thrombin. Longer-chain heparins increase antithrombin activity, leading to thrombin inhibition. Heparins that activate antithrombin are a third of those that are part of “unfractionated” heparin [22].

Heparin drugs injected into the blood remain active for 4-6 hours, during which time the substance is inactivated by the enzyme heparinase produced by the liver and kidneys. As heparinase activity of blood of patients is not defined, careful control of indicators of blood coagulation at the introduction of heparin is necessary for timely correction of a dose of drug [23]. Therefore, UFH is more convenient and safer to use in a hospital.

Low molecular weight heparins (LMWH) are a heterogeneous mixture of sulfated glycosaminoglycans with molecular weight of mostly 4,000 to 5,000 Da (ranging from 2,000 to 9,000 Da) [24]. LMWH are purified fragments of natural heparins that have anticoagulant activity. Unlike UFH, LMWH inhibit mainly the coagulation factor Xa, because LMWH bind only to antithrombin III (ATIII) in the complex [heparin + ATIII + Xa]. Inhibition of thrombin requires the formation of a triple complex involving at least 18 saccharide residues in the heparin molecule, which is possible at a molecular weight of at least 5400 Da [18, 25, 26].

LMWH have a number of advantages over UFH due to more favorable pharmacokinetics and fewer side effects. Their use can more accurately predict the dose-dependent anticoagulant effect, they have increased bioavailability when administered subcutaneously, longer half-life, low incidence of thrombocytopenia, so there is no need for regular monitoring of hemostasis and outpatient administration is possible. All LMWH have a similar mechanism of action, but different molecular weights cause different activity for factors Xa and thrombin, as well as different affinities for plasma proteins [18, 27, 28].

LMWH preparations differ in chemical structure, production methods, half-life, specificity of action and therefore they are not interchangeable. LMWH are obtained by depolymerization of heparin isolated from pig mucous membranes by various methods. As a result of the depolymerization process, the formed LMWH are mainly enriched with molecules with less than 18 monosaccharide subunits [29].

LMWH are widely used in the treatment of acute coronary syndrome, deep vein thrombosis of the lower extremities, pulmonary embolism, for the prevention of thromboembolic complications in persons at risk. In clinical practice, LMWH preparations such as enoxaparin and nadroparin are used as the first choice, followed by dalteparin, tinzaparin etc.

Enoxaparin is a LMWH with an average molecular weight of about 4500 Da. This is the first LMWH approved in the United States. It shows high anti-Xa activity and low antithrombin activity. It can ATIII-dependently inhibit factor VIIa, activate the inhibitor of tissue factor pathway (TFPI), reduce the release of von Willebrand factor (vWF) from the vascular endothelium into the circulatory tract [30].

Nadroparin is a LMWH containing glycosaminoglycans with an average molecular weight of 4300 Da. It exhibits a high level of binding to antithrombin III, which causes accelerated inhibition of factor Xa. Other mechanisms that provide the antithrombotic effect of nadroparin include activation of tissue factor pathway inhibitor (TFPI), activation of fibrinolysis by direct release of tissue plasminogen activator from endothelial cells and modification of rheological properties of blood (decrease in blood viscosity and increase in permeability of membranes of thrombocytes and granulocytes). Compared with UFH, nadroparin has less effect on platelet function and their ability to aggregate and less pronounced effect on primary hemostasis [31].

Deltaparin is a LMWH with an average molecular weight of 4000–6000 Da. It binds plasma antithrombin, thereby inhibiting some coagulation factors, primarily factor Xa, and slightly inhibits thrombin formation. It has virtually no effect on blood clotting time. It weakly affects platelet adhesion and may also act on the vascular wall and fibrinolysis system. Tinzaparin has a molecular weight of 4500–5500 Da, lower efficiency and limited indications [32, 33].

Heparins are also important regulators of the activity of protein C (PC), which cause the interaction between pC and its inhibitors, forming a complex with activated pC. The formation of such a complex occurs in three binding sites, also specific to factor Va [34].

The most common side effect of heparin therapy is bleeding, and the most serious is heparin-induced thrombocytopenia type II (HIT II), caused by the induction of antibodies to neoantigen complexes “heparin-platelet factor 4” (HPF4) which provokes serious bleeding [35]. That is why during UFH heparin therapy it is necessary to perform the platelet count at least every four days [36–38]. An important feature of LMWH is their low ability to sorb on the surface of vascular endothelium and blood cells and interact with proteins of the hemostasis system. Therefore, LMWH are much less

likely than UFH to lead to thrombocytopenia, and have no inhibitory effect on fVa [39]. The anticoagulant effect of UFH is removed if necessary by the introduction of protamine sulfate or protamine chloride. This is not always effective in the case of LMWH, but due to milder and predictable dose-dependent inhibitory effect on blood coagulation, LMWH are much less likely to cause massive bleeding and do not require frequent monitoring of the state of the blood coagulation system, so they can be safely used not only in the hospital but also in an outpatient setting [40].

It should be noted that heparin therapy may distort the results of coagulation tests. In this case, the greater the number of proteins of the blood coagulation system involved in the diagnostic test, the greater the error may be in the presence of heparin. For example, the minimum amount of unfractionated heparin introduced into the body (5000 units) leads to a decrease in prothrombin index by 30% [41].

*Synthetic inhibitors of coagulation cascade factors*

The listed shortcomings, high cross-selectivity of heparin, as well as the need for parenteral administration, led to the search for new effective means of reducing the procoagulant potential of blood plasma. To this end, compounds that selectively inhibit thrombin or activated coagulation factor Xa have been developed [42]. Direct inhibitors of these coagulation factors have been shown to be quite effective and are used increasingly in conjunction with indirect anticoagulants.

The success of coagulation inhibitors of this type is due to the fact that thrombin is key in the coagulation system due to its ability to catalyze the conversion of fibrinogen to fibrin and stimulate platelet activation and aggregation. Factor Xa combines the "external" and "internal" pathways of coagulation cascade, and converts prothrombin to the active enzyme thrombin [43].

Synthetic low molecular weight inhibitors of thrombin have a number of advantages over other anticoagulants currently used in practical medicine. These are the speed of action, high efficiency, predictable pharmacokinetics, the lack of need for continuous monitoring of hemostasis [44].

All inhibitors of factor Xa have the suffix "Xa-ban" [Ten-A-Ban] to indicate their mechanism of action.

Dabigatran is one of the best-known direct inhibitors of thrombin activity. Dabigatran inhibits free thrombin, fibrin-bound thrombin, and thrombin-initiated platelet aggregation. Dabigatran prolongs activated partial thromboplastin time (APTT), blood clotting time and thrombin time (TT). Today, several preparations have been developed on the basis of dabigatran, many of which are used clinically [45–46].

Indeed, inhibition of thrombin is one of the most obvious ways to prevent thrombosis, but there are a number of reasons why inhibition of factor Xa may be more effective. Activation of the blood coagulation system leads to the formation of a prothrombinase complex on cell membranes. Factor Xa, as part of this complex, is the only enzyme responsible for the continued formation of thrombin in the bloodstream. Unlike thrombin, which acts on various protein substrates, including fibrinogen and PAR receptors, factor Xa has one physiological substrate, namely prothrombin [47, 48].

Factor Xa is a key point of "enhancement" in the coagulation cascade: one factor Xa molecule generates activation of more than 1,000 thrombin molecules. Thus, the direct inhibition of factor Xa, as a method of indirect inhibition of thrombin formation, provides powerful control of fibrin formation (the basis of thrombus). Being part of the prothrombinase complex, factor Xa has a catalytic activity 10 times higher than the free enzyme. To achieve the anticoagulant effect, it is necessary to inhibit a much smaller amount of factor Xa than thrombin, due to the concentration of their zymogens in the blood (1.4 μM prothrombin against 150 nm factor X) [49, 50].

Indirect evidence for the hypothesis of the predominance of factor Xa as a therapeutic target over thrombin can also be found in clinical trials for the prevention of deep vein thrombosis. Fondaparinux (ATIII-dependent factor Xa inhibitor) has been shown to be superior to LMWH in anticoagulant activity [51]. The antithrombotic activity of fondaparinux is the result of selective inhibition of factor Xa mediated by antithrombin III. By selectively binding to ATIII, fondaparinux potentiates (approximately 300-fold) the initial neutralization of factor Xa by ATIII. Neutralization of factor Xa interrupts the coagulation chain and inhibits both thrombin formation and thrombus formation. Fondaparinux is not inactivated by thrombin and does not affect platelets.

Today, preparations based on factor Xa inhibitors are widely used to prevent intravascular thrombosis in most cardio-vascular and other pathologies. These drugs reduce the risk of blood clots, but in some

cases can also cause bleeding. Clinical studies suggest that they are comparable to warfarin in terms of efficacy, however, unlike indirect anticoagulants, direct anticoagulants have an antidote that makes them safer [52, 53].

The first oral factor Xa inhibitor, rivaroxaban, was approved in the United States in 2011 and is currently one of the most studied and widely used oral anticoagulants [54]. Rivaroxaban is a highly selective direct factor Xa inhibitor, which has a fairly high bioavailability when taken orally and a rapid onset of action. The preparation is used primarily for the prevention of venous thromboembolism (VTE) after elective surgery on the knee or hip joint, therapy and secondary prevention of VTE, prevention of ischemic stroke and thromboembolism in persons diagnosed with valvular fibrillation. In Europe, rivaroxaban is also used for the prevention of atherothrombotic episodes after acute coronary syndrome in patients with elevated cardiac biomarkers. Rivaroxaban is relatively easy to use compared to LMWH and fondaparinux, which require subcutaneous administration, or vitamin K antagonists (VKA), which require regular monitoring of the international normalized ratio, though dose adjustment is required in people with renal impairment [55]. Factor Xa inhibitors apixaban [56] and edoxaban [57, 58] have been approved and are still under investigation.

Apixaban inhibits free and thrombus-associated factor Xa and inhibits prothrombinase activity. Apixaban does not directly affect platelet aggregation, however it indirectly inhibits thrombin-induced platelet aggregation. By inhibiting factor Xa, apixaban prevents thrombin formation and thrombus formation. Preclinical studies of apixaban in animals have shown the effectiveness of antithrombotic action of the drug for the prevention of arterial and venous thrombosis in doses that do not disrupt hemostasis.

Edoxaban, a direct inhibitor of factor Xa, is the last of the oral anticoagulants that are not vitamin K antagonists (NOACs). Its use is now widespread in modern clinical practice, indicated for thromboprophylaxis in patients with non-valvular atrial fibrillation (NVAF) and for the treatment and prevention of venous thromboembolism (VTE).

There is ample data in the literature on the development of low molecular weight inhibitors of thrombin and factor Xa. The studies include those of molecular modeling, rational design and synthesis of a new series of carboxylate compounds of bisphenylamidine, which are inhibitors of factor Xa. Approaches to the effective search for new inhibitors using *de novo* software solutions and data on the X-ray crystal structure of factor Xa have been studied [59].

There are also attempts to create direct inhibitors of coagulation factors located in the coagulation cascade before prothrombinase. In particular, the main component of the tenase complex, factor Va, can be inhibited by a recombinant analogue of protein C — drotecogin [60]. Such preparation will have not only anticoagulant action but also anti-inflammatory properties. It is already offered as part of complex therapy for the treatment of sepsis [61, 62].

*Anticoagulant action that targets fibrinogen*

Decades ago, attempts were made to directly reduce the procoagulant potential of blood plasma by defibrination. For this purpose, it was proposed to use ancrod, an enzyme from the venom of *Calloselasma rhodostoma* [63, 64]. Created on the basis of this enzyme, the drug "Arvin" has been widely used in clinical trials [65]. As a thrombin-like enzyme, ancrod caused desA-fibrin to appear in the bloodstream, which was not stabilized by factor XIIIa and therefore had to be rapidly and efficiently removed from the bloodstream due to the fibrinolysis system [66]. However, the deliberate generation of polymeric fibrin in the bloodstream, even if unstabilized, is associated with many risks because of the possibility of its further polymerization in the presence of thrombin in the bloodstream. That is, the use of Arvin to prevent thrombosis primarily threatens thrombosis. In addition, the amount of fibrinogen in the bloodstream is so high that it is probably impossible to reduce it so much that it cannot be converted under action of thrombin to fibrin in an amount sufficient for thrombosis. In addition, defibrinogenizing the blood plasma completely is extremely risky due to the inability to stop bleeding if it occurs.

Special mention should be made of enzymes specific primarily for fibrinogen, which circulates in the bloodstream. Limited proteolysis of fibrinogen by such enzymes reduces the ability of fibrinogen to convert to fibrin and polymerize, while not leading to the removal of fibrinogen from the bloodstream, which allows the partially hydrolyzed molecule to perform other physiological functions [67, 68]. The idea of using fibrinogenases as a

means of reducing procoagulant potential has not yet received widespread support [69].

Today, many studies are concerned with creating antithrombotic drugs that would directly target the final stage of thrombosis by directly inhibiting the polymerization of monomers and oligomers of fibrin [70, 71]. In particular, it is proposed to use certain low molecular weight compounds that block fibrin polymerization centers, silver nanoparticles [72] and peptide inhibitors that completely or partially mimic fibrin polymerization centers, in particular GPRP conjugated with albumin [73], etc. [ 74]. These include calix[4]arenes, which have low molecular weight and are potentially non-immunogenic compounds of non-protein nature. Calixarenes are promising antithrombotic agents because they inhibit fibrin polymerization centers and thus inhibit the formation of polymeric fibrin network, which is the framework of thrombus [75]. The study of the action of calix[4]arene C-145 *in vivo* with intravenous administration showed a significant antipolymerizing effect [76].

*Antiplatelet agents as inhibitors of platelet aggregation*

Platelet activation required for their aggregation is a complex process regulated by changes in metabolic and biochemical mechanisms, change in the shape of platelets, activation of platelet surface receptors, and change in the orientation of membrane phospholipids [77].

Changes in the orientation of phospholipids near the plasma membrane create the possibility of association of coagulation factors on the activated surface with the formation of a catalytic prothrombinase complex. This leads to increased thrombin secretion and strengthening of the thrombus with transverse fibrin insertion [78].

Platelets are activated by collagen and the first portions of thrombin, which is formed at the site of damage to the vessel wall. The products of these reactions activate protein kinase C, as well as increase the concentration of calcium in the cytosol of platelets. In addition to thrombin, platelet activation is caused by such soluble agents as platelet activating factor (PAF) and ADP, which are released from the damaged cell, as well as catecholamines, serotonin and others. All of these agents have specific receptors on the platelet plasma membrane. The result is a series of successive reactions [79]:

1. The shape of platelets changes, they form long pseudopodia.

2. On the surface of the platelet membrane, a combined GPIIbIIIa receptor is formed from GPIIb and GPIIIa receptors, to which fibrinogen and other adhesive proteins bind, causing platelets to adhere together [80].

3. Arachidonic acid is released from membrane phospholipids. It oxidizes to form a number of derivatives, including prostaglandin PGH2, which is a platelet activating cofactor, and thromboxane A2, which is also able to activate platelets [81].

4. ADP is secreted, which has the ability to activate platelets and attract more of them to thrombus formation.

5. The membrane surfaces of platelets are reorganized, exposing phospholipids, which are necessary for the further formation of coagulation enzyme-cofactor complexes. Secretion of platelet factor V from -granules of platelets provides a key component for the formation of one of the enzyme-cofactor complexes. As a result, an additional amount of thrombin is formed, which leads to the activation of fibrinogen and the formation of fibrin threads that radially depart from the platelet aggregate and contribute to the formation of platelet thrombus, which closes the vessel [82].

6. Inside platelets, the mechanism of platelet actomyosin contraction is activated. The platelet clot compresses, providing a more effective attachment to the site of vascular damage.

Each of these stages can potentially be targeted by antiplatelet agents.

Platelet aggregation inhibitors are divided into groups depending on the targets of inhibition.

P2Y12 receptor antagonists are known to be activated by ADP [83]. Such preparations include ticagrelor, clopidogrel and prasugrel. All of these drugs are widely used in treatment of diseases associated with the risk of intravascular coagulation, including the myocardial infarction [84].

Nevertheless, the most common inhibitor of platelet activation is aspirin, a cyclooxygenase inhibitor [85]. This is due to the high efficiency, low cost and comprehensive information on possible side effects of aspirin [86]. However, some patients have innate resistance to aspirin, which is very difficult to predict [87, 88], so the search for and development of new inhibitors of platelet aggregation continues.

There are also phosphodiesterase inhibitors that inhibit the hydrolysis of cAMP and cGMP. Anti-ischemic potential was shown by drugs of this group, namely cilostazol [89] and dipyridamole [90].

Another large group of inhibitors of platelet aggregation are GPIIbIIIa receptor antagonists. An intracellular signal is required, which is induced by an external signal (ADP, collagen, etc.) and realized through G-proteins, to build and activate the glycoprotein complex of the fibrinogen receptor GPIIbIIIa on the platelet surface [91, 92]. There is an allosteric equilibrium between the affine and non-affine forms of GPIIbIIIa [93, 94]. The affinity form is able to bind fibrinogen and support aggregation. At this stage, the binding of fibrinogen to GPIIbIIIa can be reversed [95]. Interaction of fibrinogen with GPIIbIIIa causes exposure of ligand-induced binding sites that are involved in platelet aggregation — antibodies to these sites inhibit platelet aggregation [96]. Multipoint binding of fibrinogen and GPIIbIIIa leads to irreversible intermolecular interactions [97], as well as to clustering of receptors on the platelet surface and triggering a signaling that enhances platelet activation. The final stage of the interactions of fibrinogen and GPIIbIIIa is the retraction (sealing) of the fibrin-platelet thrombus [98].

The main class of GPIIbIIIa receptor antagonists are RGD-containing protein preparations [99]. However, it should be noted that they cannot inhibit the interaction of platelet receptors and fibrinogen, which is realized most effectively at the C-terminal dodecapeptide γ-chain of the fibrinogen molecule. Development of RGD-containing drugs continues [100]. This is a topical issue because disintegrins are potential antiproliferative agents, preventing the adhesion of cancer cells [101]. Some of them have been successfully tested in models of carcinogenesis in laboratory animals [102].

The drugs based on antibodies to GPIIbIIIa receptors are promising for the inhibition of platelet aggregation. In particular, abciximab is used in coronary heart disease [103]. The synthetic heterocyclic compound tirofiban was an active inhibitor of platelet aggregation [104].

Thus, it is possible to identify the main targets for the inhibition of intravascular thrombosis: factors of the blood coagulation cascade, primarily factor Xa and thrombin, as well as platelets. Thrombin or factor Xa inhibitors have been shown to be more effective in preventing thrombosis than platelet aggregation inhibitors, but the use and research of platelet aggregation inhibitors is ongoing. Specific inhibition of a single cascade or mechanism, as well as several mechanisms simultaneously, is possible. The effect on platelets does not lead to disruption of the protein part of the blood coagulation system, but reduces the overall procoagulant potential of the blood.

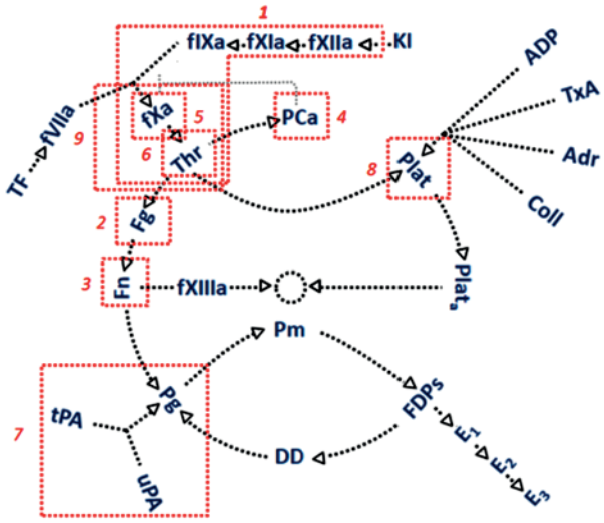


*Fig. 2.* **The generalized scheme of the hemostasis system with the indicated nodal points targeted by the most common and promising anticoagulant agents**

Fig. 2 presents a generalized scheme of the hemostasis system with the indicated nodal points, which are targeted by the action of the most common and promising anticoagulant agents: 1 — indirect anticoagulants; 2 — fibrinogenases; 3 — fibrin polymerization inhibitors; 4 — activated protein C; 5 — direct thrombin inhibitors; 6 — direct inhibitors of factor Xa; 7 — activators of fibrinolysis; 8 — inhibitors of platelet activation and aggregation; 9 — heparins.

The following symbols are used in Fig. 2: Fg — fibrinogen; Fn — fibrin; fXIIIa — activated factor XIII; Plat — platelets; $Plat_a$ — activated platelets; fIXa — activated factor IX; fXIa — activated factor XI; fXIIa — activated factor XII; KI — kallikrein; TF — tissue factor; fXa — activated factor X; fVIIa — activated factor VII; PCa — activated protein C; Coll — collagen; Adr — adrenaline; TxA — thromboxane A; ADP — adenosine diphosphate; Pg — plasminogen; Pm — plasmin; tPA — tissue plasminogen activator; uPA — urokinase; FDPs — fibrin degradation products; DD — D-dimer; $E_1$ — high molecular weight E-fragment; $E_2$, $E_3$ — hydrolyzed E-fragment.

*Methods of stimulating extravascular thrombosis*

The need to initiate thrombosis arises in the case of violation of the integrity of the walls of blood vessels, internal or external bleeding, which threaten to disrupt the blood

supply to tissues and organs and should be stopped as soon as possible. Biotechnological challenges of creating opportunities to initiate thrombosis are an important issue of disaster medicine and surgery.

There are three basic ways to solve the problem of local bleeding: the introduction of exogenous activated blood clotting factors, and the use of non-specific or highly selective activators of blood clotting cascade factors.

The first method is the base of transfusion, the only effective antidote against bleeding caused by drug overdose or congenital pathologies of blood clotting [105, 106]. The patient can be transfused with blood, platelet mass, blood plasma, but the most effective is the use of prothrombin concentrate, which contains vitamin K-dependent coagulation factors, salted from human blood plasma [107]. Recombinant or purified plasma coagulation factors are also used [108].

Non-specific and highly selective agents that activate coagulation will be considered separately.

*1. Nonspecific activators of the coagulation cascade*

Under conditions of violation of the integrity of the vascular wall or in the case of pathological activation of blood clotting, the initiation of thrombosis occurs by physiological mechanisms of the external and internal pathways of the coagulation cascade [109]. Accordingly, it is possible to activate these mechanisms using non-specific physiological activators: thromboplastin for the external blood clotting pathway, and substances that have negatively charged negative surfaces for the internal pathway.

Thromboplastin is a preparation derived from tissues of various origins (most often the brain), which has procoagulant activity and is able in a matter of seconds to cause clotting of donor blood plasma. The composition of thromboplastin includes myelin membranes, individual membranes and even cell organelles. Coagulation activity of thromboplastin is determined by tissue factor [110].

Tissue factor is a membrane glycoprotein with a molecular mass of 45 kDa, which is found in the membranes of endothelial and smooth muscle cells, as well as in the membranes of monocytes and macrophages, and always functions exclusively in complex with the phospholipid matrix. The tissue factor molecule has three domains, the hydrophilic E- and C-domains and the hydrophobic domain represented in the membrane phase [111].

The process of damage to the outer cell membrane is associated with loss of the normal asymmetric distribution of lipids between its outer and inner surfaces. At the same time, tissue factor appears on the surface of the damaged cell, which, with the participation of $Ca^{2+}$ ions, immediately forms a complex with factor VII, activating it. Bound to the lipid membrane, this complex effectively converts factors IX and X. In addition, the ability of tissue factor to form regulatory complexes is important, including the triple complex with VIIa and antithrombin III, as well as the complex with tissue factor inhibitor TFPI and factors Xa and VIIa [112].

Meanwhile, it is difficult to imagine the use of tissue factor of natural origin as a procoagulant agent. Recombinant tissue factor can be obtained, but as a transmembrane protein, it will require a lipid matrix.

Therefore, tissue factor is not considered as a basis for the creation of drugs with procoagulant action. But substances and biomaterials that can trigger the contact activation system are quite common in biomedicine.

A striking example of such preparations are kaolin-containing substances [113]. In particular, the most common APT test in clinical practice is performed using a reagent containing kaolin and ellagic acid [114]. Activation of the kallikrein-kinin system leads to the generation of tenase and prothrombinase complex and ultimately to the appearance of thrombin [115]. Moderate activation of this mechanism occurs to some extent in contact of blood with any negatively charged surface [116].

Hence, the action of thromboplastin or kaolin does not involve direct activation of prothrombin, instead, thrombin formation is carried out indirectly through a number of factors of the coagulation system, which in turn are activated in the presence of nonspecific activators (factors IX, VII, X, etc.). In view of this, the activity of these factors and their content in the studied plasma may affect the efficiency of thrombosis [117].

*2. Activators of factor Xa*

Describing specific activators, it is possible to imagine a hypothetical enzyme activator for each of the factors of the blood clotting cascade. However, selective activation of certain factors in the initial stages of the coagulation cascade will be less effective compared to the strong the avalanche-like action of kaolin or thromboplastin. In fact, activators of enzymes of the final links of the coagulation cascade,

namely the prothrombin and factor X, can be quite active procoagulants.

Activators of coagulation factor X have been found in the venoms of snakes of the families Viperidae and Crotalidae, and in the venom of some members of Elapidae. These include both serine proteinases and metalloproteinases [118]. The most well-known activator of factor X is RVV-X from the venom of Russell's viper *Daboia russelli*. RVV-X has found application in clinical laboratory diagnosis [119]. This metalloproteinase consists of a heavy chain that forms a catalytic domain and two light chains that are homologous to C-lectins [120].

Factor X activator has been described in particular in the venoms of desert cobra *Walterinnesia aegyptia* and Lebetine viper *Vipera lebetina* [121–123]. All of them act directly on factor X directly, regardless of the presence of phospholipids, which distinguishes the mechanism of their action from the mechanism of action of tenase. However, they have never been proposed for use as coagulation inducers.

*3. Prothrombin activators*

Activation of prothrombin seems to be the most promising way to stimulate thrombosis, as it leads to the generation of intravascular thrombin, which in turn not only converts soluble fibrinogen into insoluble fibrin, but also causes its covalent stabilization. However, there are ways to stop local bleeding by using directly exogenous thrombin, which is designed to immediately start the process of blood clotting, converting fibrinogen to fibrin with its subsequent polymerization and formation of a three-dimensional thrombus framework [124]. However, exogenous thrombin may be inhibited by endogenous anticoagulant proteins, including antithrombin, and lose its activity. In addition, exogenous thrombin can be applied to the wound surface only in limited quantities, which will not be sufficient to effectively stop the bleeding.

Endogenous prothrombin activators are devoid of these disadvantages and can be used to stop bleeding locally. Promising agents that can directly activate prothrombin are snake venom enzymes [125].

Among the enzymes of snake venoms that can activate prothrombin, there are factor X-like and ecarin-like proteins. Factor X-like enzymes activate prothrombin via prethrombin stage 2, cleaving the Arg274-Thr275 bond in the molecule to form fragment 1,2. Such enzymes are obtained from the poisons of Australian elapids. Ecarin-like enzymes cleave the Arg320-Ile321 bond in the prothrombin molecule to form mesothrombin, which is converted to normal α-thrombin via the mesothrombin stage [126].

The last group of prothrombin activators includes, in particular, ecarin, derived from the venom of *Echis carinatus* of the family Viperidae. This metalloproteinase is widely used in clinical practice and is a commercially viable drug, not least due to the possibility of obtaining a highly purified fraction of the enzyme, which allows for its detailed biochemical and biophysical characteristics [125, 127, 128]. An analogue of ecarin is ecamulin which is an enzyme isolated from the venom of a viper from Central Asia, *E. multisquamatis*, a species close to *Echis carinatus* [129, 130].

Non-physiological prothrombin activators activate all forms of prothrombin, even those that could not be activated by the physiological activation pathway. Ecamulin and ekarin both perform such activation through formation of an intermediate product, mesothrombin, with splitting of the Arg320-Ile321 communication without release of peptide. Mesothrombin, in turn, is autocatalytically converted to mesothrombin 1 (by cleavage of the Arg 156-Ser157 bond), and then to α-thrombin [131].

Although ecamulin and ekarin have similar functions and are synthesized by related species of snakes, there are significant differences in their structure. Thus, ekarin is a single-chain glycoprotein with a molecular mass of 63 kDa, 17% of the mass of which is a carbohydrate component. In contrast, ecamulin gives two bands with molecular masses of 67 kDa and 27 kDa in SDS electrophoresis. The highest coagulation activity is seen in fractions containing both of these components in equal proportions. The 27 kDa component contains two chains (13 kDa, 14 kDa). Separate chains of ecamulin do not have their own coagulation ability. S2 and S3 fractions are distinguished by the number of peaks that characterize ecamulin in ion exchange chromatography. One g of whole poison contains about 3 mg of S2-form and 10 mg of S3-form. The coagulation activity of S3 form is twice as high as that of S2. The isoelectric point of ecamulin is 4.3–4.5 [132].

Ecamulin, the activator of prothrombin released from the poison of *E. multisquamatis*, is used to determine the content of prothrombin and detect its functionally inactive forms. This test, called "ecamulin time", is based on the ability of ecamulin to activate both prothrombin and its functionally inactive forms — decarboxylated forms of

prothrombin, formed in the absence of vitamin K, and prethrombin. The latter appears in the bloodstream under the action of thrombin and is one of the markers of intravascular coagulation [133].

*4. Thrombin-like enzymes*

Another alternative use of exogenous thrombin to induce extravascular bleeding is the use of thrombin-like enzymes. Their main source is also snake venom [134].

Among the thrombin-like enzymes of snake venom, proteins such as ancrod (thrombin-like enzyme from the venom of *Colloselasma rhodostoma*) and batroxobin (*Bothrops moojeni*), have been relatively widely used in clinical practice for defibrination and laboratory diagnosis [135]. These enzymes belong to the class of serine proteases. Acting by a similar mechanism, they, unlike thrombin, cleave only one of the fibrinopeptides [136]. In addition, thrombin-like enzymes, unlike thrombin, do not have the ability to activate coagulation factor XIII, resulting in the formation of lacking covalent binds, and therefore unstable, fibrin clot of low strength.

In recent years, hemocoagulase, a reptilase analog, has been proposed to stop local bleeding [137–139]. The efficacy of such a hemostatic agent and its ability to initiate the formation of desA-fibrin was noted.

When comparing the prospects for the use of prothrombin activators and thrombin-like enzymes, the former should be preferred because they: a) lead to the activation of endogenous prothrombin with unlimited thrombin production potential; b) generating thrombin, trigger platelet activation; c) lead to the formation of a covalently stabilized clot.

*5. Transglutaminase*

The final stage of thrombus formation is covalent stabilization of fibrin by factor XIIIa. It is a physiological transglutaminase, activated by thrombin. In humans, nine types of transglutaminases have been described, which play an important role in maintaining homeostasis and are important in the development of certain pathological processes. Transglutaminases are enzymes that catalyze the formation of covalent isopeptide bonds between glutamyl and lysine protein residues [140].

The idea of increasing the efficiency of extravascular thrombosis through the use of transglutaminases is to stabilize endogenous fibrin polymers and bind them with adjacent tissues.

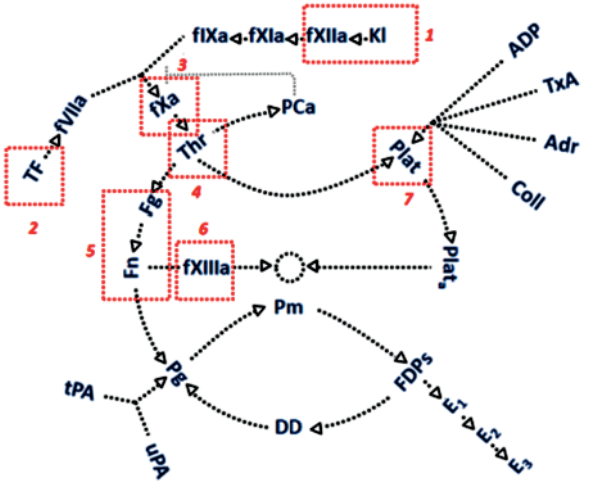


*Fig. 3.* **The generalized scheme of the hemostasis system with the indicated nodal points targeted by the action of the most common and promising procoagulant agents**

It is proposed to enhance thrombosis using factor XIIIa of the blood coagulation system [141] or its analogues with transglutaminase activity [142, 143]. However, although such enzymes act in the final stage of blood clotting, initiating covalent stabilization of polymeric fibrin, they are not able to trigger the conversion of fibrinogen to fibrin with its subsequent polymerization and formation of a three-dimensional thrombus framework. Thus, these enzymes can not serve as a basis for effective hemostatics.

Fig. 3 presents a generalized scheme of the hemostasis system with the indicated nodal points, which are targeted by the action of the most common and promising procoagulant agents: kaolin (*1*); tissue factor (*2*); factor X activators (*3*); prothrombin activators (*4*); thrombin-like enzymes (*5*); transglutaminase (*6*); platelet activators (*7*).

The following symbols are used in Fig. 3: Fg — fibrinogen; Fn — fibrin; fXIIIa — activated factor XIII; Plat — platelets; $Plat_a$ — activated platelets; fIXa — activated factor IX; fXIa — activated factor XI; fXIIa — activated factor XII; KI — kallikrein; TF — tissue factor; fXa — activated factor X; fVIIa — activated factor VII; PCa — activated protein C; Coll — collagen; Adr — adrenaline; TxA — thromboxane A; ADP — adenosine diphosphate; Pg — plasminogen; Pm — plasmin; tPA — tissue plasminogen activator; uPA — urokinase; FDPs — fibrin degradation products; DD — D-dimer; $E_1$ — high molecular weight E-fragment; $E_2$, $E_3$ — hydrolyzed E-fragments.

## Conclusions

Physiological (extravascular) thrombosis occurs in violation of the integrity of the vascular wall to prevent blood loss and is evidence of normal functioning of the hemostasis system. The pathological thrombosis (intravascular) is the result of imbalance in the hemostasis system. Both of these processes are based on the same molecular mechanisms: enzymatic coagulation cascade, platelet cell signaling, fibrinolytic and anticoagulant units. According to the basic concept, the researcher can, as needed, stimulate or inhibit the process of thrombosis, by acting on a certain part of the system.

The most effective way to inhibit intravascular thrombosis is to affect various parts of the coagulation cascade, primarily thrombin and factor Xa. The ways to affect platelet aggregation are less effective, but currently widely used. To inhibit the last link in coagulation (fibrin polymerization and the formation of a fibrin network, which is the framework of the thrombus), it is proposed to use peptide inhibitors that mimic the amino acid sequence of fibrin polymerization sites, or low molecular weight calixarenes that can block such sites.

The most effective way to stimulate extravascular thrombosis was at the stage of prothrombin activation. The use of enzymatic coagulation activators to modify biomaterials will make it possible to create effective means to prevent acute vascular and capillary bleeding.

The review was written as a part of the scientific and technical work «Creation of recombinant therapeutic protein — activator of the blood clotting system» for the State Order under the contract DZ/104–2021 from March 9, 2021, of state registration 0120U104804 (scientific supervisor — Academician S. V. Komisarenko).

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## МОЛЕКУЛЯРНІ МЕХАНІЗМИ ІНГІБУВАННЯ ВНУТРІШНЬОСУДИННОГО І СТИМУЛЯЦІЇ ЕКСТРАСУДИННОГО ТРОМБОУТВОРЕННЯ

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Система гемостазу покликана підтримувати баланс між процесами зсідання крові, антикоагуляції, а також фібринолізу, забезпечувати постійний ефективний кровообіг в організмі та швидке припинення кровотеч у разі їх виникнення. В основі забезпечення прокоагулянтного потенціалу системи гемостазу лежать молекулярні механізми, що ведуть до утворення фібрину в кров'яному руслі, який є каркасом тромбу, та до агрегації тромбоцитів — основи тіла тромбу. Антикоагулянтний потенціал плазми крові забезпечується механізмами, спрямованими на інгібування процесів коагуляції крові. Ґрунтовне вивчення та розуміння цих механізмів дозволить відкрити численні засоби лікування патологічних станів, пов'язаних як із внутрішньосудинним тромбоутворенням, так і з кровотечами різного ґенезу.

*Метою* огляду є аналіз способів запобігання внутрішньосудинному тромбоутворенню та стимулювання екстрасудинного тромбоутворення. В огляді описано та проаналізовано доступні та перспективні засоби запобігання тромбоутворення, зокрема, прямі й непрямі антикоагулянти та антиагреганти, а також способи ефективного стимулювання тромбоутворення, що необхідно у разі пошкодження судин. Результатом такого аналізу є визначення вузлових точок протеїнової мережі системи гемостазу, дія на які специфічними молекулярними ефекторами дозволить керувати процесом тромбоутворення.

***Ключові слова:*** антикоагулянти, антиагреганти, активатор, зсідання крові, тромбоутворення.

UDC 579.262 **https://doi.org/10.15407/biotech14.06.023**

# *Sinorhizobium meliloti* BACTERIUM AS A PERSPECTIVE OBJECT FOR BIOTECHNOLOGY

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Received 12.09.2021
Revised 11.12.2021
Accepted 30.12.2021

*Sinorhizobium meliloti* is a Gram-negative soil nitrogen-fixing bacterium that increases the yield of legumes. There is information in the literature about the complete genome sequence of this bacterium. In addition, the polysaccharide composition of the biofilm, which is actively involved in nitrogen fixation, has been studied. The well-known nucleotide sequence, as well as the genetic and biochemical features of *S. meliloti* bacterium makes this organism an ideal model for biotechnological research. The purpose of this work was to analyze the current data provided in the literature on the symbiotic interaction of *Sinorhizobium meliloti* bacterium with the host plant, and to characterize the main directions of the use of this bacterium in agriculture, bioremediation and medicine.

***Key words:*** *Sinorhizobium meliloti* bacterium, symbiosis, biotechnology.

More than half of all nitrogen required for successful farming is currently provided by nitrogenous chemical fertilizers [1]. However, these fertilizers are expensive both economically and environmentally [2]. The application of synthetic N fertilizers has greatly enhanced crop production but also has caused serious environmental problems, such as groundwater contamination and surface water eutrophication [3]. On the other hand, symbiotic nitrogen-fixing microorganisms are a sustainable nitrogen source for agriculture.

Nodule bacteria belong to microaerophilic microorganisms that can develop at a low partial pressure of oxygen in the environment. One of the most studied nitrogen-fixing bacteria, *Sinorhizobium meliloti*, has distinguished features of metabolism and structure. The complete genome of *S. meliloti* was sequenced and annotated in 2001. All strains analyzed so far contain three replicons: one chromosome and two inherently stable megaplasmids [4]. Plasmid pSymA is considered as a symbiotic accessory megaplasmid, as it can be cured without affecting *S. meliloti* viability. It contains genes for nodule formation and nitrogen fixation. Most sequences located on pSymA are transcribed only at the bacteroid stage. It was shown that pSymA had a role in the regulation of the expression of genes from the other replicons (3.5 Mbp chromosome and the 1.7 Mbp pSymB plasmid) presented in the *S. meliloti* cells [5]. Plasmid pSymB contains both plasmid and chromosomal features and is designed as a second chromosome. Genes involved in polysaccharide biosynthesis were identified in this megaplasmid. It was shown that 14% of the pSymB sequence is dedicated to polysaccharide synthesis [6]. Other recognizable gene clusters include many involved in catabolic activities. *S. meliloti* genome architecture was shown to be highly dynamic, as the three replicons continuously cointegrate and excise. A detailed study of the bacterial genotype allowed scientists to identify the genes responsible for the key stages of symbiosis. Thus, it was possible to purposefully influence the genetic material in order to obtain viable symbionts that showed resistance to adverse environmental conditions [7].

On the other hand, *S. meliloti* has found application in biotechnology as a source of polysaccharides, which due to their ability to change the rheological properties of water

systems, can be considered as emulsifiers, suspending and gel-forming agents [8–9].

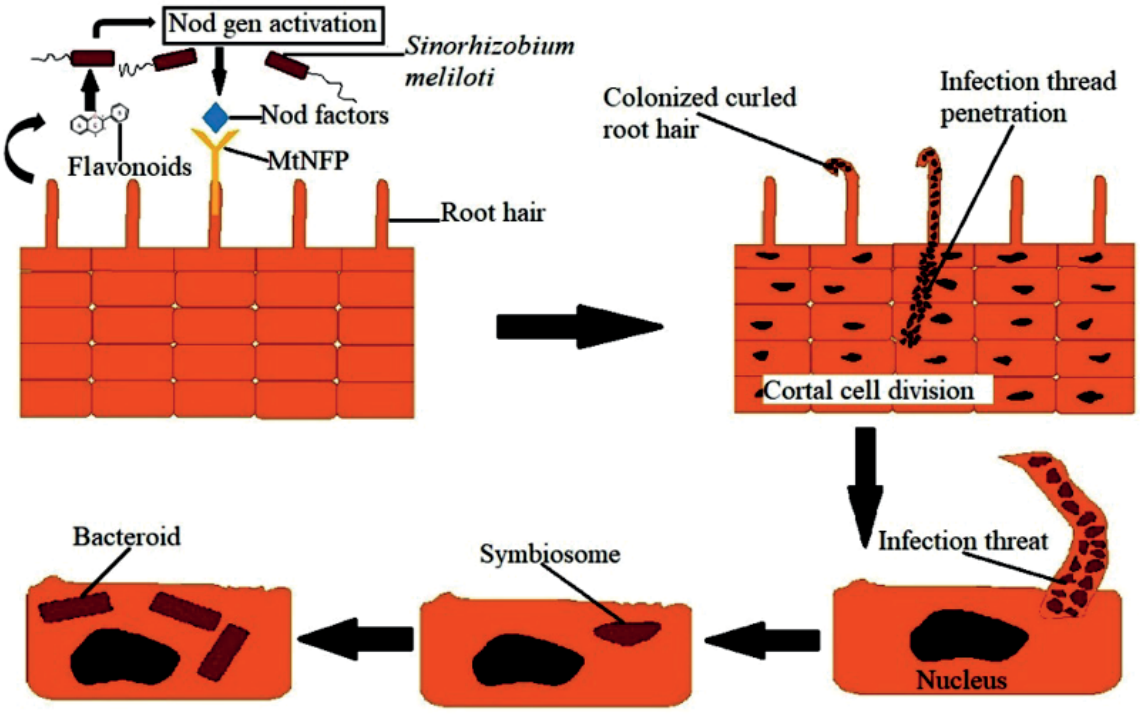
This paper describes the main steps of symbiosis of *Sinorhizobium meliloti*, taking into account the data obtained over the last decade, and concludes with application for this microorganism in biotechnology.

*The mechanism of symbiosis*

The molecular reactions underlying symbiotic relationships are well described in the modern literature, however, taking into account the latest data, the following stages can be distinguished (see Figure).

It is known that the roots of leguminous plants secrete a diverse cocktail of flavonoids and isoflavonoids into the soil. Flavonoids in the exudates of legume roots act as chemotactic signals for rhizobia in conditions of low nitrogen content [10]. It is difficult to determine, which flavonoid in the rhizosphere is perceived by a compatible bacterium, since plants, as a rule, secrete a complex mixture. It is likely that the determining factor for host specificity is the spectrum of flavonoids secreted by this legume plant. Some rhizobia are chemotactic with respect to compatible flavonoids. Hence, it is assumed that aspects of host specificity are established before the bacterium and its host physically interact [11]. Flavonoids of plant origin cause a transcriptional response in bacteria, in particular, they lead to the activation of genes that are responsible for the synthesis of nod factors (NF), the expression of these genes is suppressed in the presence of ammonium [12].

Among the rhizobial genes induced by activated flavonoids, there are mainly genes encoding enzymes necessary for the production of lipochytooligosaccharide Nod factors [13]. Nod factor is a complex signaling molecule that can be represented as a chitinous backbone modified on a non-reduced terminal residue at the C2 position by a fatty acid; however, the size and saturation state of this lipid chain vary depending on the species [14]. The NF molecule can be further modified by various chemical substituents. A certain type of rhizobia has its own mixture of node factor compounds, approximately from 2 to 60 molecules, this is especially characteristic of bacteria with a wide range of hosts [15]. *Nod* genes are divided into common and specific. The common nod genes (including *nod*A, B and C), which are found in almost all rhizobia species, are responsible for the synthesis of the NF chitin framework. On the contrary, host-specific nod genes ensure the formation of nodules in a particular host and are involved in various modifications of the chitin framework. However, the presence of the rhizobial bacterium itself is not a prerequisite. If a




**Stages of development of legume-rhizobium symbiosis**

cluster of biosynthetic genes of node factors is transferred into the Gram-negative bacterium *Escherichia coli*, then this bacterium gets the ability to cause several early reactions of the host plant to these factors, in particular a spike in the concentration of calcium ions [16]. The movement of the bacterium towards the host plant is due to the presence of chemoreceptors. The periplasmic region of transmembrane chemoreceptors of *S. meliloti* acts as a sensory input module for chemotaxis systems directly or indirectly through the binding of specific ligands. The methyl-accepting chemotaxis protein has a periplasmic sensory domain for binding the chemoeffector ligand, as well as a cytoplasmic signaling domain, which is reversibly methylated by glutamic acid residues. *S. meliloti* demonstrated positive chemotaxis against seven carboxylates found in the host alfalfa seed exudates, namely — α-ketobutyrate, citrate, glyoxylate, malate, malonate, oxalate and succinate [17]. The interaction of the receptor with the ligand leads to a rapid response at the level of the flagellar motor. Recently some interesting results, which contradict the previous investigations concerning the role of flavonoids, have been obtained. Compton K. et al. analysed exudate from germinating alfalfa seedlings for composition and quantities of different flavonoid compounds using mass spectrometry. They found four prevalent flavonoids in germinating alfalfa seed exudates (hyperoside, luteolin, luteolin-7-glucoside, and chrysoeriol). Using quantitative chemotaxis capillary assays, they did not detect chemotaxis of motile *S. meliloti* 1021 cells to these, and two other flavonoids identified in seed exudates. In support of these findings, the flavonoid fraction of seed exudates was found to be an insignificant attractant relative to the more hydrophilic fraction. Authors have proposed that the role flavonoids play in *S. meliloti* chemotaxis is insignificant relative to other components released by alfalfa seeds [11].

*The plant's response to the action of node factors*

Initial reactions of the root epidermis include cytosol alkalinization and plasma membrane depolarization within a few minutes after root inoculation. Purified NF can also cause root hair deformation and root hair twisting within a few hours after application. The deformation of the root hair probably depends on calcium-induced changes in the organization of the actin cytoskeleton, which lead to a reorientation of cell growth. These reactions are followed by twisting of the root hairs, which detains rhizobial bacteria in the tightly colonized twisted root hair [18]. At the same time, the NF stimulate the cells of the root cortex to resume mitosis, resulting in the formation of cells that will form the primary nodule and ensure the invasion of bacteria [19]. For a complete plant response to NF, several receptors containing an extracellular domain are required. One of these MtNFP receptors is a member of the LysM receptor family and is required for root hair twisting and induction of transcriptional changes. Some experiments have demonstrated the additional role of MtNFP at a later stage of bacterial penetration into the root hair, namely, the formation of an infectious thread. It is known that another receptor of the LysM family is necessary for twisting the root hair and fine-tuning plant reactions of plants to the NF during the formation of an infectious thread [20]. However, the interaction of NF with their receptors is not the only event at this stage. It has been shown that NF stimulate the formation of reactive oxygen species. Reactive oxygen species have a regulating effect on the process of symbiosis between legumes and nitrogen-fixing bacteria. Rapid and temporary production of these forms has been reported after treatment of the root hairs of leguminous plants with NF [21]. In addition to NF, bacterial surface exopolysaccharides (EPS) and their receptors play an important role in the recognition of the symbiont bacterium by the plant. Thus, the exopolysaccharide receptor LJEPR3 contains a LysM domain, which is not required for an early response to the NF, but is necessary for the formation of a microcolony. The LJEPR3 receptor is differentially regulated in the epidermis and primary nodules. However, in the question of the combined action of EPS and NF, much remains unclear [22]. It has been suggested that exopolysaccharides produced by rhizobial species are actively involved in suppressing the protective reactions of the host plant [23].

*Infection tread development*

Bacteria trapped in a twisted root hair and capable of producing NF and a symbiotically active exopolysaccharide induce ingrowth of the root hair cell membrane, which leads to bacterial invasion into the internal plant tissue. Effective invasion occurs even if the NF and the exopolysaccharide are supplied separately by *S. meliloti* strains, which are jointly captured in the same twisted hair [24].

*S. meliloti* produces the exopolysaccharides succinoglycan and galactoglucan, which contribute to the formation of infection filaments. As soon as this thread penetrates into the base of the root hair cell, the bacteria must induce new cycles of formation of the infection thread in each subsequent cell layer. Several hormon signaling pathways intersect with NF signals to control nodule formation [25]. Organogenesis of nodules is controlled by hormones. Concentrations and ratios of auxins and cytokinins determine where and when cells divide. Cytokinins regulate cell division, leading to the formation of a primary nodule, and their signals are mainly perceived by the MTCRE1/LJLHK1 cytokinin receptor [26]. Direct targets of cytokinin signaling include MTNSP2 and the main gene MtbHLH476 TF, both encoding positive regulators of nodule organogenesis. Genes for the biosynthesis and homeostasis of cytokinins are necessary for the normal development of nodules. Cytokinin biosynthesis is mediated by isopentenyltransferase and cytokinin homeostasis during nodule development is supported by cytokinin oxidase/ dehydrogenase [27]. Plant cytokinin and Nod-dependent cell cycle reinitiation are involved in the spread of infection into the root hairs of the plant. Depletion of the cytokinin receptor of *M. truncatula* leads to blocking of the re-initiation of cell division and interruption of infection filaments [28]. The earliest infection threads penetrating the growing nodule of *M. truncatula* or *M. sativa* should germinate past actively dividing cells in the developing primary nodule. As a result, stable nodule meristem is formed.

*Symbiosome and bacteroid formation*

Each bacterial cell is endocytosed by a target cell. A system consisting of a single bacterium and the surrounding endocytic membrane is known as a symbiosome. In nodules, the bacterial cell and the surrounding membrane divide synchronously before the bacteria differentiate into nitrogen-fixing bacterioids, which can fix atmospheric nitrogen into ammonia, establishing an intricate metabolic interchange with the host plant [29].

Biochemical markers of the symbiosome membrane were determined. These include identified nodule-specific proteins, energy and transport proteins, bacterial proteins and proteins that will participate in the docking of vesicles on the target membrane. According to the data concerning the structure of symbiosome membrane proteins, protein syntaxin plays an important role in this process. Syntaxin may be crucial for the transformation of a vesicle containing rhizobium into a mature symbiosome [30]. Once the bacteria are absorbed by the host cell membranes, they must survive in the symbiosomal compartment and differentiate into a nitrogen-fixing bacterioid. Both bacterial and plant factors are involved in these processes. One of the important defense mechanisms that Gram-negative bacteria use to resist the extracellular environment is lipopolysaccharide (LPS). It has been shown that *S. meliloti* produces lipid A of the appropriate structure that will ensure its survival in host cells [31].

*Symbiosis control*

The host plant controls the survival of bacteria in the symbiosome and must not only provide nutrition and the right microaerobic environment necessary for nitrogen fixation, but also provide an opportunity for bacterial differentiation. In unformed nodules, the captured bacteria and the symbiosome membrane divide simultaneously before the formation of bacteroids. The bacteroids in the nodules increase the DNA content and cell size, which may allow them to achieve a higher metabolic rate necessary for nitrogen fixation. The intensive DNA synthesis required for endoreduplication in bacterioids requires a large number of dideoxynucleotides, which must be supplied by ribonucleotide reductase. For many bacterial species, DNA synthesis in an oxygen-rich environment (an infection thread) and in an oxygen-depleted environment (a symbiosome) would pose a significant problem [32]. However, some bacterial species, including rhizobia, have adaptations that solve this problem: they have a B12-dependent ribonucleotide reductase that functions independently of oxygen concentration [33]. Bacteria that are enclosed in symbiosomes are provided with a low-oxygen environment and complete the bacteroid differentiation program. They can express the enzymes of the nitrogenase complex and begin to fix nitrogen. An oxygen-sensitive bacterial regulatory cascade controls the expression of the nitrogenase complex and microaerobic respiratory enzymes, which are necessary to provide energy and a nitrogenase reducing agent. This cascade is induced by a low oxygen content in the differentiating bacteroid. Bacterial regulators include the oxygen-sensitive two-component regulatory system FIXL and FIXJ, NIFA, σ54 and FixK98. These regulators are responsible for

many changes in the expression of genes and proteins were found during the differentiation of bacteroids [33].

As an example of the mutual adaptation of legumes and bacteria, the reaction of legumes to chitin can be considered. Chitin is a pathogen that induces innate immune responses of plants, such as an oxidative explosion. This reaction helps plants to protect themselves from fungal attack, since chitin is a component of the cell walls of fungi [34] Interestingly, the chitin receptor of rice (*Oryza sativa*) is a protein containing the LysM domain, as are Nod factor receptors such as MTNFP and MtLYK3131. Perhaps in the same way legumes recognize bacterially produced lipochitooligosaccharide NF [35]. At the same time, there are protective reactions of plants that limit the number of nodules on the colonized root. After the initial round of nodule formation has begun, subsequent nodule formation events undergo auto-inhibition. Signaling is mediated by plant hormones ethylene and jasmonic acid, which are involved in protective reactions during other interactions of plants and microorganisms. There is an assumption that auto-inhibition is controlled by the plant by interrupting infection threads [36].

*Defensive reactions of the plant*

In parallel with the choice of a partner dependent on the NF, the immune system of plants helps to exclude other soil microorganisms from the roots of legumes [37]. The host legume plant uses multiple control points throughout this process to differentiate between symbionts and pathogens. Protective receptor kinase complexes, including LRR-RLKS and LysM-RLKS, recognize microbial molecules on the surface of plant cells, while microbial "effectors" injected into cells to remove plant protection are recognized and neutralized by proteins NBS-LRR ((nucleotide binding site leucine-rich repeat) R (Resistance) proteins) R [38]. It has been shown that the compatibility of bacteria and plants is regulated by peptides with a high content of cysteine named NCR (nodule-specific cysteine-rich), which are produced in the nodules of the plant. The role of these peptides as effectors of differentiation of endosymbionts into nitrogen-fixing bacteroids was previously shown. NCR peptides were detected as a result of transcriptomic analysis of *M. truncatula.* In non-lethal doses, NCR peptides have several effects on rhizobial cells: they promote genome endoreduplication and stimulate cell branching, promote efficient nitrogen uptake, nutrient exchange and inhibition of rhizobia proliferation [39]. It has recently been shown that a genetic disorder of specific genes encoding NCR peptides leads to a violation of the ability of the bacteroid to fix nitrogen [40]. The effect of host-derived NCR peptides on nodule-associated bacteria has led to the creation of a model, in which the corresponding bacterial peptidases can modulate this effect [41].

It was later discovered that NCR peptides cause bacterial cell death and early nodule aging in the case of incompatible microsymbionts [42]. The reaction of host plants to lipopolysaccharide (LPS) of bacteria deserves special attention. In most plant species, the reaction to bacterial LPS differs from the toxic shock that can be caused in animals. The lipid component A of *S. meliloti* can suppress both the oxidative spike and the expression of protective genes in cultured host cells of *M. truncatula* and *M. sativa* [43]. A promising area of research is to determine, which LPS epitopes from rhizobial bacteria and plant pathogens cause or suppress protective reactions in plants of various lines.

*Bacterial exopolysaccharides and their role in symbiosis*

Speaking about the symbiotic relationship of *S. meliloti* with plants, special emphasis should be placed on the elements of exopolysaccharide nature, their regulation of their biosynthesis. It is known that *S. meliloti* secretes two acidic exopolysaccharides (EPSs), succinoglycan (EPSI) and galactoglucan (EPSII), which differentially enable it to adapt to a changing environment. Succinoglycan is essential for invasion of plant hosts and, thus, for the formation of nitrogen-fixing root nodules. Galactoglucan is critical for population-based behavior such as swarming and biofilm formation and can facilitate invasion in the absence of succinoglycan on some host plants. The biosynthesis of galactoglucan is not as completely understood as that of succinoglycan. NMR analysis of EPS isolated from a mutant of the WGAE gene revealed a new pyruvyltransferase that modifies galactoglucan. [44].

It was suggested that succinoglycan plays an important role in the survival of *S. meliloti* at low pH levels. When *S. meliloti* Rm 1021 was grown at pH 5.75, synthesis of succinoglycan increased, whereas synthesis of galactoglucan decreased. Succinoglycan that was isolated from cultures grown at low pH had a lower degree of polymerization relative to that, which was isolated from cultures grown at

neutral pH, suggesting that low–molecular weight (LMW) succinoglycan might play a role in adaptation to low pH. The data suggest that the role for LMW succinoglycan in nodule development may be to enhance survival in the colonized curled root hair [45].

The SYRM and PHOB proteins are positive regulators of EPS I and EPS II production, respectively [46]. Among the identified regulators, MUCR appears to be a global regulatory protein that plays a key role in both the positive regulation of EPS I synthesis and the negative regulation of EPS II synthesis, thus linking these two biosynthetic pathways [46].

The production of *S. meliloti* EPS is affected by several nutritional and stress conditions. Limitations of some non-carbon nutrients, such as nitrogen and sulfur, very high concentrations of phosphates and hyperosmotic stress stimulate the synthesis of EPS I [47].

On the other hand, phosphate starvation stimulates the production of EPS II, indicating that the concentration of this nutrient is an important signal affecting, which type of EPS will be produced by *S. meliloti*. In addition, different osmotic conditions alter the biosynthesis of EPS in this bacterium. Low osmotic pressure leads to the formation of mainly low-molecular EPS I, while the production of the high-molecular fraction of this polymer is stimulated by increased osmotic pressure. Jofre and Becker reported that the polymerization of EPS I is influenced by the ionic strength of the medium, and not by osmolarity [48].

Although there are quite a lot of studies that consider the role of EPS in symbiosis, it is still far from a complete understanding of the functions of EPS in the formation and functioning of symbiotic systems. It has been shown that the quality and quantity of polysaccharides, especially EPS and lipopolysaccharides produced by rhizobia, can affect both their agglutination of these bacteria and the formation of biofilms on the surface of plant roots, which plays a crucial role in the initial stages of symbiosis [49,50].

Mutants that do not produce EPS could cause twisting of root hairs, but do not form infectious filaments and nodules. EPS has also been shown to be involved in various stages of the development of the infection thread, bacteroids and suppression of the immune response of the host plant [51, 52]. There are several papers that have been devoted to the specificity of bean-rhizobial symbiosis due to the structure of EPS [22, 53]. It has been suggested that the amount of EPS produced by rhizobia is associated with the optimization of the interaction of the microsymbiont with the macrosymbiont [54]. One of the latest achievements in this field, using optical control of the expression of EPS II biosynthesis genes, demonstrated spatial control of structured biofilm formation [55]. Thus, EPS is one of the key factors for achieving successful interaction between symbiotic partners [56].

However, there are still no effective ways to modify the synthesis of EPS in rhizobial cells for the purpose of further application of transformed strains. Most articles describe mutant strains, in which the expression of certain genes involved in EPS biosynthesis is blocked. Often, such strains lose their competitiveness and cannot enter into a full-fledged symbiotic relationship [57].

*Application of Sinorhizobium meliloti in biotechnology*

There are several directions for the use of nitrogen-fixing bacteria, in particular, *S. meliloti*, in biotechnology. The first direction is the creation of strains that provide the plant with a sufficient amount of nitrogen and the enrichment of the soil with nitrogen. However, as noted by a number of authors, in the practical use of the results the scientists face some problems. Despite the advantages of microbial inoculant technology, there are some success-limiting factors against a universal utilization [58]. In fact, the efficiency of microbe-based biofertilizers depends on many factors including the targeted crop, edaphic (pH, salinity, and soil type), biotic (competition between introduced and indigenous strains, microbial parasites and predators), and climatic factors [59, 60]. Competition among microbial strains for resources and plant nodulation, partner fidelity and specificity mediated by genetic and molecular mechanisms are among the success-limiting factors against a universal utilization of microbial inoculants [61, 62]. On the other hand, commercial inoculants were often made with one or at most two strains, while under field conditions, plants are associated with many strains, which provide them diverse benefits through functional complementarity. Nevertheless, the poor performance of biofertilizers is primarily linked to inappropriate strains and inefficient production technology. Herrmann et al., studying the microbial quality of 65 commercial inoculants manufactured in seven different countries, showed that only 36% of the products could be considered as “pure”.

Among the remaining 64% some contained one or several strains of contaminants and some products did not contain any strains [63].

Particular attention is paid to the use of symbiotic bacteria in various climatic zones and under the influence of stress factors, such as soil salinity. The effectiveness of symbiotic interaction under salinization conditions depends on the effectiveness of isolates under standard conditions, on the number of nodules formed by rhizobia on the roots of the host plant, but did not correlate with the source of rhizobia release (soil, nodule) and their salt resistance [64]. The data obtained indicate the possibility of identifying strains of nodule bacteria that provide high efficiency of symbiosis in conditions of salinity of the soil.

However, the use of nodule bacteria is not limited to the earth's soil. Nodule plants and their N-fixing symbionts may play a role in increasing the fertility of the Martian soil. This approach is due to an increase in population growth and a reduction in arable land on Earth. It is known that on Earth clover (*Melilotus officinalis*) forms a symbiotic relationship with the N-fixing bacteria *S. meliloti*. It was assumed that an increase in plant biomass would be observed in the Martian regolith inoculated with the corresponding N-fixing bacteria, and the excess nitrogen available to plants would be deposited in the surrounding regolith, as is the case on Earth. Experiments have shown that the growth of shoots and roots of plants increased by more than 75% with inoculation of *S. meliloti* 1021compared with plants grown in non-inoculated regolith. This study highlights the importance of nitrogen as the main limiting factor for plant growth in regolith, suggesting that nitrogen-fixing bacteria can be used to reduce this restriction. The authors of the study suggest that their experience can become the basis for future research on food production in the conditions of the Martian soil [65].

The second most significant and developed direction of biotechnological use of *S. meliloti* is bioremediation. It means the elimination, neutralization or conversion into a less toxic form of eco-pollutants using biological processes. This method is often used in cases of soil contamination with heavy metals, using microorganisms and plants to restore the biological productivity of the ecosystem. Soil contamination by heavy metals has become a serious worldwide environmental problem. Nitrogen-fixing rhizobia with high intrinsic metal resistance have been investigated widely for their potential to improve plant growth, reduce metal toxicity, and change metal availability in soil, which may help in the development of microbe-assisted phytoremediation [66]. In recent years, more attention has been paid to the potential use of legume-rhizobium symbiosis for bioremediation of contaminated soil and to the responsible biochemical and molecular mechanisms [67]. *S. meliloti* CCNWSX0020 displayed tolerance to high levels of multiple metals, such as Cu, Zn, Cd, and Pb. Moreover, it could promote the growth, metal uptake, and antioxidant responses of *M. lupulina* in copper-contaminated soil [68, 66]. Extracellular polymeric substances were found to immobilize Cu ions and were predicted to play a role as a first protective barrier to prevent copper from reaching the cytoplasm [69]. Several genes conferring copper resistance were identified and a putative copper-transporting P1B-type ATPase and a zinc-transporting P1B-type ATPase were identified and shown to be involved in Zn, Cd, and Pb resistance [70]. Additionally, the genome sequence of *S. meliloti* CCNWSX0020 revealed several putative molecular chaperones, metal binding proteins, and unspecific divalent cation transporters predicted to have a role in Cu and Zn resistance [71]. The transcriptome profiles of the *S. meliloti* CCNWSX0020 responses to Cu and Zn stresses were analyzed to investigate *S. meliloti* CCNWSX0020 Cu and Zn resistance mechanisms [72].

Szewczuk-Karpisz et al. made the attempt to determine flocculating properties of exopolysaccharide (EPS) synthesized by the bacteria *Sinorhizobium meliloti* 1021, which would increase the efficiency of chromium(III) oxide removal from sewages and wastewaters [8]. Chromium(III) oxide is an amphoteric, dark green solid. This most stable dye is widely used in construction and ceramic industries as well as in painting. Due to its extensive use in many industries, the $Cr_2O_3$ presence in wastewaters is inevitable. The obtained results showed that EPS of *Sinorhizobium meliloti* 1021causes chromium(III) oxide suspension destabilization in the whole examined pH range. The largest change in the system stability before and after the polymer addition was observed at pH 9. It is probable that under these conditions bridging flocculation occurs in the examined system [73].

EPSs of *S. meliloti* can have a protective role against the exposure to toxic metals. In this sense, in presence of either As or Hg, the Rm8530 WT strain was able to reach OD or cfu·mL$^{-1}$ similar to control condition

without metal, whereas mutants defective on the synthesis of EPSs were not capable, in presence of metals, of achieving the growth parameters reached under control conditions. These results support that the EPS II would be more relevant that the EPS I in dealing with the toxicity of heavy metals/metalloids [74]. These results are quite promising, but the following things should be taken into consideration. There must be strict monitoring and regulation of EPS-metal ion sorption experimental conditions in order to yield maximum possible removal. The subsequent notable point is the reusability and selectivity of this polymeric adsorbent. Also, it has been evident that the sorption through EPS is generally non specific. Overcoming this pitfall requires technological advancements as well as deeper understanding about the polymer and its mechanism of metal uptake. EPS modification and immobilization can be a good idea, but it is still much unexplored field [75].

Another approach is the use of genetically modified strains to expand the range of bioremediation action. This approach allows *S. meliloti* to be used for bioremediation of polychlorinated biphenyls (PCBs). Polychlorinated biphenyls are a class of potent environmental toxicants. The toxicological properties of a class of PCB congeners are largely influenced by the aqueous solubility and subsequent bioavailability. A variety of human health effects have been attributed to PCB exposure, including reproductive and birth defects, damage to the kidney, the nervous system and the immune system, and cancer. It is known that the genome of *S. meliloti* does not possess genes for bioremediation of aromatic pollutants. However, the genetically modified bacterium has the ability to enhance fertility of soil in association with the leguminous alfalfa plant while simultaneously enhancing bioremediation of PCB-contaminated soils. Enhanced bioremediation of PCB and robust alfalfa plant growth was also noted when uncharacterized mixed cultures containing alfalfa plant nodule formers were used [76].

The third direction of biotechnological use of *S. meliloti* has been developed relatively recently. It is known that lipolysaccharides of Gram-negative bacteria have a pronounced biological activity, including therapeutic activity. The experimental study of hematopoietic activity of four lipopolysaccharide (LPS) fractions isolated from *S. meliloti* L2 under induced immunodeficiency was carried out in mice. It was shown that administration of the lipolysaccharide preparation to mice with secondary experimental immunodeficiency was associated with decreased count of stab neutrophils and monocytopenia; LPS-1 fraction increased the count of segmented neutrophils; LPS-2 decreased the count of stab neutrophils and induced lymphocytosis; LPS-3 decreased the count of stab neutrophils and induced lymphocytosis associated with a significant increase in the count of segmented neutrophils; LPS-4 induced basophilia, decreased count of stab neutrophils, and lymphocytosis associated with a significant increase in the count of segmented neutrophils. It was made the conclusion that lipolysaccharide fractions of *S. meliloti* L2 exerted modulating effects similar to the mechanisms of "emergency myelopoiesis" in the physiological course of bacterial infections [77].

On the other hand, the study of rhizobial succinoglycan for its application in biotechnological and biomedical developments deserves attention. It can be used in drug delivery, biomedical imaging and nano-biosensor. A number of studies on biomedical applications of dextran-nanoparticle conjugate indicated a paradigm shift in bacterial exopolysaccharide based nanobiotechnology [78]. These conjugates are widely used in organ specific drug delivery, biosensor, drug carrier and encapsulation, haemoglobin-conjugate as blood substitute etc. [79]. Recently, modifications of succinoglycan using alginate beads with functionalized polydiacetylene vesicles are developed to assess barium (II) as a tangible fluorogenic sensor system [80]. As bacterial exopolysaccharides are unique group of biopolymers, which is both biodegradable and non-toxic, thus more research on application of succinoglycan as nanobiomaterial may open a new era in biomedical field. Till date, due to the non-toxic and good viscosifying nature, succinoglycan is used in food or cosmetic industry as commercial emulsifying agent. Production and commercialization of raw EPS is an intensive process; thus, easy downstream processing technique is needed for fast and better sales [81].

## Conclusions

Analyzing the research of *S. meliloti*, some prospective directions of the further investigations can be considered:

- in the field of agricultural research: selection of optimal genotypes of bacterial strains and host plants for effective symbiosis;

**Application of Sinorhizobium meliloti in biotechnology**

| Strain of *S. meliloti* | Application area | References |
|---|---|---|
| *Sinorhizobium meliloti* p221 | Destructor of polycyclic aromatic hydrocarbons | [82] |
| *Sinorhizobium meliloti* АК130 | Nitrogen fixator for normal and saline soils | [83] |
| *Sinorhizobium meliloti* АК55 | Nitrogen fixator for various agro-climatic conditions | [84] |
| *Sinorhizobium meliloti* 1021 | Chromium(III) oxide removal from sewages and wastewaters | [73] |
| *Sinorhizobium meliloti* 1021 | Cosmobiology | [65] |
| *Sinorhizobium meliloti* Rm8530 WT | Binding toxic metals (As or Hg) | [74] |
| *Sinorhizobium meliloti* MS-125 | Heavy metals adsorption (Pb, Ni, Zn) | [75, 85] |
| transformed *Sinorhizobium meliloti* (pE43) containing PCB-degrading genes | Bioremediation of polychlorinated biphenyls | [76] |
| *Sinorhizobium meliloti* L-14 | Medecine | [77] |
| *Sinorhizobium meliloti* 1021 | Stabilizing agent | [81, 86] |
| *Sinorhizobium meliloti* A2 | Emulsifier | [87] |

the study of the genetic regulation of the resistance of symbiont bacteria and host plants to stress factors for the targeted design of symbiotic systems with a given adaptive potential;

- in bioremediation field: strict monitoring and regulation of EPS-metal ion sorption;
- in medicine: modifications of bacterial exopolysaccharides to create the effective nanobiomaterials.

The data on the practical application of this bacterium in biotechnology are summarized in Table.

This work was supported by the State Program 10-H by 12.03.2021.

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## БАКТЕРІЯ *Sinorhizobium meliloti* ЯК ПЕРСПЕКТИВНИЙ ОБ'ЄКТ ДЛЯ БІОТЕХНОЛОГІЇ

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Бактерія *Sinorhizobium meliloti* належить до Грам-негативних грунтових азотфіксувальних бактерій, здатних підвищувати врожайність бобових рослин. У літературі є відомості про повну послідовність геному цієї бактерії, крім того, вивчено полісахаридний склад біоплівки, яка бере активну участь у фіксації нітрогену. Відома нуклеотидна послідовність, а також генетичні та біохімічні особливості бактерій *S. meliloti* роблять цей організм ідеальною моделлю для біотехнологічних досліджень. Метою роботи було проаналізувати сучасні дані, наведені в літературі щодо симбіотичної взаємодії бактерій *Sinorhizobium meliloti* з рослиною-господарем, й охарактеризувати основні напрями їх використання в сільському господарстві, біоремедіації та медицині.

***Ключові слова:*** *Sinorhizobium meliloti*, симбіоз, біотехнологія.

UDC 577.151.6 https://doi.org/10.15407/biotech14.06.037

# DIRECTED PROTEOLYSIS OF FIBRINOGEN BY PROTEASE OF *Gloydius halys halys* VENOM

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Received 24.09.2021
Revised 18.11.2021
Accepted 30.12.2021

One of the approaches for studying structure and functions of proteins is their limited proteolysis. Proteolytic fragments of macromolecules can preserve the biological activity and can be used for the study of their structural and functional peculiarities. Thus, the characterization of new proteolytic enzymes and determination of the specificity of their action can be of interest for exploration. In the present work, we focused on the action of protease from the venom of *Gloydius halys halys* on fibrinogen, the crucial protein of blood coagulation system.

*Methods.* Products of fibrinogen hydrolysis by protease from the venom of *G. halys halys* were studied by SDS-PAGE electrophoresis and western-blot analysis using monoclonal antibodies II-5 Cand 1-5A targeted to 20–78 and 549–610 fragments of fibrinogen Aα-chain. Molecular weights of hydrolytic products were determined using MALDI-TOF analysis on Voyager DE PRO (USA). Sequence of hydrolytic products were predicted by "Peptide Mass Calculator" soft ware.

*Results.* SDS-PAGE showed that protease from the venom of *Gloydius halys halys* initially cleaved Aα-chain of fibrinogen molecule. Western-blot analysis confirmed that this protease specifically cleaves off fragment of C-terminal parts of Aα-chain with apparent molecular weight of 22 kDa. Cleaved fragment was identified by MALDI-TOF analysis as the 21.1 kDa polypeptide. "Peptide Mass Calculator" predicted that such a fragment corresponded to Aα414-610 residue of fibrinogen molecule. Thus, we showed that studied protease cleaved peptide bond AαK413-L414 with the formation of stable partly hydrolyzed fibrinogen desAα414-610.

*Conclusions.* The use of protease from the venom of *Gloydius halys halys* would allow obtaining the unique partly hydrolyzed fibrinogen des Aα 414–610 that is suitable for the study of structure and functions of fibrinogen αC-regions.

***Key words:*** fibrinogen, limited proteolysis, protease, fibrin polymerization, hemostasis.

Proteinases that exhibit fibrinogenolytic activity can be used to study the structure and function of fibrin(ogen). Limited proteolysis allows to obtain unique fragments of fibrinogen, studying which we can ascertain the functional importance of cleaved sites in the processes of fibrin polymerization, platelet aggregation, endothelial cell proliferation, leukocyte interaction, etc. [1]. Fragments of fibrinogen obtained by limited proteolysis can retain biological activity, information about which is important in medicine primarily for understanding the course of pathophysiological processes [2]. In addition, proteinases targeting fibrinogen and/or fibrin are also of interest as potential agents for direct defibrination *in vivo* [3, 4]. One of the sources of fibrinogenolytic proteinases is snake venom, which mostly contains α- or β-fibrinogenases [5, 6].

The vast majority of α-fibrinogenases isolated from snake venoms are metalloproteinases with an average molecular weight of 20–26 kDa. During longer incubation, they are also able to break down the β-chain of fibrinogen [7]. Such proteinases have been isolated from venoms of snakes of the genera *Gloydius*, *Daboia* [8], *Deinagkistrodon* [9], *Crotalus* [10], *Trimeresurus* [11], *Agkistrodon* [12], and *Naja* [13].

Most of β-fibrinogenases are serine proteinases. They are able to break down the Aα chain of the fibrinogen molecule at a lower rate [6]. Such proteinases have been found in snakes of many genera, such as *Trimeresurus* [14], *Bothrops*, *Lachesis* [15], *Gloydius* [16] and *Hydrophis* [17].

Serine proteinases also include a special group of so-called thrombin-like enzymes. These enzymes hydrolyze fibrinogen, and their action is directed at those bonds that are affected by thrombin, which leads to the formation of fibrin [18]. Such enzymes are known in snakes of the genera *Agkistrodon*, *Bitis*, *Trimeresurus*, *Cerastes*, *Bothrops*, and *Lachesis* [18, 19, 20].

Previously, a fibrinogen-specific serine proteinase with molecular weight of 28 kDa was obtained from the venom of *Gloydius halys halys* [21]. The aim of our study was to study the specificity of its action on fibrinogen, to identify the generated partially hydrolyzed form of fibrinogen and to determine the hydrolyzed bond.

## Materials and Methods

*Materials*

Proteinase with fibrinogenolytic activity was collected from *G. halys halys* by a previously developed method [21]. Fibrinogen was purified from human blood plasma obtained by adding sodium citrate according to the method described by T. V. Varetskaya [22]. Marker proteins (Thermo Fisher Scientific, USA) and anti-mouse goat antibodies labeled with horse reddishper oxidase (Sigma, USA) were used. Mouse monoclonal antibodies II-5C (anti-Aα20-78) and 1-5A (anti-Aα537-595) were developed and purified in the Department of Protein Structure and Function of the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine [23, 24].

*Directed proteolysis of fibrinogen*

Hydrolysis of fibrinogen was performed in the ratio of enzyme:substrate = 1:300 in 0.05 M Tris(2-amino-2-hydroxymethyl-propane-1,3-diol)-HCl buffer, pH 7.4, containing 0.13 M NaCl, at a temperature of 37 °C. The hydrolysis reaction was stopped by adding electrophoresis sample buffer containing 2% SDS, 5% glycerol and 2% β-mercaptoethanol and then boiling the resulting mixture.

*SDS-PAGE/ Westernblot*

Electrophoresis was performed by the Laemmli method [25] using the Tris-glycine system. Separation of proteins was performed at a current of 19 mA in the concentrating and 35 mA for the separating gels. Samples for electrophoresis were prepared by adding the protein solution to buffer sample, which was prepared by adding 5% sucrose, 2% SDS and 0,2% bromophenol to the electrode buffer. Before applying to the gel, the samples were heated to boiling.

After SDS-PAGE, the proteins were transferred to the nitrocellulose membrane by electric current (voltage 100 V) for 2 hours. The membrane was blocked with 5% skim milk solution in TBS (tris-buffered saline) for 1 hour. Then the proteins were incubated with mouse monoclonal antibodies II-5C (anti-Aα20-78) or 1-5A (anti-Aα537-595) for 2 hours, and after washingin TBS, incubated with the secondary secondary anti-mouse goat antibodies labeled with peroxidase. The strips were incubatedin a solution of 0.001 M 4-chloro-1-naphthol in 0.5 M Tris pH 7.5 and 0.03% $H_2O_2$ to develop specific immunostaining.

*Obtaining low molecular weight products of hydrolysis*

To obtain a low molecular weight productsof hydrolysis, a solution of fibrinogen (15 mg/ml) in Tris-HCl buffer pH 7.4 with 0.13 M NaCl was incubated for 30 min with proteinase from the venom of *Gloydius halys halys* (0.02 mg/ml) at temperature 37 °C. The hydrolysis reaction was stopped by salting out fibrinogen in an equal volume of 16% $Na_2SO_4$. After centrifugation at 3 000 rpm for 30 min, the supernatant containing the enzyme and the cleaved polypeptide was collected and examinedusing mass spectrometry analysis.

*Mass spectrometry*

Mass spectrometry analysis was performed on a MALDI-TOF spectrometer Voyager DE PRO (Applied Biosystems, USA). H+-matrix ionization was performed by laser irradiation. The concentration of sinapine acid (Sigma, USA) in the matrix reagent was 1 mg/ml. The reagent was dissolved in a solution containing equal volumes of acetonitrile (Sigma, USA) and 1% aqueous trifluoroacetic acid (Sigma, USA). The obtained spectra were processed by Data Explorer 4.0.0.0 (Applied Biosystems) [26].

*Statistical processing and bioinformatics analysis*

Statistical processing of the results was performed using standard statistical software "Microsoft Excel" for Windows 2000.

Determination of the amino acid sequence, cleaved from fibrinogen by the action of proteinase from the venom of *Gloydius halys halys*, was performed with the program "Peptide Mass Calculator" (https://www.peptidesynthetics.co.uk/tools/).

## Results and Discussion

Fibrinogen-specific proteinase from the venom of *G. halys halys* was obtained by a method previously developed at the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. It was found that the studied enzyme has a molecular weight of approximately 28 kDa and belongs to serine proteases, because the activity of the enzyme is inhibited by DFP (diisopropylfluorophosphates) and benzamidine [21].

SDS-PAGE showed that the enzyme from the venom obtained from *G. halys halys* is specific to the Aα chain of fibrinogen, which was hydrolyzed within 90 min. No cleavage of Bβ- and γ-chains was observed during the incubation period.The reaction revealed the accumulation of hydrolysis products of the Aα-chain of fibrinogen, the molecular weights of which are approximately 44, 20 and 17 kDa (Fig. 1).

Western blot analysis was performed to determine which part of the Aα-chain of fibrinogen is cleaved by proteinase obtained from *G. halys halys*. Accumulation of a high molecular weight polypeptide with a mass of approximately 43 kDa was shown using II-5C monoclonal antibody to the N-terminal portion of the Aα chain of the fibrinogen molecule (Fig. 2). When using antibody1-5A to the C-terminus of the Aα-chain of fibrinogen, the formation of two products was observed, with molecular weights of approximately 22 and 18 kDa (Fig. 3).

The obtained results indicate that this proteinase cleaves a fragment with a molecular weight of approximately 22 kDa from the C-terminus of the Aα-chain of fibrinogen.

Detection of two products by Western blot analysis using antibody 1-5A to the C-terminal region of the Aα-chain of fibrinogen is due to partial hydrolysis of fibrinogen with low amounts of plasmin impurities. In this case, plasmin cleaves the sequence which includes 27 amino acid residues. As a result, the solution contains a small amount of partially hydrolyzed fibrinogen, the molecular weight of which is 2.8 kDa less than the weight of native fibrinogen [27]. Therefore, two polypeptides can be detected among the hydrolysis products under the action of proteinases that are specific for one peptide bond of the C-terminal region of the Aα-chain.

To determine the site of enzymatic hydrolysis of fibrinogen, the exact mass of the obtained polypeptides was determined using MALDI-TOF analysis (Fig. 4). It was found that the hydrolysis produces peaks in which the ratio of mass to charge (M/Z) is 21109 and 18375, which indicates the appearance of polypeptides with masses of 21.1 kDa and 18.4 kDa.

According to calculations in the Peptide Mass Calculator program, this molecular weight corresponds to the peptides Aα414-610 and Aα414-583, which are formed by hydrolysis of the AαLys413-Leu414 bond of the fibrinogen molecule.

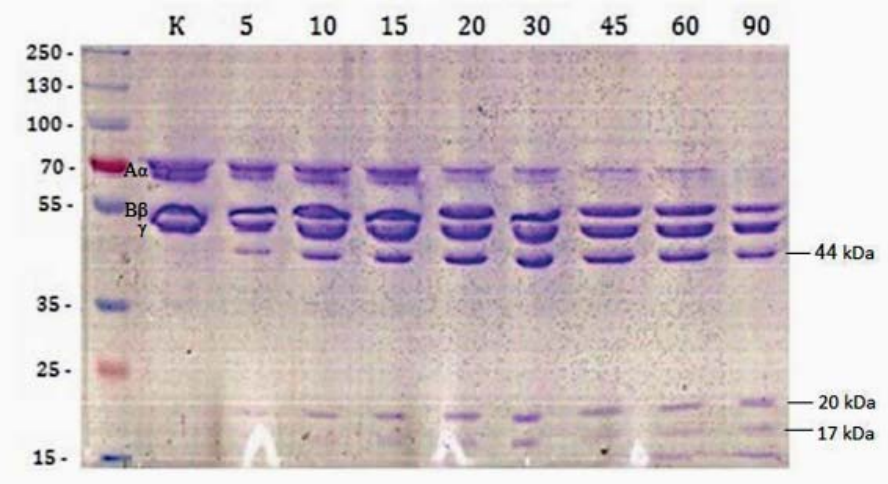


*Fig. 1.* **Electrophoregram of fibrinogen hydrolysis products by proteinase from the venom of *G. halys halys*:**
K — native fibrinogen; 5, 10, 15, 20, 30, 45, 60, 90 minutes of exposure to the enzyme. The samples were prepared in the presence of 0.2% β-mercaptoethanol. The gel was stained with Coomassie blue

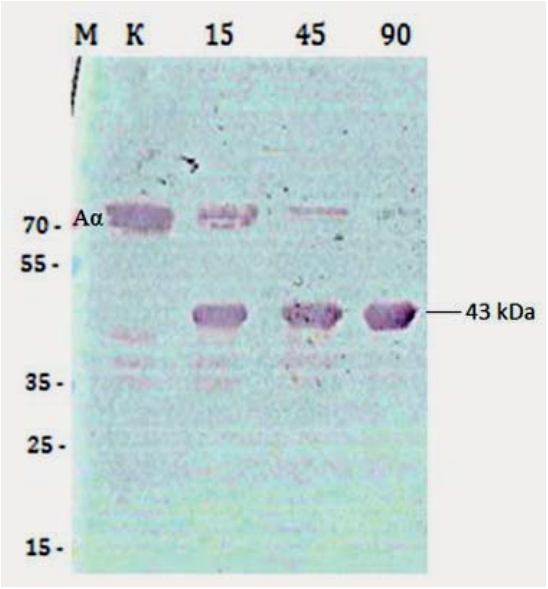


*Fig. 2.* **Western blot analysis of products of fibrinogen hydrolysis by proteinase from the venom of *G. halys halys* using monoclonal antibody II-5C to the N-terminal regions of the Aα–chain of fibrinogen:**
K — native fibrinogen; 15, 45, 90 minutes of exposure to the enzyme. Samples were prepared in the presence of 0.2% β-mercaptoethanol

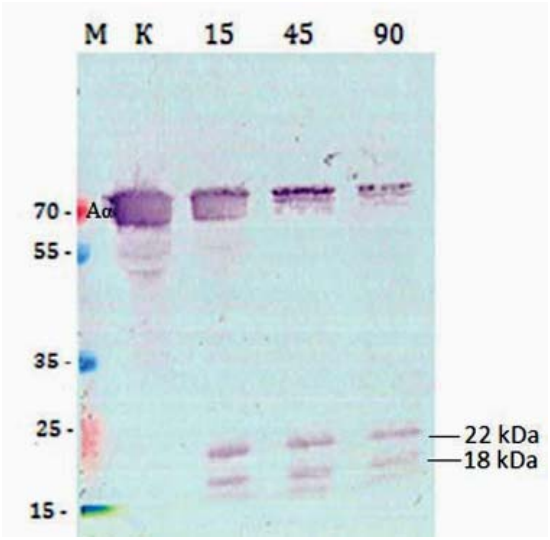


*Fig. 3.* **Western blot analysis of products of fibrinogen hydrolysis by proteinase from the venom of *G. halys halys* using monoclonal antibodyI-5A to the C-terminal regions of the Aα-chain of fibrinogen:**
K — native fibrinogen; 15, 45, 90 min of exposure to the enzyme. Samples were prepared in the presence of 0.2% β-mercaptoethanol

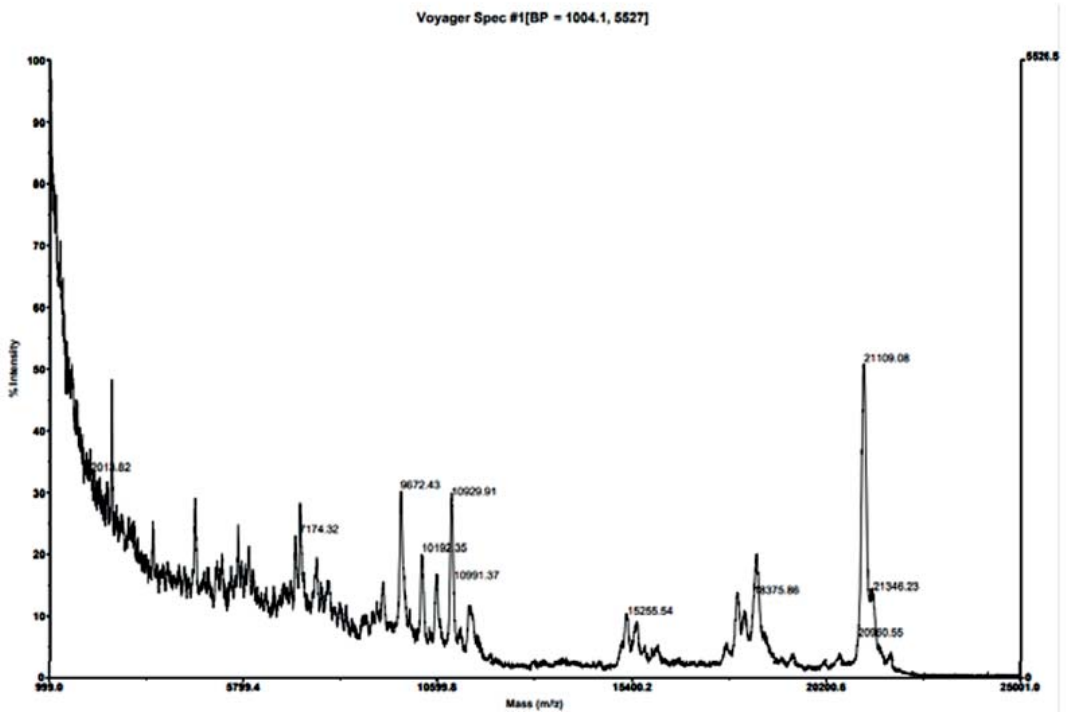


*Fig. 4.* **MALDI-TOF spectrum of hydrolysis products formed by incubation of fibrinogen with proteinase from the poison of *G. halys halys***
The arrows indicate the peaks with a mass to charge ratio of 21109 and 18375

The sequence of the fibrinogen molecule Aα221-610 belongs to the αC-region. In this region, the connector sequence (Aα221-391) and αC-domain (Aα392-610) are distinguished [28]. It is known that αC = domains are involved in intermolecular interactions in the lateral association of protofibrils and fibrils [29]. Also, this sequence is involved in the processes of platelet aggregation [30], migration and proliferation of endothelial and other cells [31, 32].

Our results show that proteinase from the poison of *G. halys halys* cleaves the Aα413-414 bond. Therefore, we can obtain a fragment of fibrinogen Aα414-610 and a form of fibrinogen desAα414-610 with the help of this proteinase. This fibrinogen completely preserves the connector sequence of the αC-region, as well as the sequence Aα392-413 of the αC-domain. Study of the peculiarities of the functioning of the partially hydrolyzed form of fibrinogen and the influence of the fragment Aα414-610 on the functioning of native fibrinogen can reveal the role of Aα221-413 and Aα414-610 αC-region in the functioning of fibrinogen.

The proteinase of *G. halys halys* is specific for the AαLys413-Leu414 peptide bond. Using this enzyme, a partially hydrolyzed form of fibrinogen desAα414-610 can be obtained from the native fibrinogen of human blood plasma to study the functional role of individual sites of the αC region of fibrinogen.

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# СПРЯМОВАНИЙ ПРОТЕОЛІЗ ФІБРИНОГЕНУ ПРОТЕЇНАЗОЮ З ОТРУТИ *Gloydius halys halys*

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*Мета.* Одним із підходів до вивчення структури та функції протеїнів є їх обмежений протеоліз. Отримані шляхом гідролізу фрагменти протеїнів також можуть мати біологічну активність, що може бути використано в дослідженні структурно-функціональних особливостей цих макромолекул. Тому актуальним є пошук селективних протеїназ та визначення специфічності їхньої дії. В цій роботі досліджувалася дія протеїнази з отрути *Gloydius halys halys* (щитомордника звичайного) на фібриноген — основний протеїн системи згортання крові.

*Методи.* Продукти гідролізу фібриногену протеїназою отрути *G. halys halys* досліджували за допомогою методів електрофорезу у поліакриламідному гелі та вестерн-блоту з використанням моноклональних антитіл II-5C (анти-Aα20-78) та 1-5A (анти-Aα549-610). Молекулярну масу продуктів гідролізу визначали за допомогою MALDI-TOF мас-спектрометрії на Voyager DE PRO (США). Послідовності, які відщеплюються від фібриногену досліджуваною протеїназою, встановлювали за допомогою програмного забезпечення «PeptideMassCalculator».

*Результати.* Електрофоретичний аналіз показав, що протеїназа з отрути *G. halys halys* найбільш специфічно розщеплює Aα-ланцюг молекули фібриногену. За допомогою вестерн-блот аналізу було виявлено, що протеїназа відщеплює фрагмент з молекулярною масою приблизно 22 кDa від С-кінця Aα-ланцюга молекули фібриногену. Відщеплюваний фрагмент було ідентифіковано MALDI-TOF аналізом як поліпептид з масою 21,1 кDa. Згідно програми «PeptideMassCalculator», цей фрагмент відповідає послідовності Aα414-610. Отже, протеїназа, виділена з отрути *G. halys halys*, специфічно гідролізує пептидний зв'язок AαK413-L414 з утворенням стабільної частково гідролізованої форми — фібриногену desAα414-610.

*Висновок.* Використовуючи досліджувану протеїназу, виділену з отрути *G. halys halys*, можна отримати унікальний частково гідролізований фрагмент фібриногену, позбавлений ділянки Aα414-610, яка дозволить дослідити структурно-функціональні особливості αC-регіонів фібрин(оген)у.

***Ключові слова:*** спрямований протеоліз, фібриноген, протеїназа, полімеризація фібрину, гемостаз.

UDC 611.018.51. 013.68. 612.592.111 https://doi.org/10.15407/biotech14.06.044

# RESTORATION OF THE STRUCTURAL AND FUNCTIONAL STATE OF ERYTHROCYTES AFTER HYPOTHERMIC STORAGE USING HUMAN CORD BLOOD LOW-MOLECULAR FRACTION AND THE DRUG ACTOVEGIN

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Received 27.09.2021
Revised 26.11.2021
Accepted 31.12.2021

One of the modern transfusiology tasks is to preserve the properties of erythrocytes after hypothermic storage. The transfusion medium quality and efficiency depend on their functional state. Plausible protective effects of a human cord blood low-molecular fraction (CBF) and the drug Actovegin were evaluated in the study in order to use them as components of recovery medium.

*Aim.* The purpose of this study was to investigate the CBF and the drug Actovegin effect on erythrocytes morphology, energy balance, and oxygen transport function of erythrocytes after their hypothermic storage.

*Materials and methods.* Erythrocyte mass was stored in hypothermia for 7–21 days. Every 7 days, the CBF or Actovegin (final concentration 0.6 mg/ml) were added to the samples and incubated for 1 h at 37 °C. CBF was obtained from human cord blood by ultrafiltration. The erythrocytes morphology was assessed using light microscopy. The content of ATP and 2.3-DPG was determined by photoelectrocalorimetric method. The saturation, $O_2$ and $CO_2$ tension were assessed using an analyzer of blood gases. The ratio of hemoglobin forms was studied by photometry.

*Results.* It has been shown that both CBF and the drug Actovegin helped to restore the morpho-functional characteristics of erythrocytes after 21 days of their storage at 2–4 °C. This was indicative as an increase in the normocytes number increase and restoration of $O_2$ tension, saturation, ATP and 2.3-DFG content, and normalization of the hemoglobin forms ratio. It is suggested that the mechanisms of the drags action was are associated with ATP synthesis activation and 2.3-DFG formation.

*Conclusions.* The use of media containing CBF or the drug Actovegin enabled to effectively restore the properties of erythrocytes disturbed after their prolonged storage.

***Key words***: cord blood, Actovegin, erythrocytes, hypothermia, recovery medium.

In practical transfusiology, preservation of donor blood erythrocytes under low temperatures and with special preservative solutions is widely used for their long-term storage. This method enables not only to provide strategic reserves of red blood cells, but also to accumulate several doses from one donor for transfusions under the scheme “one donor — one recipient” [1]. However, since the 70’s it is known that the violation of the main indicators of erythrocytes’oxygen transport function, such as saturation and ATP (adenosine triphosphate) and 2,3-DFG (2,3-diphosphoglycerate) content, occurs already on the third day of hypothermic storage (2–4 °C) [2]. Given this, the efforts of scientists and hematologists over the last decades were aimed at solving the problem of restoring the structural and functional integrity of erythrocytes after hypothermic storage, and much attention was paid to the methods for extending the storage period of thawed erythrocytes after cryopreservation [1, 3, 4]. To do this, numerous attempts

were made to develop hemoconservatives and rehabilitation media, which helped stabilize the membrane of deconserved erythrocytes due to non-penetrating components (sucrose, proteins, polymers) and increase their energy potential due to penetrating metabolites of carbon phosphate metabolism [5–9]. As a result of these studies, it was managed to extend the hypothermic storage period fordeconserved erythrocytes from 24 hours to 5–21 days, provided the use of the "closed systems" of preservation, hardware methods and additional solutions [1]. However, a common disadvantage of such additional solutions is that their components have usually a negative side effect on the recipient's body [6]. In addition, to obtain the desired effect, most of these media should be used in a ratio where the donor erythrocytes volume in the sample is less than the rehabilitation solutionvolume, which provoke additional stress in the recipient.

We have previously shown the possibility of using a low molecular weight fraction (up to 5 kDa) from cord blood or a similar pharmaceutical product Actovegin, which is known to be a low molecular weight blood fraction (up to 5 kDa) of dairy calves, to rehabilitate leukoconcentrate cells after cryopreservation. It has been proven that low molecular weight fraction (up to 5 kDa) from cord blood or the drug Actovegin, which is a deproteinized hemoderivative from dairy calves, can significantly increase the energy potential of cells, have a balanced composition of biomolecules and do not have toxic effects on the animals' body and different cell cultures [10–13]. Based on the above, the aim of this study was to investigate the restorative effect of low molecular weight fraction (up to 10 kDa) from human cord blood (CBF) and the drug Actovegin on the main indicators of erythrocytes'oxygen transport function after long-term hypothermic storage.

## Materials and Methods

*Ethical approval.* Human cord and donor blood samples were collected and manipulated in accordance with the recommendations of the Helsinki Declaration of the World Medical Association for Biomedical Research on Humans (Helsinki, 1964, Somerset West, 1996), as well as the recommendations of the European Convention for the Vertebrates Protection used for research and other scientific purposes (Strasbourg, 1986).

*The method of CBF obtaining.* The low molecular weight fraction with molecular weight components up to 10 kDa was obtained from human cord blood after cryodestruction using the method of multistage ultrafiltration using equipment from Sartorius (Germany) [14]. Lyophilization of the ultrafiltrate was performed in a freeze-drier at a temperature of −40 °C for 18–20 hours under average pressure in a chamber of 102 Pa. Ready made CBF samples have the form of a hygroscopic substance of yellowish color. Before use, a drug portion was dissolved in sterile physiological solution (Lekhim-Kharkiv, Ukraine).

*Scheme of experiment*. Erythrocyte mass was obtained from the whole human donated blood preserved with the hemopreservative Glugicir or CPDA-1 at the Kharkiv Blood Transfusion Center by removing the leukothromocytelayer after centrifugation (3 000 g, 5 min). The obtained erythrocyte mass was stored under sterile conditions at a temperature of 2–4 °C. In control periods (1, 7, 14 and 21 days) aliquots of erythrocyte mass (950 µl) were taken and CBF (50 µl of solution to a final concentration in samples of 0.6 mg/ml) or the drug Actovegin for a comparative series of experiments (solution for injection, 40 mg/ml "Takeda" (Austria), the final concentration in the samples was 0.6 mg/ml) were added to them. 50 µl of sterile physiological solution (Lekhim-Kharkiv, Ukraine) was added to the control samples. Thus, 3 experimental groups were formed. The obtained samples were incubated for 1 hour at a temperature of 37 °C, after which further studies were performed.

S*tudy of erythrocyte morphology*. After incubation of erythrocyte mass samples with the studied preparations, clinical smears were prepared, which were fixed according to May-Grunwald and stained according to Romanovsky [15]. Light microscope (PZO-Warszava, Poland) under immersion (eyepiece 8, objective 100) was used for erythrocyte morphology analyze, which included counting cells in 10–12 fields of view (at least 1000 cells per smear) and determining the ratio of normocytes and echinocytes number.

*ATP and 2,3-DFG content determining*. The ATP and 2,3-DFG content was studied by non-enzymatic method evaluating inorganic phosphorus in erythrocyte hydrolysates. The amount of inorganic phosphorus was calculated by photoelectrocalorimetric method [16–18]. The amount of ATP and 2,3-DFG was monitored on the 7$^{th}$, the 14$^{th}$ and the 21$^{st}$

day of storage (2–4 °C). To do this, after each specified period of hypothermic storage, CBF or the drug Actovegin were added to the samples of erythrocyte mass at a final concentration of 0.6 mg/ml and incubated for 1 h at 37 °C. The concentration of ATP and 2,3-DFG was then measured. To determine the mechanism of the studied preparations action, before their addition the cells were additionally incubated with a glycolysis inhibitor sodium iodoacetate (Serva, Germany) for 5 min at 37 °C at a final concentration of 1 mM.

*Parameters of erythrocytes oxygen transport function research.* To assess the oxygen transport function of erythrocytes, the dynamics of changes in such indicators as saturation (percentage of oxygen in the blood) and oxygen and carbon dioxide tension in experimental samples of erythromass from human donor blood were analyzed using a cartridge analyzer IL GEM Premier — 3000.

*Determination of hemoglobin forms ratio.* To study the effect of CBF and the drug Actovegin on the hemoglobin forms ratio, erythromass from human donor blood was incubated with the studied drugs, as described above. After that, the samples were subjected to three-time washing with physiological solution at 3000 rpm for 15, 5 and 5 min, respectively. In the obtained erythrocyte mass, the ratio of hemoglobin forms was determined by the photometric method according to [19].

*Statistical analysis* of the obtained data was performed using the software package "StatGraphics Plus 2.1". Verification of the data distribution normality was performed according to the Shapiro-Wilk W-test. Student's t-test was used to compare two independent groups on one basis. The value of the significance level $P$ was taken equal to 0.05, which meets the criteria adopted in biomedical studies. Data are presented as arithmetic mean ± standard deviation.

## Results and Discussion

*Study of changes in erythrocyte morphology.* The effect of CBF and the drug Actovegin on the change in erythrocyte morphology after hypothermic storage was primarily studied. In control samples of donated blood, the number of normocytes decreased rapidly during the first 7 days of hypothermic storage from 80% to 21% (Fig. 1, *A*), while the number of echinocytes increased from 12% to 70% (Fig. 1, *B*). Incubation of cells in medium containing CBF or the comparison drug Actovegin for 1 h at 37 °C to the same extent and significantly contributed to the restoration of erythrocytes morphological parameters, most pronounced at the 21$^{st}$ day of hypothermic storage (Fig. 1).

Thus, the analysis of the obtained data showed that starting from the 7$^{th}$ day of hypothermic storage after incubation in a rehabilitation medium containing CBF (up

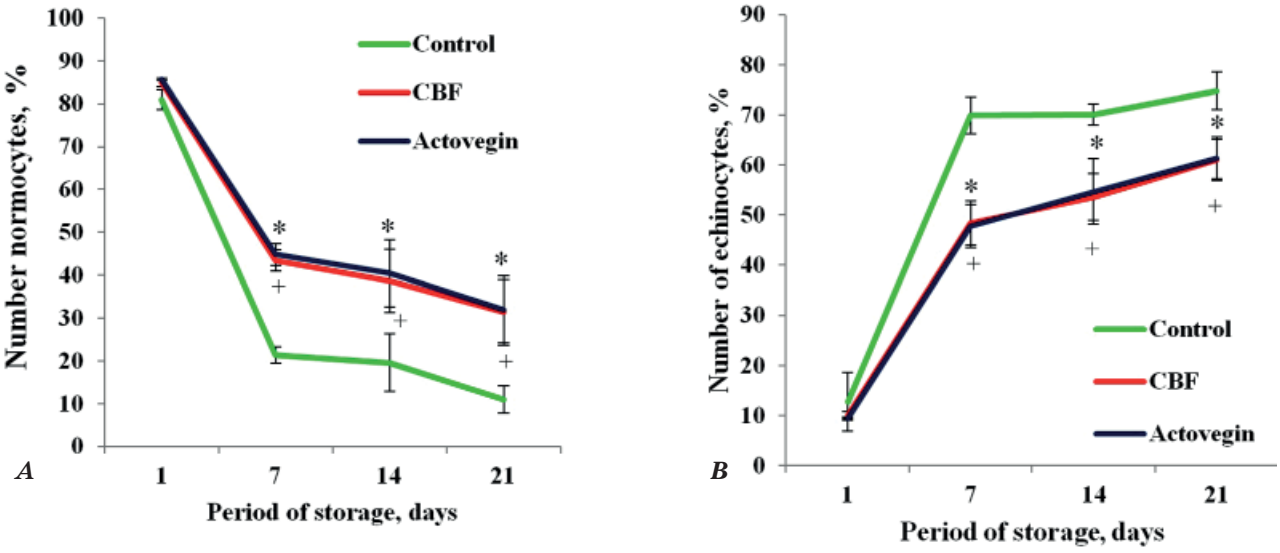


*Fig. 1.* **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the number of normocytes (*A*) and echinocytes (*B*) in human donor blood depending on the duration of hypothermic storage**

*, + — differences are significant in comparison with control values in the corresponding day of hypothermic storage ($P \leq 0.05$); $n = 6$

to 10 kDa) or the drug Actovegin, there was a significant improvement in morphological parameters of erythrocytes compared to control samples: normocytes content increased 2 times, while the number of echinocytes decreased by 30–40% depending on the hypothermic storage duration. The ability of CBF and the drug Actovegin to help normalize the erythrocytes shape was observed even after 21 days of these cells' storage in hypothermic conditions.

*The content of ATP and 2,3-DFG in erythrocytes*. In parallel with the study of erythrocytes morphological changes during hypothermic storage, the effect of CBF and the drug Actovegin on the content of phosphorus-containing compounds in erythrocytes was studied. The results of the study showed that the content of ATP in the control erythrocytes samples gradually and significantly decreased. In general, during the entire storage period, the initial value decreased by 2.3 times. Incubation of cells in media containing CBF or the drug Actovegin helped to restore ATP levels to those that were close to baseline. Addition to the incubation medium of the glycolysis inhibitor sodium iodoacetate blocked the stimulating effect of both investigated drugs. As a result, the ATP content remained at the level of control values (Table 1).

Similar changes were observed in the study of the 2,3-DFG content.During the storage of erythrocytes, this indicator significantly decreased and it was only 62% of the initial valueby the final date. After cells incubation in rehabilitation media with CBF or the drug Actovegin, the 2,3-DFG content was restored (Tabl. 2). It should also be noted that the glycolysis inhibitor blocked the 2,3-DFG accumulation.

*Criterion of erythrocytes oxygen transport function*. At the next stage of the research, the effect of CBF on the erythrocytes oxygen transport function was studied according to standard indicators: oxygen tension ($PO_2$), carbon dioxide tension ($PCO_2$) and saturation ($SPO_2$). The results of these parameters study are given in the Table 3. In the process of erythrocytes hypothermic storage, the $O_2$tension remained virtually unchanged, but the $CO_2$ tension increased and saturation decreased to critical values. Addition of CBF to the erythrocytes incubation medium contributed to a probable increase in $O_2$tension and a decrease in $CO_2$tension at each of the time control points. Throughout the study period, a pronounced effect of CBF on the saturation index was also recorded.

*The ratio of hemoglobin forms.* Investigation of the hemoglobin forms ratio in donor blood erythrocytes stored in hypothermic conditions have shown that in the control there is a redistribution between the content of oxy-, deoxy- and methemoglobin in the direction of the share of the latter two increasing. After incubation in rehabilitation medium with CBF, there was an increase in oxyhemoglobin and a parallel decrease in deoxy- and methemoglobin, which resulted in the restoration of the relationship between these indicators at each of the studied storage periods (Table 4).

A similar regularity was found when adding the drug Actovegin to the incubation medium. Thus, erythrocytes incubation in rehabilitation medium with the drug Actovegin contributed to a significant increase in oxyhemoglobin content at each of the observation points. The content of deoxy- and methemoglobin was significantly reduced, which contributed to the $O_2$Hb: DeoxyHb: MetHb ratio normalization.

Fig. 2 shows the change in the oxygenation coefficient, which is the ratio of the deoxyhemoglobin amount to the oxyhemoglobin

*Table 1.* **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the ATP content in donor human erythrocytes, μmol/ml of erythromass**

| Period of storage, days | Control | CBF | Actovegin | CBF + Ia | Actovegin + Ia |
|---|---|---|---|---|---|
| 1 | 5.38 ± 0.07 | – | – | – | – |
| 7 | 3.3 ± 0.32* | 5.3 ± 0.29$^{+}$ | 5.31 ± 0.44$^{+}$ | 3.5 ± 0.16** | 3.48 ± 0.11** |
| 14 | 2.98 ± 0.19* | 4.9 ± 0.23*$^{+}$ | 4.93 ± 0.28*$^{+}$ | 3.02 ± 0.31** | 3.09 ± 0.34** |
| 21 | 2.3 ± 0.31* | 4.05 ± 0.51*$^{+}$ | 4.21 ± 0.25*$^{+}$ | 2.38 ± 0.47** | 2.4 ± 0.47** |

*Note*: Ia — sodium iodoacetate; * — differences are significant compared to the control indicator for the 1st day of storage ($P < 0.05$); $^{+}$ — differences are significant compared to the corresponding indicator for control ($P < 0.05$); ** — differences are significant compared to the options for erythrocytes incubation in media containing CBF and the drug Actovegin, respectively ($P < 0.05$); $n = 5$.

*Table 2.* **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the 2,3-DFG content in human donor erythrocytes, μmol/ml of erythromass**

| Period of storage, days | Control | CBF | Actovegin | CBF + Ia | Actovegin + Ia |
|---|---|---|---|---|---|
| 1 | 5.61 ± 0.18 | – | – | – | – |
| 7 | 4.8 ± 0.32* | 5.4 ± 0.09$^{+}$ | 5.5 ± 0.13$^{+}$ | 4.9 ± 0.11** | 5.05 ± 0.18** |
| 14 | 4.05 ± 0.35* | 4.9 ± 0.13*$^{+}$ | 5.06 ± 0.22$^{+}$ | 4.11 ± 0.17** | 4.17 ± 0.28** |
| 21 | 3.5 ± 0.41* | 4.9 ± 0.34*$^{+}$ | 5.36 ± 0.4$^{+}$ | 3.58 ± 0.38** | 3.77 ± 0.21** |

*Note:* Ia — sodium iodoacetate. * — differences are significant compared to the control indicator for the 1$^{st}$ day of storage ($P < 0.05$); $^{+}$ — differences are significant compared to the corresponding indicator for control ($P < 0.05$); ** — differences are significant compared to the options for erythrocytes incubation in media containing CBF and the drug Actovegin, respectively ($P < 0.05$); $n = 5$.

*Table 3.* **Change in the parameters of erythrocytes oxygen transport function depending on the storage period and the presence in the incubation medium of the human cord blood low-molecular fraction**

| Period of storage, days | Criterion of oxygen transport function | | | | | |
|---|---|---|---|---|---|---|
| | $PO_2$ | | $PCO_2$ | | $SPO_2$, % | |
| | Control | CBF | Control | CBF | Control | CBF |
| 1 | 32 ± 2.0 | 45.5 ± 1.5* | 82.45 ±0.65 | 77.05 ±1.55* | 46 ± 1.1 | 63.05 ±0,75* |
| 7 | 29 ± 1.0 | 51.5 ± 0.5* | 96.6 ±1.3 | 87 ± 1.0* | 43.7±1.5 | 60.3 ±0.4* |
| 14 | 41 ± 1.0 | 77 ± 3.0* | 111 ±0.3 | 104.5±1.25* | 40.2 ±0.3 | 58.1 ±0.4* |
| 21 | 35 ± 2.0 | 72 ± 1.0* | 116 ±1.5 | 105.2±1.3* | 37.9±0.65 | 55.9±0.5* |

*Note*: * — differences are significant compared to the corresponding control indicator ($P < 0.05$); $n = 6$.

*Table 4.* **The effect of the human cord blood low-molecular fraction and the drug Actovegin on the hemoglobin forms ratio**

| Period of storage, days | Incubationmedium | $O_2Hb$, % | DeoxyHb, % | MetHb, % |
|---|---|---|---|---|
| 1 | Control | 73.2 ± 1.32 | 25.8 ± 2.18 | 1.1 ± 0.14 |
| 7 | Control<br>CBF<br>Actovegin | 66.3 ± 2.23<br>73.0 ± 1.9*<br>79.1 ± 3.07* | 27.3 ± 2.44<br>22.1 ± 2.94*<br>19.8 ± 0.58* | 6.4 ± 0.43<br>4.9 ± 0.66*<br>1.1 ± 0.72* |
| 14 | Control<br>CBF<br>Actovegin | 64.6 ± 3.67<br>73.9 ± 2.87*<br>72.3 ± 2.18* | 30.0 ± 2.98<br>23.0 ± 2.48*<br>21.7 ± 2.09* | 7.75 ± 0.54<br>3.1 ± 0.47*<br>6.0 ± 0.88* |
| 21 | Control<br>CBF<br>Actovegin | 60.4 ± 2.49<br>69.8 ± 2.38*<br>71.7 ± 2.9* | 31.1 ± 1.77<br>25.8 ± 2.15*<br>21.9 ± 1.35* | 8.5 ± 0.5<br>4.5 ± 0.08*<br>6.4 ± 1.13* |

*Note*: * — differences are significant compared to the corresponding control indicator ($P < 0.05$); $n = 6$.

amount. In control erythrocytes samples there was a gradual decrease in the oxygenation coefficient throughout the observation period and on the 21st day of hypothermic storage the indicator decreased by 1.5 times (Fig. 2). CBF increased the oxygenation coefficient after 7 days of erythrocytes hypothermic storage by 37.5%, after 14 days — by 52%, and after 21 days — by 42%. Stimulating effect on the oxygenation coefficient was observed also when adding the drug Actovegin to the incubation medium. Thus, after 7, 14 and 21 days of storage, cell rehabilitation with the drug Actovegin led to an increase in the studied coefficient by 67%, 57% and 73%, respectively (Fig. 2).

Under physiological conditions, human erythrocytes have the shape of a biconcave disk. The shape of erythrocytes has a high sensitivity to changes in medium conditions and composition. So, for many years it is considered a parameter that reflects the normal state of cells [20]. It is known that under conditions of erythrocytes storage at 4–6 °C there is a change in their shape from discocytes (normocytes) to echinocytes, and then to spherocytes. Such deformation is irreversible even after transfusion [21].

In our studies, the shape of erythrocytes that were subjected to hypothermic storage was restored by adding to their incubation medium of CBF or the drug Actovegin at a final concentration of 0.6 mg/ml. Discussing the possible mechanisms of the detected rehabilitative effect of CBF and the drug Actovegin on the erythrocytes morphology, we could make the following assumptions. First, the erythrocytes shape restoration may be associated with an improvement in the cells energy potential as a result of the glycolysis process stimulation and a corresponding increase in phosphorus-containing metabolites in erythrocytes. A similar mechanism was found for the CBF effect on energy metabolism in leukocytes of donated blood [10, 12, 13]. In turn, increasing the ATP level enables to maintain the cytoskeleton structure by regulating the protein kinases activity that phosphorylate actin, spectrin and ankyrin, thereby maintaining the discoid form of erythrocytes [22, 23]. Secondly, this phenomenon may be associated with the direct action of the components contained in the CBF on the erythrocytes' cytoskeleton. This assumption is based on our experiments on the effect of fraction on donor blood leukocytes, using cytocholasin B, which is known to inhibit the cytoskeleton and inhibit glucose transport in cells by blocking glucose transporters of the Glut family [24, 25].

The first assumption is confirmed by studying the ATP and 2,3-DFG content in

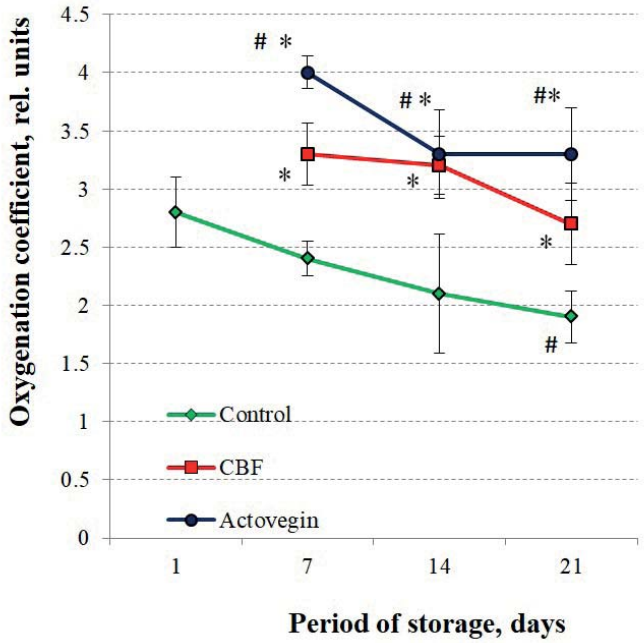


*Fig. 2.* **The effect of the human cord blood low-molecular fractionand the drug Actovegin on the oxygenation coefficient**

* — differences are significant in comparison with the corresponding control ($P < 0.05$); # — the difference is significant in comparison with the control for the 1st day of storage ($P < 0.05$)

erythrocytes. It has been found that CBF and the drug Actovegin contribute to the increase the content of these compounds in erythrocytes. It is important to note that the glycolysis inhibitor sodium iodoacetate blocks the stimulating effect of both drugs. The obtained data of inhibitory analysis indicate that with the help of the investigated drugs it is possible to influence the intensity of glycolysis reactions and thus stimulate the ATP synthesis and 2,3-DFG formation, as the latter is formed in glycolysis from 1,3-diphosphoglycerate.

As a result of the conducted experiments it has been also found that by CBF adding to the erythrocytes incubation medium it is possible to increase the indicators of their oxygen transport function. Studies of hemoglobin affinity for oxygen in donor blood erythrocytes stored in hypothermic conditions showed that in the control this indicator decreases by 30% for 21 days, and after incubation in a rehabilitation medium with CBF, it increases by 30–50% depending on storage period. This effect can be compared with the stimulating effect of CBF with a molecular weight of up to 5 kDa, found in our previous research [26].

In addition, CBF and the drug Actovegin have been shown to affect the ratio of erythrocytes' hemoglobin forms. It is known that under physiological conditions, hemoglobin in erythrocytes can be in one of three forms — deoxyhemoglobin, methemoglobin or oxyhemoglobin [27].

In our experiments, incubation of erythrocytes stored under hypothermic conditions in the medium with CBF or the drug Actovegin was able to increase significantly the oxyhemoglobin proportion. The CBF and the comparison drug Actovegin had the same effect on the ratio of hemoglobin forms (Table 4, Fig. 2). In total, the obtained data indicate the normalization of erythrocytes oxygen transport function, as deoxyhemoglobin does not contain oxygen molecules, and methemoglobin is not able to perform the function of an oxygen carrier. Based on these data, it can be concluded that both rehabilitation media increase blood oxygen capacity.

The revealed facts can be explained by several reasons. First, it has been found that CBF and the drug Actovegin increase the ATP and 2,3-DFG content (Tables 1 and 2), which regulate the hemoglobin conformation and the reaction of oxygen attachment to it [28]. Secondly, it is possible that the studied drugs contain components that are able to change the hemoglobin conformation independently or as a result of a combined complex action towards its affinity increasing for oxygen.

## Conclusions

As a result of the research it was found by the first time that the CBF (5-10 kDa) or the drug Actovegin presence in the incubation medium promoted to restore the morpho-functional characteristics of erythrocytes after hypothermic storage at 2–4 °C for 21 days, which is expressed in a probable increase in normocytes and restoring indicators of their functional state, namely oxygen tension, saturation, ATP and 2,3-DFG and normalization of hemoglobin different forms ratio.

The article was prepared within the framework of the scientific work "Investigation of the dependence of the composition and biological activity of cord blood fractions on the conditions of cold exposure" (State registration number 0115U000093, deadline 2015–2019), which is funded from the budget of the National Academy of Sciences of Ukraine.

The authors declare that any pharmaceutical or other companies did not influence the treatment conditions, study design, data collection and analysis, preparation of manuscript, and choice of journal.

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## ВІДНОВЛЕННЯ СТРУКТУРНО-ФУНКЦІОНАЛЬНОГО СТАНУ ЕРИТРОЦИТІВ ПІСЛЯ ГІПОТЕРМІЧНОГО ЗБЕРІГАННЯ ЗА ДОПОМОГОЮ НИЗЬКОМОЛЕКУЛЯРНОЇ ФРАКЦІЇ КОРДОВОЇ КРОВІ ЛЮДИНИ І ПРЕПАРАТУ АКТОВЕГІН

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Однією із задач сучасної трансфузіології є збереження властивостей еритроцитів після гіпотермічного зберігання. Від їхнього функціонального стану залежить якість та ефективність трансфузійного середовища. Досліджено можливість використання як субстанції для реабілітувального середовища низькомолекулярної фракції кордової крові людини (ФКК) і препарату Актовегін.

*Мета.* Дослідити вплив ФКК і препарату Актовегін на морфологію, енергетичний баланс і киснево-транспортну функцію еритроцитів.

*Матеріали та методи.* Еритроцитарну масу зберігали за умов гіпотермії впродовж 7–21 доби. Кожні 7 діб до зразків додавали ФКК або препарат Актовегін (кінцева концентрація 0.6 мг/мл) та інкубували 1 год при 37 °С. ФКК отримували з кордової крові людини методом ультрафільтрації. Морфологію еритроцитів оцінювали за допомогою світлової мікроскопії. Вміст АТФ та 2,3-ДФГ визначали фотоелектрокалориметричним методом. Сатурацію, напруженість $O_2$ і $CO_2$ оцінювали за допомогою аналізатора газів крові. Співвідношення форм гемоглобіну досліджували фотометрично.

*Результати.* Показано, що ФКК або препарат Актовегін сприяють відновленню морфофункціональних характеристик еритроцитів після 21 доби зберігання при 2-4 °С. Це виражалось у збільшенні кількості нормоцитів і відновленні показників напруженості $O_2$, сатурації, вмісту АТФ і 2,3-ДФГ та нормалізації співвідношення форм гемоглобіну. Механізм дії цих препаратів пов'язаний з активацією синтезу АТФ та утворенням 2,3-ДФГ.

*Висновки.* Використання середовищ з ФКК або препаратом Актовегін, дозволило ефективно відновлювати властивості еритроцитів після тривалого зберігання.

***Ключові слова:*** кордова кров, Актовегін, еритроцити, гіпотермія, відновлююче середовище.

UDC [577.13+543.645]:581.143.6 **https://doi.org/10.15407/biotech14.06.053**

# OBTAINING AND BIOCHEMICAL ANALYSIS OF TISSUE CULTURE *Scutellaria baicalensis* Georgi.

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Received 12.09.2021
Revised 21.11.2021
Accepted 30.12.2021

*Aim.* To obtain a tissue culture of *S. baicalensis* as a possible source of biologically active compounds (BAC) with a wide range of pharmacological action.

*Methods.* Photocolorimetric method, reversed-phase high performance liquid chromatography (HPLC) method.

*Results.* Two stably productive tissue culture strains (16SB3 and 20SB4) of *S. baicalensis* were obtained from fragments of roots seedling on a specially developed agar 5C01 nutrient medium. The yield of dry biomass from 1 liter of this medium per passage (21st day of growth) for strain 16SB3 is 25–30 g, for strain 20SB4 — 30–40 g. The total content of flavonoids in dry biomass was in terms of routine for strains 16SB3 and 20SB4 — 0.6–0.9 and 0.7–0.9 mg/g, respectively, and the yield of flavonoids — 18–27 and 21–36 mg/l of nutrient medium, respectively. BAC, typical for plants in nature, in particular, flavonoids vogonin, baikalein, neobaikalein, skulkapfavon and their derivatives, were found in the studied biomass of both strains.

*Conclusions.* It was found that the biomass of the two strains of *S. baicalensis* tissue culture accumulated the same BAC, in particular, flavonoids, as do plants in natural conditions. The resulting tissue culture is promising as a possible source of Baikal skullcap BAC.

***Key words:*** *Scutellaria baicalensis* Georgi., plant tissue culture, flavonoids, strains — producers of biologically active compounds.

*Scutellaria baicalensis* Georgi. is a Daurian endemic, a popular medicinal plant. Its roots are used in traditional Chinese medicine, which is recorded in Chinese, European and British pharmacopoeias. It is a natural source of flavonoids, flavonoid glycosides, polysaccharides — substances with sedative, hemostimulating, antimutagenic, antitumor, neuroprotective, antihypoxic, nootropic, anxiolytic, antineurotic, hepatoprotective and chondroprotective properties [1, 2].

However, the natural sources of raw materials of *S. baicalensis* are almost exhausted, and the natural raw materials themselves are not always high quality and stable in their composition. An alternative source of biologically active compounds (BAC) of plant origin may be tissue and cell culture, as already shown for a number of valuable and rare medicinal plants [3, 4]. That is, the culture of Baikal sagebrush tissues can be an alternative source of practically important BAC. In Russia, Japan and some other countries, tissue cultures of *S. baicalensis* have been obtained, which are of interest as sources of the corresponding compounds [5, 6]. There is no such tissue culture in Ukraine.

We introduced into culture *in vitro* *S. baicalensis* and identified two strains of tissue culture, biochemical analysis of which showed that they were promising as a source of raw materials for flavonoids, flavonoid glycosides, polysaccharides.

## Materials and Methods

*Seed.* Seeds of *S. baicalensis* from the 2015 harvest from the Tartu University Botanical Garden in Estonia were used for the research.

*Preparation and disinfection.* The seeds of Baikal skullcap were treated with 1% gibberellin solution for 22 h, then the seeds were treated with detergent (2 g of household soap was dissolved in 100 ml of cold water) for 30 min, washed with running water for 60 min, sterilization of seeds was performed under sterile conditions. The seeds were treated with 96% ethyl alcohol for 10 seconds, sterilized in 15% hydrogen peroxide solution for 20 min, washed three times in sterile distilled water and planted one seed to germinate in test tubes on a specially designed nutrient medium 5C01 with mineral base described in [7].

*Cultivation.* Plant tissue culture was obtained from fragments of roots seedling on the same composition of the nutrient medium 5C01. Seeds were germinated and callus was initiated in 20 ml and 1 cm diameter tubes containing 1 ml of agar medium. The resulting callus (tissue culture) was grown in glass vessels (jars) with a volume of 250 ml, containing 40 ml of medium, at a temperature of 24–26 °C without lighting at a relative humidity of 70–80%. All materials and components of nutrient media were domestically produced.

*Materials, methods and equipment for biochemical research.* The total content of flavonoids was determined on a photoelectrocalorimeter Photometer KFK3 according to the method [8]. The used reagents were of domestic production.

*Preparation of samples.* The samples of callus tissue, dried at 54–56 °C, were ground in an agate mortar and filled with methanol (10 ml per 1 g of sample).

Analysis of secondary compounds was carried out by reversed-phase high performance liquid chromatography. Separation of the samples was performed on an Agilent 1100 chromatographic system with 4-channel pump, vacuum degasser, autosampler, column thermostat and diode-matrix detector. It was used a two-eluent scheme (eluent A = 0.05 M aqueous solution of orthophosphoric acid $H_3PO_4$; B = methanol/all eluents and additives Sigma-Aldrich, gradation of purity HPLC) on a column Poroshell C18, 2.7 µm, 2.1×150 mm with a passport resolution of more than 18,000 t.t. We made use of sample volume 5 µl, column temperature was 20 °C, flow rate — 0.15 ml/min, analysis time up to 70 min. Detection at wavelengths of 206, 254, 300, 350 and 450 nm was carried out to determine the most organic compounds (including terpenoids), most aromatic substances, phenylpropanoids (oxycinnamic acids and lignans), flavonoids (flavones and flavonols), carotenoids and chlorophyll, respectively. Absorption spectra were recorded for all substances in the ultraviolet and visible ranges in order to establish the nature of secondary metabolites and assign chromatographic peaks to certain groups of substances [9]. Vogonin was identified based on chromatographic data of the standard.

*Statistical analysis.* The calculation of the Student's test was performed using the Origin program.

## Results and Discussion

*Primary calluses obtaining.* Five days later, 4 seeds out of 10 planted germinated (Fig. 1). With a sterile scalpel, the seedlings roots of *S. baicalensis* were cut into pieces 1–3 mm long placed in the test tubes on nutrient medium 5C01, and left in a thermostat at a temperature of 24–28 °C in dark until the formation of callus tissue. A month later, a callus was formed, well visually accessible, the size of the pieces of callus was 3–4 mm in diameter. The frequency of callus formation reached 100%. Pieces of callus were transferred to Petri dishes with a nutrient medium of the same composition. Then callus culture was grown under the same conditions for another month. The selected light, actively growing pieces of callus culture were transferred to 40 ml and 2 cm diameter tubes containing 2 ml of nutrient medium.

Three weeks later, the most productive light yellow and yellow, pink-colored pieces of callus culture were collected and transferred to 250 ml glass jars containing 40–45 ml of nutrient medium of the same composition. The passage duration was reduced to 3 weeks. After several consecutive passages, two strains (variants) of callus culture of tissues 16SB3 and 20SB4 of Baikal skullcap of two genotypes (from two different original seedlings) were obtained.

Cultivation of strains 16SB3 and 20SB4 of *S. baicalensis* plant tissue culture was performed at a temperature of 24–28 °C, relative humidity of 70–80% in dark in a one-step method on agar nutrient medium 5C01, prepared according to the recipe of [5]. The medium was poured into glass jars with a

*Fig. 1.* **One of the four *S. baicalensis* seedlings**

volume of 250 ml of 40–45 ml, covered with foil, autoclaved for 15 min at a pressure of 1 atmosphere.

The biomass of both strains consisted of cells and cell aggregates of medium density. Strain 16SB3 was homogeneous, medium density, culture color from light yellow to dark yellow with a pink tinge (Fig. 2). Strain 20SB4 was homogeneous, loose, culture color from light yellow to dark yellow (Fig. 3).

The duration of the passage of both strains was 21 days. The weight of the inoculum when transplanted to fresh nutrient medium was 100–150 g of living biomass per 1 liter of nutrient medium.

The type of growth was disorganized, the growth index for strain 16SB3 — 4, for 20SB4 — 5. The yield of dry biomass on the 21$^{st}$ day of growth for strain 16SB3 was 25–30 g/l of medium, and for strain 20SB4 — 30–40 g/l (Table 1).

Biochemical analysis of the total content of flavonoids showed that the dry biomass contained flavonoids in terms of rutin for 16SB3 — 0.6–0.9 mg/g of dry tissue or 18–27 mg/l of nutrient medium on day 21 of growth, for 20SB4 — 0.7–0.9 mg/g of dry tissue or 21–36 mg/l of nutrient medium for 21 days (Table 1).

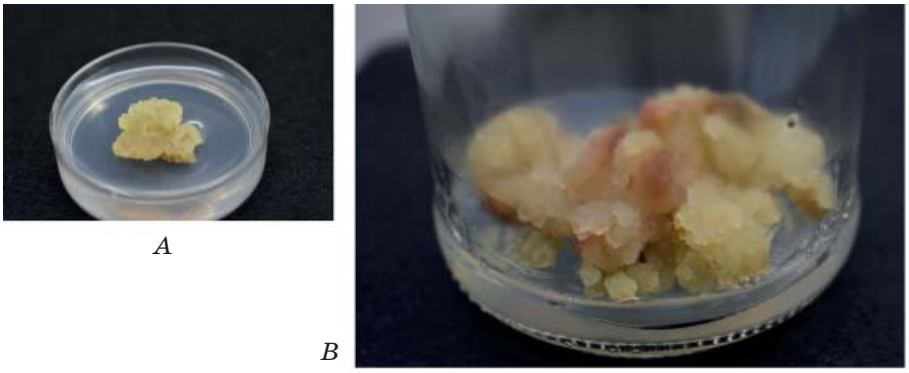
Strains 16SB3 and 20SB4 of Baikal skullcap tissue culture are stored in the collection of plant tissue cultures of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine. At the time of writing, both strains were cultivated for 1.5 years, passed 20 passages and were characterized by the stability of the studied characteristics.

*Analysis of the spectrum of secondary compounds.* Qualitative biochemical analysis of the spectrum of secondary compounds of both strains (16SB3 and 20SB4) of *S. baicalensis* tissue culture by high-performance liquid chromatography revealed substances characteristic of the plant in nature [5, 6], for example, the flavonoids neobaikalein, skulcapflavone, baicalein, wogonin and their derivatives.

Figures 4 and 5 show the chromatographic profiles of dry biomass extract of *S. baicalensis* tissue culture of both strains 16SB3 and 20SB4. Comparison of profiles shows that the strains differ only in the number of secondary compounds accumulated by culture cells (Table 2–5).

Thus, strain 20SB4 predominates in the content of derivatives of benzoic and oxybenzoic acids, flavanes, and the content of individual flavonoids of neobaikalein, baikalein and wogonin, while strain 16SB3 contains more flavonoid skulcapflavone.

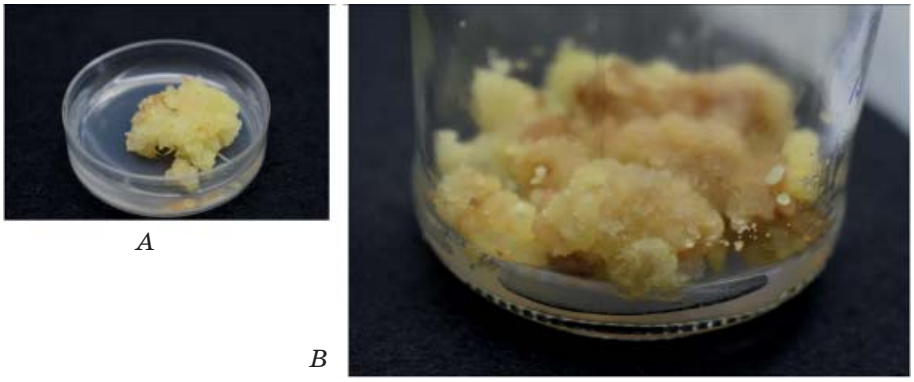
Further study of the obtained plant tissue culture will allow to determine a promising version of the culture.



*A*

*B*

*Fig. 2.* **Strain 16SB3 of *S. baicalensis* plant tissue culture for cultivation in Petri dishes (*A*) and in glass vessels with a volume of 250 ml (*B*)**



*A*

*B*

*Fig. 3.* **Strain 20SB4 *S. baicalensis* plant tissue culture for cultivation in Petri dishes (*A*) and in glass vessels with a volume of 250 ml (*B*)**

*Table 1.* **Productivity of strains 16SB3 and 20SB4 of *S. baicalensis* plant tissue culture**

| Strain | Passage | Yield of dry weight, g/l environment | Yield of dry weight, % from the living | Growth index | The content of flavonoids, mg/g of dry tissue |
|---|---|---|---|---|---|
| 16SB3 | 5<br>9<br>16 | 35.0 ± 0.07<br>30.6 ± 0.10<br>30.9 ± 0.05 | 4.9 ± 0.21<br>5.5 ± 0.09<br>6.2 ± 0.06 | 4<br>4<br>4 | 0.73 ± 0.01<br>1.46 ± 0.01<br>1.15 ± 0.03 |
| 20SB4 | 5<br>9<br>16 | 33.6 ± 0.09<br>27.3 ± 0.08<br>32.0 ± 0.06 | 4.8 ± 0.12<br>4.6 ± 0.10<br>4.2 ± 0.08 | 5<br>5<br>5 | 0.75 ± 0.01<br>0.94 ± 0.07<br>1.04 ± 0.01 |

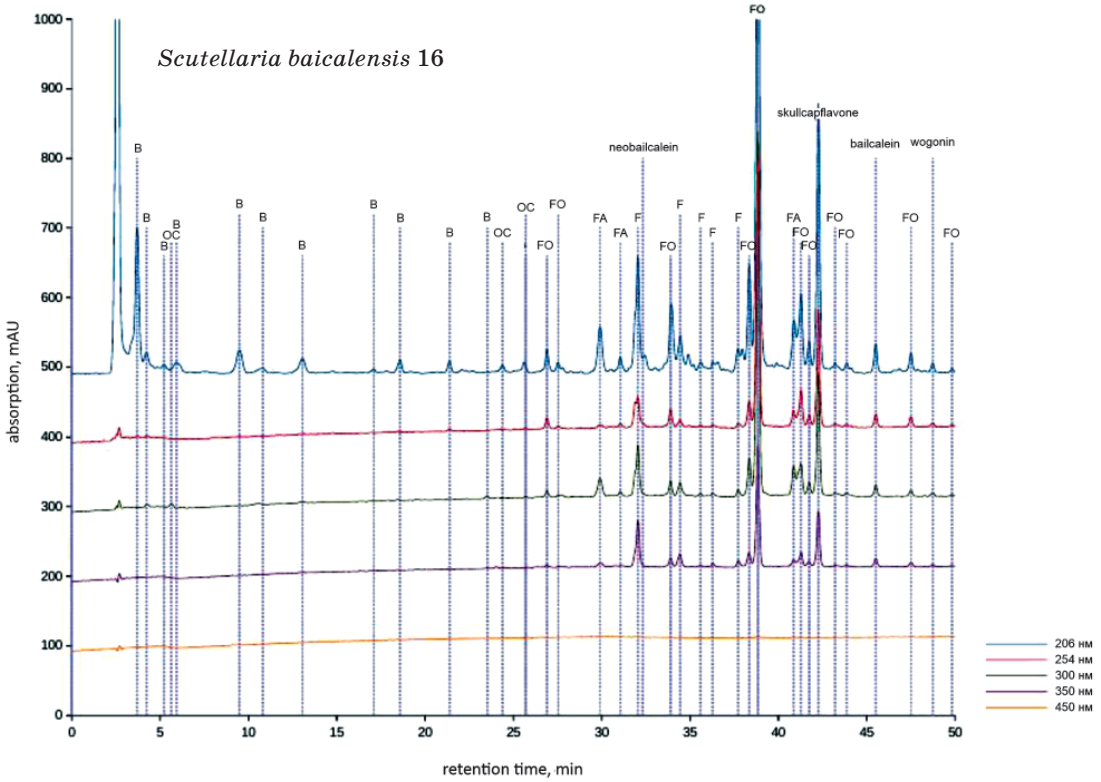



*Fig. 4.* **Chromatographic profiles of dry biomass extract of strain 16SB3 of *S. baicalensis* plant tissue culture**
Labels of substances: A — unrestrained pool of hydrophilic substances (free organic acids, amino acids, etc.) + solvent; B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid: F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxy fragments); F — other flavonoids; flavones: neobaicalein, skullcapflavone, baicalein, wogonin

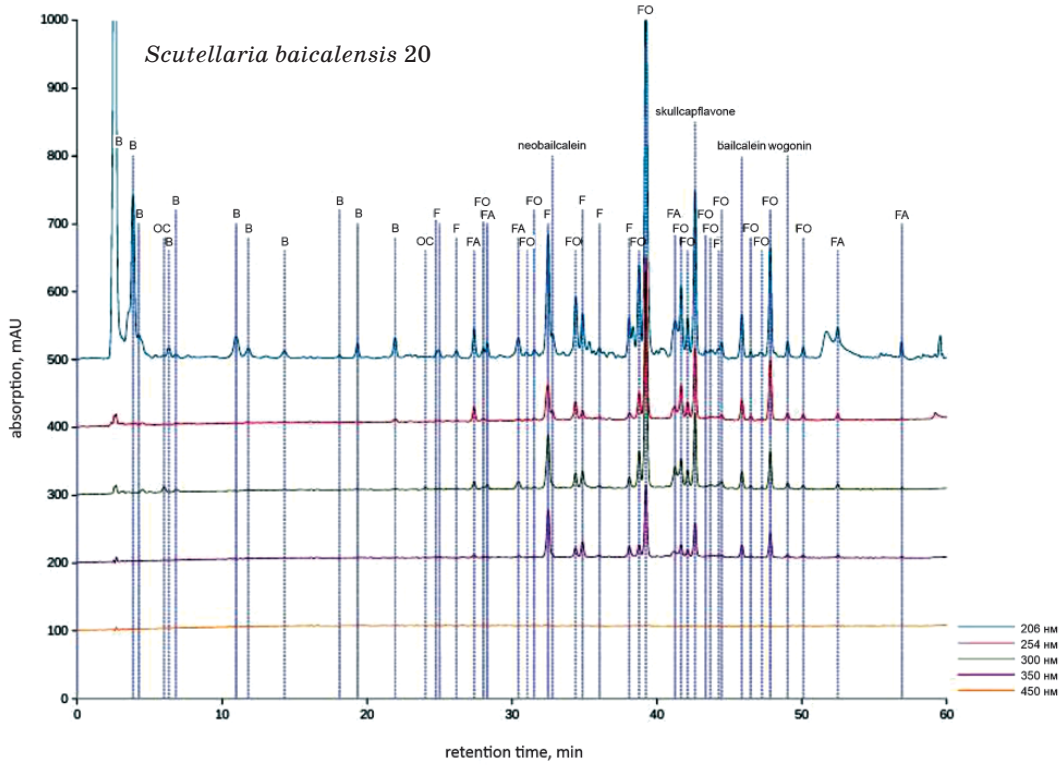



*Fig. 5.* **Chromatographic profiles of dry biomass extract of strain 20SB4 of *S. baicalensis* plant tissue culture**

**Labels of substances:** A — unrestrained pool of hydrophilic substances (free organic acids, amino acids, etc.) + solvent; B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid; F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxy fragments); F — other flavonoids; flavones: neobaicalein, skullcapflavone, baicalein, wogonin

*Table 2.* **The content of certain classes of organic substances in the dry biomass of strain 16SB3 of *S. baicalensis* plant tissue culture, (μg/g), the measurement error of the device ≤ 10%**

| Substances* | Content, μg/g of biomass |
|---|---|
| B | 2630 |
| OC | 174 |
| F | 890 |
| FA | 527 |
| FO | 1510 |

*Note:* * B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid; F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxyl fragments); F — other flavonoids.

*Table 3.* **The content of individual flavones in the dry biomass of strain 16SB3 plant tissue culture *S. baicalensis* (μg/g), the measurement error of the device ≤ 10%**

| Flavon | Content, μg/g of biomass |
|---|---|
| Neobaicalein | 5 |
| Skullcapflavone | 354 |
| Baicalein | 36 |
| Wogonin | 17 |

The peaks in the chromatograms were assigned to specific compounds based on the literature UV spectra. Vogonin was identified based on chromatographic data of the standard.

*Table 4.* **The content of certain classes of organic substances in the dry biomass of strain 20SB4 of *S. baicalensis* plant tissue culture, (μg/g), the measurement error of the device ≤ 10%**

| Речовини* | Content, μg/g of biomass |
|---|---|
| B | 3080 |
| OC | 89 |
| F | 1120 |
| FA | 499 |
| FO | 1180 |

*Note:** B — derivatives of benzoic and oxybenzoic acids; OS — derivatives of oxycinnamic acid; F — flavans; FA — flavones; FA — flavones similar to neobaikalein (there are hydroxyls in the phenyl moiety); FO — flavones similar to baicalein and vogonin (without hydroxy fragments); F — other flavonoids.

*Table 5.* **The content of individual flavones in the dry biomass of strain 20SB4 plant tissue culture *S. baicalensis* (μg/g), the measurement error of the device ≤ 10%**

| Flavon: | Content, μg/g of biomass |
|---|---|
| Neobaicalein | 12 |
| Skullcapflavone | 227 |
| Baicalein | 54 |
| Wogonin | 21 |

The peaks in the chromatograms were assigned to specific compounds based on the literature UV spectra. Vogonin was identified based on chromatographic data of the standard.

## Conclusions

Thus, Baikal skullcap *Scutellaria baicalensis* Georgi was introduced into *in vitro* tissue culture. Primary calluses were obtained from fragments seed seedlings roots of on a nutrient medium 5C01, specially created for plant cultures of tissues — superproducers.

The promising strains (16SB3 and 20SB4) of *S. baicalensis* tissue cultures were selected and the total content of flavonoids in dry tissue was determined. The yield of dry biomass from 1 liter of medium on the 21st day of growth for strains 16SB3 and 20SB4 is 25–30 g and 30–40 g, respectively. The content of flavonoids in dry biomass was in terms of rutin for strains 16SB3 and 20SB4 — 0.6–0.9 and 0.7–0.9 mg/g of tissue or 18–27 and 21–36 mg/l of nutrient medium for 21 day respectively.

The biochemical spectrum of flavonoids synthesized by cells of the obtained strains was determined. Cultivated callus cells were found to accumulate the same BAC, in particular, wogonin, baicalein, neobaikalein, and skulkapfavone, as *S. baicalensis* plants in nature.

The obtained plant tissue culture is the first stage in the development of cellular biotechnology for the production of practically valuable Baikal skullcap compounds in Ukraine.

*Acknowledgement.* Special thanks to the employe of the biotechnology department of the A. V. Fomin Botanical Garden Ph.D. A. V. Golubenko for transferred for work seeds Baikal skullcap *Scutellaria baicalensis* Georgi. and I. G. Bernatska, an employee of the department of genetics of cell populations of the Institute of Molecular Biology and Genetics of NAS of Ukraine for technical assistance in conducting experiments.

The study was funded within the Targeted program of the NAS of Ukraine "Genomic, molecular and cellular bases for the development of innovative biotechnologies", research project No. 0120U103216 "Development of cell culture technologies for production of plant-derived biologically active compounds for pharmaceutical and food industries" (2020–2024).

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## ОТРИМАННЯ ТА БІОХІМІЧНИЙ АНАЛІЗ КУЛЬТУРИ ТКАНИН *Scutellaria baicalensis* Georgi


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*Мета.* Отримати культуру тканин шоломниці байкальської *S. baicalensis* як можливого джерела біологічно активних сполук (БАС) із широким спектром фармакологічної дії.

*Методи.* Фотоколориметричний метод, метод обернено-фазової високоефективної рідинної хроматографії (ВЕРХ).

*Результати.* Отримано два стабільно продуктивні штами культури тканин (16SB3 та 20SB4) *S. baicalensis* із фрагментів корінців насіннєвих проростків на спеціально розробленому агаризованому живильному середовищі 5С01. Вихід сухої біомаси з 1 л цього середовища за пасаж (21-й день росту) для штаму 16SB3 становить 25–30 г, для штаму 20SB4 — 30–40 г. Загальний вміст флавоноїдів у сухій біомасі становив у перерахунку на рутин для штамів 16SB3 та 20SB4 — 0,6–0,9 та 0,7–0,9 мг/г відповідно, а вихід флавоноїдів — 18–27 та 21–36 мг/л живильного середовища відповідно. У дослідженій біомасі обох штамів виявлено БАС, властиві рослинам у природі, зокрема, флавоноїди вогонін, байкалеїн, необайкалеїн, скулкапфавон та їхні похідні.

*Висновки.* Встановлено, що в біомасі двох отриманих нами штамів культури тканин *S. baicalensis* накопичуються ті самі БАС, зокрема, флавоноїди, що й у рослин у природі. Отримана культура тканин є перспективною як можливе джерело БАС шоломниці байкальської.



***Ключові слова:*** *Scutellaria baicalensis* Georgi., культура тканин рослин, флавоноїди, клітинні штами — продуценти біологічно активних сполук.

UDC 543.544+547.655.6+577.1 https://doi.org/10.15407/biotech14.05.060

# SIMULTANEOUS IDENTIFICATION, QUANTIFICATION, AND MAIN COMPONENTS ANALYSIS OF *Artemisia annua* AND *Artemisia tilesii* "HAIRY" ROOT EXTRACTS

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Received 18.09.2020
Revised 28.11.2021
Accepted 30.12.2021

*Aim.* The profiles of polyphenolic phytochemicals in extracts of "hairy" roots of *Artemisia tilesii* Ledeb. and *Artemisia annua* L. were studied. Analytical separation and quantification of main components in extracts were evaluated.

*Methods.* "Hairy" roots were grown *in vitro* on Murashige and Skoog medium. High-performance chromatography coupled with different types of detection (photo diode array detection (DAD) and electrospray ionization with ultra-high resolution Qq-Time-of-Flight mass spectrometry) was used to identify and quantify the main biologically active components in ethanol extracts of "hairy" roots.

*Results.* The amount of flavonoids was 94.71–144.33 mg RE/g DW and 33.52–78.00 mg RE/g DW in "hairy" roots of *A. annua* and *A. tilesii*, respectively. In most samples of "hairy" roots, the amount of flavonoids was higher than the content in the control plant roots. The presence of Apigenin (0.168 ± 0.003 mg/L and 0.178 ± 0.006 mg/L), Quercetin (0.282 ± 0.005 mg/L and 0.174 ± 0.005 mg/L) in the extracts of *A. annua* and *A. tilesii* was shown by reverse-phase HPLC-DAD method. Chlorogenic acid, Kaempferol, and other flavonoids were detected.

*Conclusions.* Developed HPLC-DAD method demonstrated the high percentage of recovery, low limit of detection and quantification (9,11 ng/ml ≤ LOQ ≤ 16,51 ng/ml). Thus, the method is suitable for the simultaneous quantification of phenolic acids and flavonoids in various plant extracts with short time and high efficiency.

***Key words:*** *Artemisia tilesii*, *Artemisia annua*, polyphenols, flavonoids, "hairy" roots, reversed-phase HPLC with diode matrix detector.

In recent years, polyphenolic compounds have aroused increasing interest in the food and medical industries due to their positive and "mild" effects on human health [1]. Phenolic compounds accumulated in crops, fruits, and vegetables are associated with a wide range of health benefits, such as antioxidant, antidiabetic, anti-inflammatory, and antitumor properties. That is why an extraction of bioactive compounds from plant raw materials is intensively studied now [2]. Increased interest in this area is due to the broad spectrum for medical applications or plant-derived chemicals. These compounds play an important role in maintaining the balance of redox processes in cells and prevent the damaging effects of free radicals. In particular, the flavonoid epicatechin is a powerful antioxidant [3]. Compounds such as rutin [4], quercetin [5], luteolin [6, 7], apigenin [8] have antioxidant properties, and also demonstrate anti-inflammatory effects. Thus, flavonoids synthesized in plants can be used in the treatment of inflammation of various origins.

Chemical analysis of ethanolic and aqueous extracts of various *Artemisia* genus plants showed the presence of active compounds

such as flavonoids, terpenes, proteins, polysaccharides, coumarins, and alkaloids [9]. For example, *A. annua* plants are known to produce artemisinin with antimalarial properties [10]. Our research demonstrated the accumulation of polyphenols in plants of *A. tilesii*, the study of which is of particular interest due to the peculiarities of their physiology. At the same time the chemical composition, quantitative analysis of ethanolic extracts of *A. tilesii* plants, and also the effect of *Agrobacterium*-mediated transformation on the synthesis of different metabolites in the transgenic roots of these plants are insufficiently studied.

Genetic transformation using *Agrobacterium rhizogenes* is a well-known method used to obtain "hairy" root culture. In the process of transformation, bacterial *rol* ABCD genes are transferred to plant cells. These bacterial genes are known as inducers of changes in secondary metabolism [11, 12]. Due to this feature of *rol* genes, the process of genetic transformation can be used as a way to obtain superproducer lines for "green" synthesis of valuable biologically active compounds. The possibility of long-term cultivation of "hairy" roots, characterized by the ability to grow in the medium without the addition of specific regulators, opens the way to mass production of such roots in bioreactors and thus obtaining valuable raw plant materials. Cultivation in bioreactors allows to produce these roots throughout the year, to eliminate the dependence of the synthesis of bioactive compounds on the vagaries of weather and environment (such biosynthesis in nature depends on temperature, humidity, soil pH, lighting, etc.), reduce the cost of raw materials because high-cost reagents, lighting, and additional heating are not required in the process of cultivation.

The increase in the total content of polyphenols in plant tissues due to the peculiarities of the influence of the named above bacterial *rol* genes on secondary metabolism attracts considerable attention of researchers. Though, it is important to optimize the method of efficient extraction (solvent type, temperature of extraction process, etc.) and quantify the composition of extracts from plant material of different origins in order to qualitatively and quantitatively analyze them. To quantify polyphenols and phenolic acids in plant extracts, a significant number of high-performance liquid chromatographic (HPLC) techniques are proposed, which include UV and MS detection (HPLC-MS) [13, 14]. However, the high cost of HPLC-MS makes HPLC techniques with a UV or diode array detection (HPLC-DAD) more convenient [15–17]. Though, the techniques used to analyze plant extracts by HPLC have several disadvantages, such as long run times, low resolution, and low efficiency.

The study of polyphenols content and development of a method of effective extraction and quantification of bioactive compounds in the *A. tilesii* and *A. annua* "hairy" roots were the aim of the work. Ultra high-performance chromatography coupled with electrospray ionization with ultra-high resolution Qq-Time-of-Flight mass spectrometry (UPLC-ESI-UHR-Qq-TOF-MS method) was used for the identification of biologically active compounds, including flavonoids and other polyphenols. Simultaneous quantification of polyphenols in the ethanol extracts of "hairy" roots was investigated by reversed phase HPLC-DAD.

## Materials and Methods

*Reagents.* Standard references (>98%) were used, including chlorogenic acid (00500590-25mg, primary reference standard), apigenin (10798-25 mg, ≥ 97.0% (HPLC)), rutin (78095-25 mg, analytical standard), quercetin (PHR1488-1g, pharmaceutical secondary Standard; Certified Reference Material), luteolin (72511-10 mg, analytical standard), kaempferol (60010-25 mg, ≥ 97.0% (HPLC)), kaempferol-3-glucoside (6843-5 mg, analytical standard) ) obtained from Sigma-Aldrich. All standards were prepared in 2% formic acid in MeOH (1 mg/mL). Working solutions were prepared by diluting standard solutions to a concentration of 0.01–0.05 mg/mL. The solvents ($CH_3OH$, $CH_2Cl_2$, EtAc, $CH_3CN$) used for the chromatographic analysis were HPLC-grade (Sigma-Aldrich, Spain). Ultrapure water was provided by a Milli-Q® purification system (Millipore, USA).

*Plant material.* The "hairy" roots of wormwood (*Artemisia tilesii* and *A. annua*) from the collection of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used. The roots were cultivated *in vitro* on solidified Murashige and Skoog nutrient medium with halved macrosalt content. The cultivation time was three weeks at temperature of +24 °C. The roots were separated from the medium, washed with distilled water, lyophilized and powdered.

*Preparation of extracts.* To prepare the extracts, previously dried and powdered

"hairy" roots were extracted with 70 vol. % ethanol (100 mg (EtOH)/20 ml ($H_2O$)) during two days on a rotary shaker at +28 °C.

*Total flavonoids content assay*. The standard spectrophotometric method [18] was employed using the Fluorate-02-Panorama (Russia). 0.25 ml of the sample of each extract was mixed with 1 ml of double-distilled water and 0.075 ml of 5% $NaNO_2$ solution, and allowed to react for 5 min at room temperature. After that, 0.075 ml of 10% $AlCl_3$ solution was added. After another 5 min of incubation, 0.5 ml of 1 M NaOH solution and 0.6 ml of double-distilled water were added to the reaction mixture. The absorbance of the sample was measured at 510 nm. The total flavonoid content was expressed as milligrams per gram of dry root weight in rutin equivalent (mg RE/g DW). The amount of flavonoids in extracts was performed by calibration curves in the concentration range from 50 μg/ml to 500 μg/ml: $y = 0{,}8842x - 0{,}0606$ ($R^2 = 0{,}9988$).

*Sample preparation of extracts for chromatographic studies*. Ethanol extracts from "hairy" roots were centrifuged at 5000 rpm for 10 min to precipitate a solid fraction (if it was presented). Acid hydrolysis was performed by adding an equal amount of 1M HCl to the methanolic extract (1 ml: 1 ml) for 1 hour without heating. The extracts were diluted with 0.5 ml of 1% (v/v) solution of formic acid in MeOH, and filtered using a membrane filter (nylon) (diameter: 13 mm, pore size: 0.45 mm) and stored at 6 °C until HPLC analysis.

*Identification of biologically active compounds in the extracts*. The UPLC system (Dionex Ultimate 3000) with electrospray ionization and ultra-high resolution Qq-Time-of-Flight mass spectrometry (Bruker Impact II) was used for chemical compositions analysis of the ethanol extract of "hairy" roots. The mass spectrometer was operated in the negative ESI mode with Duo-Spray source, and the mass scan range was set at m/z 50–2 500 for both TOF-MS and TOF-MS/MS scan with 2 700 resolution. The following parameter conditions were used: ion spray voltage, 3500 V; ion source heater, 500 °C; curtain gas, 25 psi; collision energy, 10 eV; declustering potential, 100. The identification of polyphenolic compounds in extracts was determined based on their mass fragmentation pattern, low mass error within the acceptance range of ± 5 mDa, and ion response. The analyst TF software (version 1.7) combined with the information-dependent acquisition packing was used to acquire the MS/MS data.

The mobile phase was composed of 0.1% formic acid in water (elution A) and methanol (elution B) using a gradient elution: 30% B (0–5 min), from 30% to 50% B (5–20 min), from 50% to 90% B (20–40 min), and from 90% to 100% B (40–45 min).

*Procedure of quantitative determination of polyphenolic compounds in the extracts*. Chromatograms of the extracts were recorded using a HPLC-DAD method (Shimadzu LC-20). Optimized chromatographic conditions for the quantification of flavonoids and phenolic acids in the gradient mode were used with a solvent system: mobile phase A: 1% (v/v) formic acid in methanol/water (25/75, v/v); mobile phase B: acetonitrile. Gradient: from 0 to 30 min, 100–64% A; from 30 to 31.2 min, 64–0% A; from 31.2 to 40.2 min, 0% A; from 40.2 to 42.5 min, 0–100% A; from 42.5 to 47.4 min, 100% A. The flow rate was 0.5–1.0 ml/min and the injection volume was 12 μl. The absorption spectra were recorded at 255 nm. Column Zorbax Eclipse Plus® C18 (15 cm, 3.0 mm, 3 μm) was used with Phenomenex pre-column (Gemini® NX C18 (4×3.0 mm)). The analysis was performed with column temperature at 28 °C. The total analysis time of the sample was 65 min.

*Validation of the method*. According to the recommendations of ICH/2005/Q2/R1 [19], there are various parameters for testing the reproducibility of the method, namely: efficiency, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, and precision. Calibration curves were constructed based on the average peak areas of the five concentrations (0.1, 0.05, 0.01, 0.005, and 0.001 mg/l) of each of the six standard solutions (in three injections) measured in three parallels at wavelength 255 nm. We used concentration ranges 0.025–100 mg/l and 0.05–200.0 mg/l.

To evaluate the accuracy of the method for the determination of polyphenolic compounds recovery-test was used. Two different amounts of polyphenolic compounds (1.5 and 0.15 mg, a solution of 1% formic acid in methanol) were added to extracts before extraction. The analyte content was evaluated in three volumes for each added amount. Preliminarily, the absence of detectable components in the extracts was evaluated. Recovery (%) values were calculated using formula:

$$R = [(C_{found} - C_{contained}) / C_{added}] \times 100.$$

The high recovery-test was observed at 96–103%.

The slopes, intercepts, and the determination coefficients of each polyphenols

were calculated using last square linear regression analysis. LOD and LOQ values for each of studied compounds were evaluated according to ICH guideline [19] and were calculated using the following formulas:

$$LOD = 3\sigma/b;$$

$$LOQ = 10\sigma/b$$

where σ is the standard error of the intercept α; and b — slope of the calibration curve.

*Statistical analysis.* The analytical data were shown as means of triplicates and subjected to variance analysis using R software (version 4.0.4). The results were expressed as mean ± standard deviation at $P = 0.95$. Linear regression method was applied and coefficient of determination ($R^2$) was calculated for establishing the relationship between the values.

## Results and Discussion

*Estimation of the chemical composition of "hairy" root extracts.*

Four lines of "hairy" roots of *A. annua* and six lines of *A. tilesii* were used in the study. The "hairy" root samples of *Artemisia tilesii* and *Artemisia annua* are presented in Fig. 1.

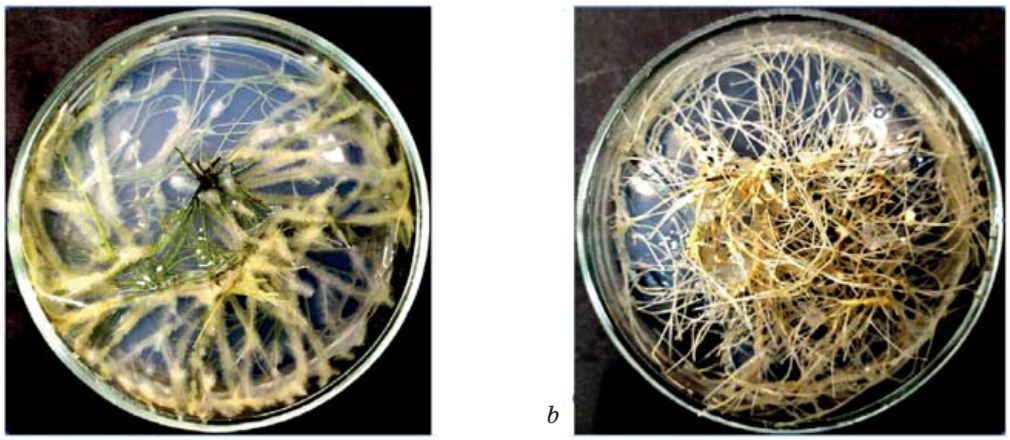
Various methods are used now for the extraction of bioactive compounds, including flavonoids, from plant raw materials to obtain the maximum possible concentrations of the target components by liquid extraction. Several solvents are commonly used to extract these phytochemicals. Ethanol and methanol are the most common ones to extract flavonoids, while water is often used to remove polar compounds from plant material. Ethanol is preferred in most studies, probably because of its safety, availability, and efficacy [20].

Due to the fact that there may be significant differences in the content of polyphenolic compounds in different lines of "hairy" roots owing to the indeterminate site of introduction of *rol* genes, it is necessary to conduct screening studies of the chemical composition of extracts including total flavonoids content to select the most productive samples (Fig. 2).

The primary analysis of the total flavonoid content in extracts from "hairy" root lines revealed significant variability in this parameter. Thus, the content of flavonoids in the roots of *A. annua* and *A. tilesii* varied within 94.71 ± 14.7–144.33 ± 28.1 mg/g dry weight (DW) and 33.52 ± 1.9–78.00 ± 4.9 mg/g DW, respectively. The content of these compounds in the "hairy" roots of *A. annua* was 2.17–3.32 fold higher than the content in the roots of the control plants grown in the same conditions *in vitro* (Fig. 2, samples Aa 1–4).

In three of the studied samples of *A. tilesii*, the total content of flavonoids differed little from the control parameter (Fig. 2, samples At 1–3). However, in other lines of "hairy" roots, the concentration of flavonoids was 1.78–2.43 fold higher than in the control sample (Fig. 2, samples At 4–6). Thus, transformation using *Agrobacterium rhizogenes* has indeed led to a significant stimulation of flavonoid synthesis in some "hairy" root lines. Since wormwood is a medicinal plant, it was of the special interest to study the obtained extracts and determine the qualitative and quantitative content of polyphenolic compounds extracted from this plant material.

*Identification of biologically active compounds in ethanolic extracts of "hairy" roots.* Among the available modern chromatographic methods, high-performance chromatography in combination with a hybrid pulsed tandem quadrupole time-of-flight mass spectrometer was recognized as a promising analytical method for chemical



*a* *b*

*Fig. 1.* **The "hairy" roots of *Artemisia tilesii* (*a*) and *Artemisia annua* (*b*) grown *in vitro***

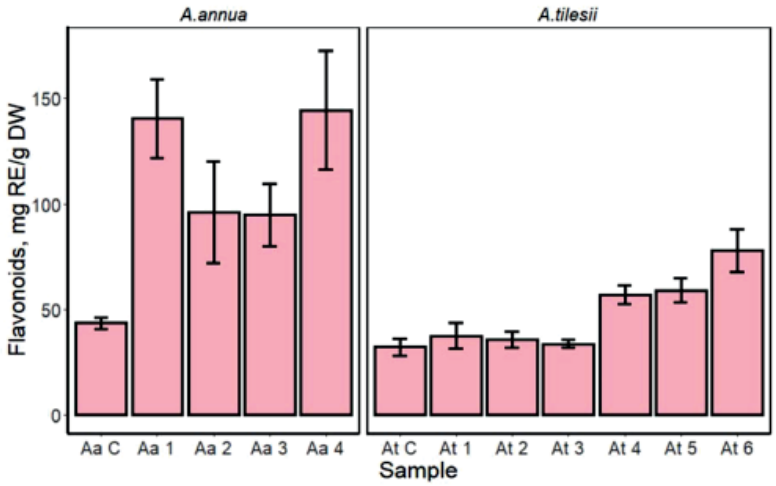



*Fig. 2.* **Total flavonoids content in ethanol extracts of different lines of *Artemisia annua* and *A. tilesii* "hairy" roots**

screening and identification of major chemical components of plants [21]. In addition, during one analysis, the detection of positive and negative ions is possible by switching the polarity of the ionizer, conductive system and Qq-TOF analyzer. Using this method, it is possible to detect simultaneously and identify bioactive compounds in the extracts according to accurate measurements of the mass of fragments of the components of the extract, as well as the system of library analysis of the obtained mass spectrometric data. Electron spray ionization and Qq-TOF, which allows measurements in TOF mode and MS mode, were used to detect and identify bioactive compounds. Measurements using TOF mode were performed with high resolution and accurate mass determination, as well as with a high speed of mass array analysis in their entire spectrum. In this mode, the detector registered the time and number of ions as they leave the flight tube, which improves the separation of the components that are part of the extract and need different times to reach the detector. Using the MS-MS mode with quadrupole and hexapole (hexanol high-frequency collision cell), mass spectra measurements were performed in front of the spanning tube and detector to obtain full spectra of high-sensitivity product ions. Due to these functional capabilities, biologically active substances that are part of the extracts of *A. tilesii* and *A. annua* were identified and evaluated (Fig. 3).

It was shown that the scanning speed of the mass spectrometer significantly affects the resolution of chromatography, and the scanning time of the whole mass spectrum should be less than the elution time of the chromatographic peak. It was found that one chromatographic peak of ethanol extract of "hairy" roots should have at least 5 complete mass spectra, which allows to obtain a spectrum with excellent statistical ions for reliable identification of compounds.

The *MassHunter Workstation* program was used to identify biologically active compounds by mass spectrum and to quantify their content even at very low concentrations (Table 1).

As it can be seen from table 1, extracts of *A. tilesii* and *A. annua* have a similar set of bioactive compounds, but they differed in the presence of some flavonoids and phenolic acids. Among phenolic acids: chlorogenic acid (peak 15), caffeoylquinic acid (peak 21), and gallic acid (peak 12) were presented both in *A. tilesii* and *A. annua* extracts (Table 1). Low amount of caffeic acid was observed only in *A. annua*. These results suggest that the distribution of phenolic acids in transgenic roots may be influenced by a variety of factors, including the nature of the "hairy" roots or extraction conditions.

Compounds found in ethanolic extracts of "hairy" roots have well-known medicinal properties. For example, antidiabetic and anti-inflammatory activity of sitosterol has been studied [22]. The positive effects of apigenin in the treatment of diabetes, neurodegenerative disorders such as Alzheimer's and Parkinson's disease, depression, insomnia have been reported [23]. Antioxidant, anti-inflammatory, anti-amyloidogenic, and antitumor activity of named polyphenols were demonstrated [24].

*High-performance liquid chromatography for the simultaneous quantification of*

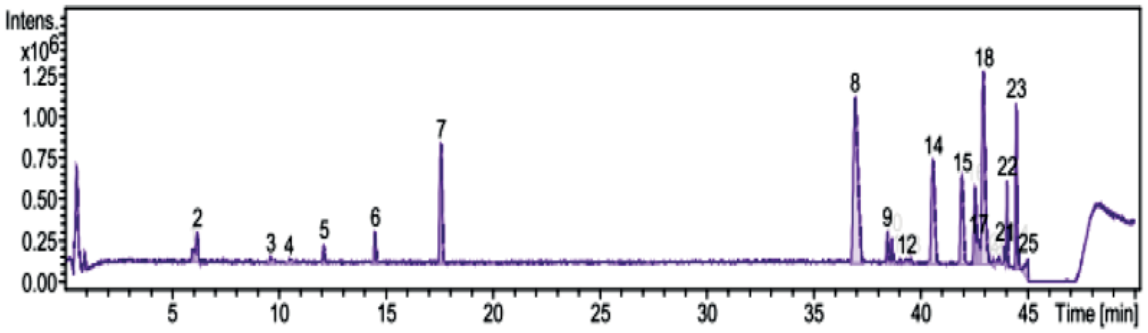



*Fig. 3.* **Representative chromatogram of ethanolic extract of *A. tilesii* "hairy" roots obtained by UPLC-ESI-UHR-Qq-TOF-MS (negative ion mode)**

*Table 1.* **Bioactive compounds in ethanolic extracts of *Artemisia tilesii* and *A. annua* "hairy" roots according to the UPLC-ESI-UHR-Qq-TOF-MS method (RT — retention time)**

| Identified compound | Molecular ion in the MS spectra (m/z) | *A. tilesii* | | *A. annua* | |
|---|---|---|---|---|---|
| | | RT, min | Presence of compound | RT, min | Presence of compound |
| Caffeic acid | 169.8956 | – | – | 38.7 | + |
| Chlorogenic acid | 353.2015 | 41.9 | + | 41.7 | + |
| Caffeoylquinic acid | 353.1438 | 43.9 | + | 43.9 | + |
| Galic acid | 170.0241 | 39.3 | + | 39.3 | + |
| Quercetin, [M-H]- | 300.1751 | 40.6 | + | 40.6 | + |
| Luteolin-7-β-D-glucopyranoside | 475.1245 | 14.5 | + | 14.5 | + |
| Arginine | 269.1486 | 36.9 | + | 37.0 | + |
| Isorhamnetin 3-O-glucoside, [M+H]+ | 476.2794 | 38.4 | + | – | – |
| Baikalein-7-O-glucuronide | 445.1860 | 17.6 | + | – | – |
| Sucrose, [M+K]+ | 381.1744 | 44.6 | + | 14.5 | + |
| Sitosterol [M−$H_2O$]+ | 397.2278 | 42.5 | + | 42.7 | + |
| Caffeoylshiqimic acids [M+H]+ Sterebin J/ Sterebin I, M+ | 327.1280 | 43.7 | + | 43.7 | + |
| Kaempferol-3-O-galactoside-rhamnoside-7-O-rhamnoside | 739.1577 | 45.0 | + | – | – |
| Apigenin-7-O-glucoside | 433.1140 | 12.1 | + | 11.9 | + |

*biologically active components in extracts of "hairy" roots.*

To quantify polyphenols and phenolic acids as components of the extracts of "hairy" roots, several chromatographic techniques described in the literature were tested [13, 15]. Generally, two mobile phases can be used in the reversed-phase HPLC polyphenol analyses for the separation of bioactive components in a mixture. However, the HPLC chromatogram of ethanol extract according to the well-known method for the determination of polyphenols [13] showed poor resolution of the chromatographic peaks of the detected substances (Fig. 4).

However, preference of the mobile phase in HPLC separation depends on the type and nature of the polyphenol compounds in the extract. HPLC techniques for the simultaneous determination of flavonoids and phenolic acids need to be improved to determine the quantitative composition of the components of ethanolic extracts of *A. tilesii* and *A. annua* "hairy" roots. Total fractions of extracts of "hairy" roots were previously purified from the solid phase. For comparison, the extract

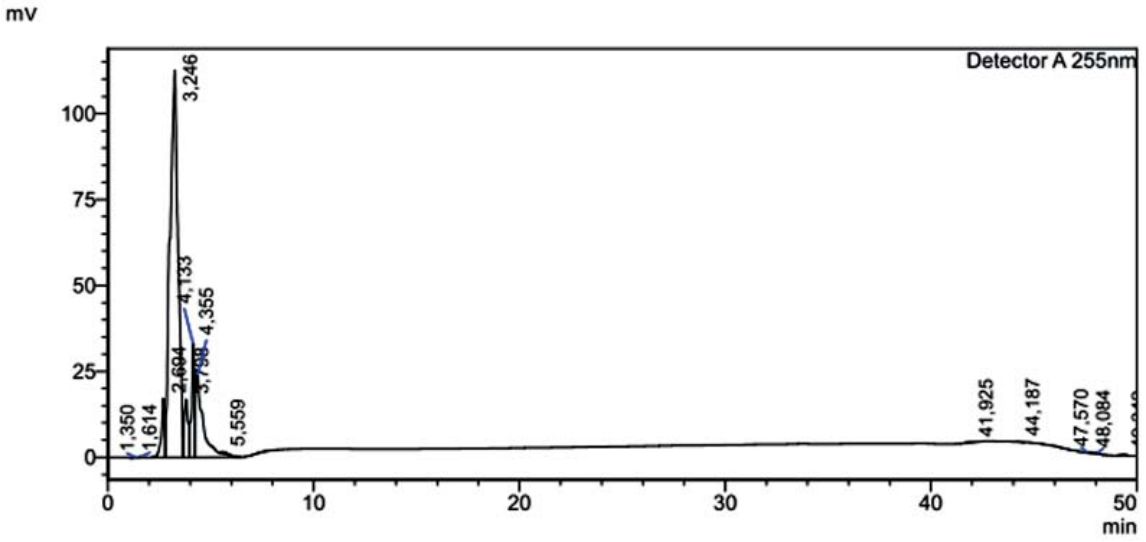



*Fig. 4.* **Chromatogram of the ethanol extract of "hairy" roots of *A. annua* obtained by the method described in [13]**

fraction and a mixture of polyphenol standards were chromatographed.

*Optimization of extraction chromatography conditions in the determination of flavonoids and polyphenolic acids content.*

Usually, the one mobile phase (Solvent A) contains water with organic acid (acetic acid, formic acid, and trifluoroacetic acid) and the other mobile phase (Solvent B) — pure organic solvents (methanol or acetonitrile). The organic solvents are necessary to reduce the chromatographic peak giving sharper peaks [13]. The ratio of methanol and water is very important when measuring isolated peaks of flavonoids by chromatography method. In this study, the effect of this parameter was evaluated using various conditions including methanol-water (10:90), acetonitrile-water (10:90), methanol-HCOOH, and other mobile phases.

We used the mobile phase containing 0.1–2% formic acid solution in different ratios $CH_3OH/H_2O$ (Solvent A) and acetonitrile (Solvent B). In the study the flow rate was adjusted in the range of 0.5–1.0 ml/min, the column was thermostated at 25–35 °C, and the volume for other injections ranged from 10 to 30 µl. Gradient elution was performed by changing the proportion of solvent B to solvent A. The gradient was changed from 10% to 40% B linearly for 28 minutes, from 40 to 60% B for 39 min, from 60 to 90% B for 50 min. The mobile phase composition was returned to its original state (solvent B:solvent A — 10:90) after 55 min and allowed to work for another 10 min before the introduction of another sample. The total analysis time of the sample was 65 min. Chromatograms were detected by HPLC-DAD at three different wavelengths ranging from 200 to 300 nm, which allowed maximum absorption, improved the resolution, and smoothed the baseline. The analysis was performed with or without a pre-column.

It should be noted that flavonoids, which are relatively less polar, were found mainly in less polar media, and their concentration is somewhat increased in polar media, given the synergistic effect of the components present in the extract, which restricts the freedom of OH-groups in the complex biomatrix. On the contrary, the increased influence of the polarity of the solvent together with the concomitant cleavage of the ether bond led to the improved separation of phenolic acids. The use of methanol with different ratios to water as a mobile phase was the compromise approach. It was found that the best ratio of methanol/water is 25/75 (v/v), which allowed to separate the maximum number of components in the mixture, as well as to quantify them. Optimization of the chromatographic conditions of the extract in gradient mode using methanol improved the separation of bioactive components, as shown in Fig. 5.

After optimization of the chromatography conditions, the extracts were analyzed in gradient mode using the following solvent system: mobile phase *A*: 0.1% (v/v) formic acid in methanol/water (25/75, v/v); mobile phase *B*: acetonitrile. The absorption spectra were recorded at 255 nm. The column temperature was 28 °C and the injection volume was 20 µl.

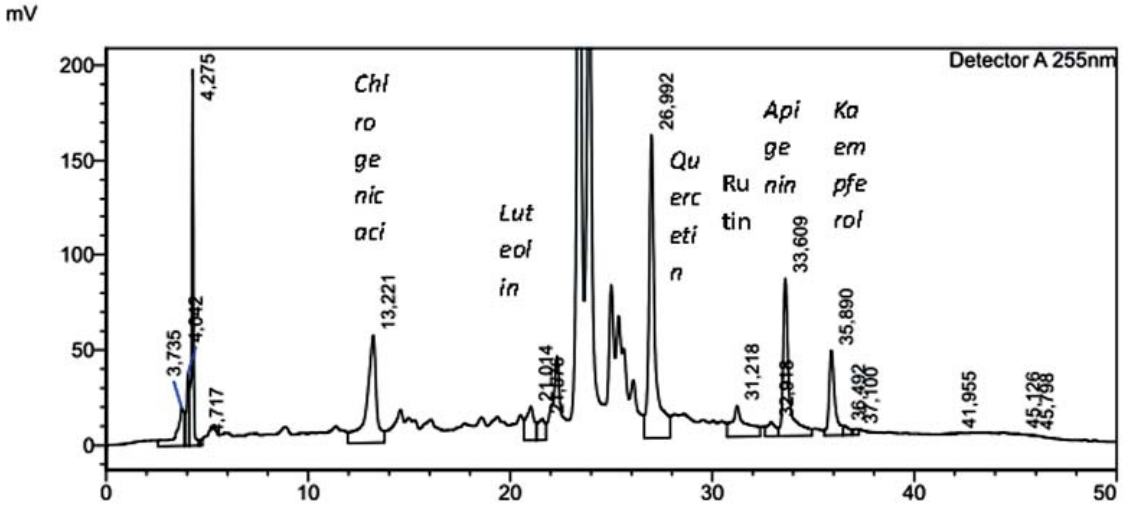



*Fig. 5.* **Chromatogram of ethanolic extract of *A. annua* "hairy" roots under optimized chromatographic conditions**

*Validation of the method of quantitative determination of phenolic acids and flavonoids.* The quantitative content of flavonoids and phenolic acids was determined using an external standard by constructing calibration curves for six representative polyphenolic compounds of the appropriate classes (chlorogenic acid, apigenin, luteolin, quercetin, kaempferol, and rutin).

Metrological parameters of the developed chromatographic method for the determination of principal components in ethanolic extracts of *A. tilesii* and *A. annua* "hairy" roots are given in Table 3.

The developed chromatographic method for the determination of biologically active compounds showed good separation of components in the mixture (Fig. 5). The linearity of the developed method was determined with the reference standard solutions with the concentration range of 0.025–100.00 mg/L at five concentration levels with correlation coefficients R2 from 0.9997 to 0.9999 (Table 3). LOD for six studied bioactive compounds ranged from 2.73 to 4.95 ng/mL, while LOQ ranged from 9.11 to 16.51 ng/mL, which is the evidence of the sensitivity of the used procedure. High values of recovery (in the range of 97.27–98.95%) indicated the correctness of the developed methodology. Therefore, studies of validation parameters of the method confirmed the validity of quantitative analysis of detectable polyphenolic compounds.

Ethanol extracts of *A. tilesii* and *A. annua* "hairy" roots were analyzed using the developed method (Table 4).

Biologically active compounds were detected in samples of extracts at concentrations of 0–0.282 mg/ml. The presence of Apigenin (0.168 ± 0.003 mg/ml and 0.178 ± 0.006 mg/ml), Quercetin (0.282 ± 0.005 mg/l and 0.174 ± 0.005 mg/ml) in the extracts of *A. annua* and *A. tilesii* was shown. It was found that the "hairy" roots of plants of two species of *Artemisia* genus differed in

*Table 3.* **Metrological parameters of the developed chromatographic method for determination of flavonoids and phenolic acids in ethanolic extracts of *A. tilesii* and *A. annua* "hairy" roots**

| Component | Linearity, mg/L | Calibration curves | $R_2$ | LOD, ng/mL | LOQ, ng/mL | Recovery, % |
|---|---|---|---|---|---|---|
| Chlorogenic acid | 0.025–100.00 | y = 4538.6x + 5.0975 | 0.9997 | 3.19 | 10.63 | 96.05 |
| Apigenin | | y = 1053.6x +0.5834 | 0.9997 | 4.95 | 16.51 | 98.76 |
| Luteolin | | y = 7808.1x + 9.7779 | 0.9998 | 3.28 | 10.94 | 97.45 |
| Rutin | | y = 2405.1x + 3.2862 | 0.9997 | 4.62 | 15.41 | 98.01 |
| Quercetin | | y = 6019.6x + 7.6469 | 0.9997 | 3.49 | 11.63 | 97.27 |
| Kempferol | | y = 5899.4x + 9.4264 | 0.9999 | 2.73 | 9.11 | 98.95 |

*Table 4*. **Quantification of phenolic acids and flavonoids (mg/ml) in ethanolic extracts of *Artemisia tilesii* and *A. annua* "hairy" roots**

| No | Compound | Content in *A. annua* | RSD, % | Content in *A. tilesii* | RSD, % |
|---|---|---|---|---|---|
| 1 | Chlorogenic acid | 0.192 ± 0.004 | 1.04 | 0.107 ± 0.005 | 1.10 |
| 2 | Apigenin | 0.168 ± 0.003 | 0.98 | 0.178 ± 0.006 | 1.12 |
| 3 | Luteolin | 0.037 ± 0.003 | 0.95 | 0.112 ± 0.004 | 0.96 |
| 4 | Quercetin | 0.282 ± 0.005 | 1.11 | 0.174 ± 0.005 | 1.10 |
| 5 | Rutin | 0.075 ± 0.005 | 1.10 | 0.056 ± 0.007 | 1.11 |
| 6 | Kaempferol | 0.108 ± 0.003 | 0.96 | 0.142 ± 0.003 | 0.95 |

polyphenolic compounds concentration. The study indicated the presence of high amounts of chlorogenic acid and quercetin in extracts of *A. annua* roots, while *A. tilesii* roots had a higher content of luteolin and kaempferol (Table 4). In a complex multicomponent extract, chlorogenic acid remains either in the free state or in combination in the form of ether and acts as a powerful antioxidant. Stability tests showed that the extracts of "hairy" roots were stable for 2 months. The relative standard deviation (RSD) of polyphenols was from 0.95 to 1.12%, respectively, indicating a good precision of measurements.

The presence of the determined flavonoids and phenolic acids was linked to certain positive health effects and other bioactive functions that have already been reported in the literature to highlight the potential functional activity of the analyzed "hairy" roots [4, 6, 7].

## Conclusions

Thus, the profile of polyphenolic compounds in extracts from *A. tilesii* and *A. annua* "hairy" roots was studied. The total content of polyphenols was used as a control parameter for the efficiency of the extracts study. The identification of a significant amount of valuable biologically active components in the studied wormwood "hairy" roots and variation in the amount determined based on the polarity of the solvent taken for the extraction process provides a clear recommendation to use the proposed method of extraction and determining the content of polyphenolic compounds in extracts in pharmacy and medical practice. The proposed UPLC-ESI-UHR-Qq-TOF-MS method was studied as a useful tool for the rapid detection and structural characterization of polyphenolic compounds in complex matrix such as ethanolic extract. Six flavonoid glycosides, 2 non-glycosylated flavonoids with several phenolic acids were detected in ethanolic extracts of *A. tilesii* and *A. annua* "hairy" roots.

The reversed-phase HPLC-DAD technique has been developed to quantify phenolic acids and flavonoids in ethanolic extracts of *A. tilesii* and *A. annua* "hairy" roots. The developed HPLC method for the determination of biologically active compounds showed good separation of components in the plant extracts. It was characterized by a wide range of linearity, was sensitive and accurate. So, the method is suitable for the simultaneous determination of phenolic acids and flavonoids with optimal analysis time and high efficiency. The developed technique can be used for the analysis of extracts of different *Artemisia* species and in the preparation of monographs for the pharmacopoeia.

This work was partially supported by the Ukrainian-Bilorusian grant (2021-2022, No. M/103-2021) from the Ministry of Education and Science of Ukraine.

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# ОДНОЧАСНА ІДЕНТИФІКАЦІЯ, КІЛЬКІСНЕ ВИЗНАЧЕННЯ ТА АНАЛІЗ ОСНОВНИХ КОМПОНЕНТІВ ЕКСТРАКТІВ «БОРОДАТИХ» КОРЕНІВ *Artemisia annua* ТА *Artemisia tilesii*

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*Мета* — дослідження профілів поліфенольних сполук в екстрактах «бородатих» коренів *Artemisia tilesii* Ledeb. і *A. annua* L., аналітичне розділення і кількісне визначення компонентів.

*Методи*. «Бородаті» корені вирощували *in vitro* на середовищі Мурасіге і Скуга. Високоефективна хроматографія з різними видами детектування (тандемним квадруполь-часопролітним мас-спектрометром та діодно-матричним детектуванням) було використано для ідентифікації та кількісного визначення основних компонентів у складі етанольних екстрактів «бородатих» коренів.

*Результати*. У більшості зразків «бородатих» коренів вміст флавоноїдів був вищим за вміст у коренях контрольних рослин. Показано наявність в етанольних екстрактах «бородатих» коренів *A. annua* та *A. tilesii* апігеніна (0,168 ± 0,003 мг/л і 0,178 ± 0,006 мг/л), кверцетина (0,282 ± 0,005 мг/л і 0,174 ± 0,005 мг/л), виявлено хлорогенову кислоту, кемпферол та інші флавоноїди.

*Висновки*. Розроблено методику ВЕРХ з діодно-матричним детектуванням для кількісного визначення основних компонентів в екстрактах «бородатих» коренів, що характеризується широким діапазоном лінійності, високою чутливістю (9,11 нг/мл ≤ LOQ ≤ 16,51 нг/мл), точністю та коректністю. Методика дозволяє робити одночасне визначення фенольних кислот та флавоноїдів з оптимальним часом і високою ефективністю. Розроблена методика може бути використана для аналізу екстрактів рослин різних видів.

***Ключові слова:*** *Artemisia tilesii*, *Artemisia annua*, поліфеноли, флавоноїди, «бородаті» корені, обернено-фазова ВЕРХ із діодним матричним детектором.

UDC 602.64 https://doi.org/10.15407/biotech14.06.071

# *Agrobacterium rhizogenes* — MEDIATED TRANSFORMATION AS AN APPROACH OF STIMULATING THE SYNTHESIS OF ANTIOXIDANT COMPOUNDS IN *Artemisia absinthium* L.

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Received 15.10.2020
Revised 29.11.2021
Accepted 30.12.2021

*Artemisia absinthium* L. plants are known as producers of substances with antioxidant properties. Among others, phenols and flavonoids are found in these plants. The synthesis of these bioactive compounds can be activated by genetic transformation. This process can be carried out even without the transfer of specific genes involved in the synthesis of flavonoids. Thus, "hairy" roots, obtained after *Agrobacterium rhizogenes* — mediated transformation, can produce a variety of valuable substances.

*The aim* of this study was obtaining *A. absinthium* "hairy" roots with high phenolic content.

*Methods*. "Hairy" roots were obtained by co-cultivation leaves with suspension of *A. rhizogenes* with pCB124 vector. The presence of transferred genes was confirmed by PCR. The reactions with $AlCl_3$ and Folin-Ciocalteu reagent were used to determine the total flavonoids and phenols content. The antioxidant activity of extracts was evaluated by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

*Results*. PCR analysis detected the presence of bacterial *rol* genes and the absence of pCB124 plasmid genes. Root lines differed in growth rate. "Hairy" roots were characterized by a higher phenolic content, particularly flavonoids (up to 4.784 ± 0.10 mg/g FW) compared to control (3.861±0.13 mg/g FW). Also, extracts from transgenic roots demonstrated higher antioxidant activity in the reaction with DPPH reagent ($EC_{50}$ = 3.657 mg) when compared with extracts from control plants ($EC_{50}$ = 6,716 mg).

*Conclusions*. *A. rhizogenes*-mediated transformation of *A. absinthium* can be applied for obtaining transgenic root lines with increased phenolic content and higher antioxidant activity.

***Key words:*** *Artemisia absinthium L., Agrobacterium rhizogenes*-mediated transformation, "hairy" roots, flavonoids, phenolic compounds, antioxidant activity.

*Artemisia absinthium* L. (wormwood) is a perennial shrubby plant of the Compositae family. It is native to Asia, the Middle East, Europe and North America [1].

*A. absinthium* extract is known to have antioxidant [2], immunomodulatory [3], wound-healing [4], anti-inflammatory, analgesic [5], antitumor [6], antiulcer [7], antibacterial, antifungal [8], antiparasitic [9] activities, as well as neuroprotective [10], hepatoprotective [11], hypoglycemic [12] effects. Such a wide range of properties is related to the *A. absinthium* chemical composition. Such compounds as lactones, terpenoids, essential oils, organic acids, resins, tannins and phenols were previously detected in the extracts of this plant [1]. For example, isolated dimeric guaianolides, that were found in *A. absinthium*, exhibited cytotoxic activity, inhibited cyclooxygenase-2, and had anti-HIV-1 protease activity [13].

Flavonoids, which are important secondary plant metabolites, are also synthesized in *A. absinthium*. Flavonoids are composed of two aromatic rings and one heterocyclic ring with an oxygen atom. Flavonoids are characterized by high antioxidant activity due to such chemical structure [14]. Thus, they can protect cells from oxidative stress, which can cause in human such pathological diseases as cancer,

atherosclerosis and cardiovascular diseases, neurodegenerative diseases, diabetes and inflammation [15]. Therefore, *A. absinthium* can be a valuable source of bioactive compounds, particularly flavonoids.

Plant bioactive compounds are usually obtained from natural raw materials. However, this method has a number of disadvantages. This traditional way of biosynthesis depends on the growing temperature, rainfall, soil contamination, etc. At the same time, "hairy" roots, which are formed after plants contact with phytopathogenic bacteria *Agrobacterium rhizogenes*, are supposed to be a better source of plant chemical compounds [16–18].

During transformation a fragment of the bacterial Ri-plasmid (T-DNA) is transferred into the genome of the host plant. It contains plant-expressed genes that control synthesis of phytohormones, such as auxin and cytokinin. T-DNA integration gives transformed roots the ability to hormone-independent growth [19]. The *A. rhizogenes rol*-genes induce synthesis of secondary metabolites, including flavonoids [20]. It allows to select the lines producing valuable compounds. "Hairy" root culture is characterized by genetic stability, the ability to grow in a nutrient medium without costly compounds. That enables to scale-up the root growth process and to obtain bioactive compounds in different bioreactors [21, 22].

Plants of the *Artemisia* genus have previously been used in genetic transformation studies. For example, obtained *Artemisia vulgaris* L. "hairy" roots produced β-caryophyllene [23], *Artemisia annua* and *Artemisia pallens* Wall. transgenic roots synthesized artemisinin [24, 25]. However, there is currently only one publication about *A. absinthium* transformation using *A. rhizogenes*. Nin et al [26] obtained wormwood "hairy" root culture by transformation via 1855 and LBA 9402 *A. rhizogenes* strains. They showed a significant difference in the qualitative and quantitative content of essential oils from transgenic roots compared to the control. Thus, there was absence of β-thujone in "hairy" roots, and linalyl 3-methylbutanoate was about 37% of the transgenic roots essential oil.

The aim of our work was to optimize the method of *A. absinthium* genetic transformation to obtain "hairy" roots. The other purpose was to evaluate their biosynthetic potential as producers of compounds with antioxidant properties.

## Materials and Methods

*1. A. rhizogenes cultivation.*

Bacteria were cultivated 24 hours in liquid LB medium [27] at 28 °C and 180 rpm using rotation shaker SpeedVac Savant AES 2010 (Labconco, USA). Next the bacterial suspension was centrifuged at 3000 *g* for 10 min. Precipitated cells were resuspended in 10 mM $MgSO_4$ solution. Obtained suspension was used for *A. absinthium* transformation.

*2. A. rhizogenes-mediated transformation of A. absinthium.*

*A. rhizogenes* A4 agropine strain with pCB124 vector (Fig. 1) was used for genetic transformation. This vector was constructed at Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine. It contained a selective neomycin phosphotransferase II gene (*npt*II) with NOS promoter and terminator regulatory sequences, as well as a human interferon-α2b gene (*ifn*-α2b) under control of 35S promoter.

*A. absinthium* leaves were used as the explants for genetic transformation. Explants were sliced and co-cultivated during 30 min with an overnight bacterial suspension. Then they were soaked using filter paper and cultivated on 1/2 MS agar-solidified medium (Duchera Biochemie) for 3 days. Next explants were transferred to the 1/2 MS agar-solidified medium with 600 mg/l cefotaxime (Borshchahivskiy CPP). Obtained roots were subcultivated every 10 days on the same medium. Each root was considered as a separate transformation event.

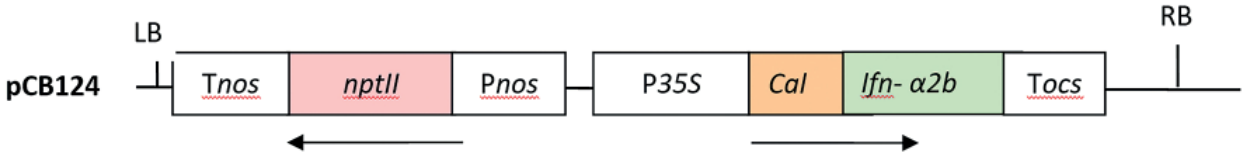


*Fig. 1.* **Schematic representation of the T-DNA site of pCB124 vector:**
LB i RB — left and right border sequences; *nptII* –*nptII* gene (NOS promoter and terminator); *ifn*-α2b – *ifn*- α2b gene (35S promoter and OCS terminator); *cal* — leader sequence that provides transport of the target protein to the apoplast

*3. PCR analysis.*

The presence of *rol*B, *rol*C, *vir*D, *ifn*-α2b, *nptII* genes in obtained "hairy" roots, leaves and roots of control plants was studied by PCR analysis. DNA extraction was carried out according to CTAB-method [28]. The presence of genes was determined in multiplex reaction using Mastercycle personal 5332 amplifier (Eppendorf). DNA amplification was carried out in a total volume of 20 μl. The reaction mixture contained 80–100 ng DNA, 1x DreamTaq reaction buffer (Thermo Scientific, proprietary composition, contains 20 mM $MgCl_2$), 0.5 U DreamTaq DNA Polymerase (Thermo Scientific), 0.2 mM deoxynucleotide triphosphates, 0.25 mM of each primers.

The conditions of amplification for all primers were as followed: primary denaturation — 94 °C, 3 min; 30 cycles of amplification (94 °C, 30 sec → 60 °C, 30 sec → 72 °C, 30 sec); final polymerization — 72 °C, 5 min. Reaction products were separated in 1.0% agarose gel with 0.005% (V/V) ethidium bromide (Sigma). O`GeneRuler 1 kb Plus DNA Ladder was used for the sizing of amplified fragments.

Primers 5′-ctc act cca gca tgg agc ca-3′ and 5′-att gtg tgg tgc cgc aag cta-3′ (592 bp — size of the amplified fragment), 5′-atg tcg caa ggc agt aag ccc a -3′ and 5′-gga gtc ttt cag cat gga gca a-3′ (432 bp), 5′-tgg agg atg tga caa gca gc-3′ i 5′-atg cct cac caa ctc acc agg-3′ (473 bp) were used to study the presence/absence of *rol*B, *vir*D, *rol*C genes in "hairy" root clones and control plants.

We used primers 5′-cct gaa tga act cca gga cga ggc a-3′ and 5′-gct cta gat cca gag tcc cgc tca gaa g-3′ for amplification of *nptII* (622 bp) and *ifn*-α2b (396 bp) gene fragment respectively.

The presence/absence of *rol*B, *vir*D, *ifn*-α2b, *nptII* genes in *A. rhizogenes* strain was studied similarly. 0.2 μl of overnight bacterial suspension was added to the reaction mixture.

*4. The study of "hairy" roots growth rate.*

Explants of the apical part of "hairy" roots (1cm long, each one considered as one growth point) were cultivated on 1/2 MS agar-solidified medium (Duchera Biochemie) at +25 °C during 34 days. Roots of untransformed wormwood plants were used as a control. Then we determined the weight gain from one growth point for the entire cultivation period.

*5. Determination of total flavonoids and phenolic content.*

Fresh plants material of two "hairy" root lines (No 3 and No 4), leaves and roots of control plants were homogenized in mortar with the appropriate amount of 70% ethanol. The ratio of the fresh plants weight (g) to the solvent volume (mL) was 1:10. Extract was centrifuged (Eppendorf Centrifuge 5415 C) at 16 000 g for 14 min. Supernatant was collected and used for analyses.

Total flavonoids content in obtained extracts was estimated by modified $AlCl_3$ method [29]. Therefore, 1 ml of deionized water and 0.075 ml of 5% $NaNO_2$ (Sigma) were added to 0.25 ml of extract. The mixture was resuspended and incubated for 5 min. Then 0.075 ml of 10% $AlCl_3$ (Sigma) was added. Next we added 0.5 ml of 1 M NaOH (Sigma) and 0.6 ml of deionized water. Optical density (OD) was measured at 510 nm on the Panorama Fluorate-2. Rutin solution in 70% ethanol was used to create the calibration plot. Total flavonoids content (C, mg/ml) was determined according to the calibration graph ($C = 0.8842*OD$, $R^2 = 0.9926$).

Total phenolic content was determined by photoelectric calorimetric method [30]. Therefore, 0.2 ml of ethanol plant extract was added to 1 ml of 10% Folin-Ciocalteu reagent (Sigma). This mixture was resuspended and incubated for 6 min. Then 0.8 ml of 7.5% $Na_2CO_3$ solution (Sigma) was added. The mixture was incubated for 2 h. The optical density was measured at 740 nm. Total phenolic content (C) was determined according to the rutin calibration graph ($C = 427.41*OD$, $R^2 = 0.9286$). We used rutin solution in 70% ethanol at concentrations of 0.05-1 mg/ml to make a calibration plot.

*6. Determination of antioxidant activity.*

Antioxidant activity of the samples was studied by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [28]. Three dilutions in 0.004% DPPH solution (Sigma) were prepared for each extract. The total volume of extract with DPPH solution was 2 ml. The optical density of the mixture was determined at 515 nm on the Panorama Fluorate-2 spectrophotometer. The percentage inhibition of DPPH extract was determined by the formula:

$$\%_{\text{inhibition}} = \frac{OD_0 - OD_1}{OD_0} \times 100\%,$$

where $OD_0$ — optical density of DPPH solution, $OD_1$ — optical density of DPPH solution after adding extract.

The graph of the percentage inhibition versus the fresh weight concentration was plotted for each sample. $EC_{50}$ was determined from this graph. $EC_{50}$ is the weight of fresh plant material (mg), which reduces the DPPH solution by 50%.

*7. Data analysis.*

All analyses were performed in triplicate. All data, except antioxidant activity results, are presented as the mean value accompanied by standard deviation. Obtained data were analyzed by one-way ANOVA to identify significant differences between the samples. Multiple comparisons were performed using the Bonferroni post-hoc test ($P < 0.05$). Correlation analysis was performed by determining the Pearson correlation coefficient. The calculations were performed using Microsoft Office Excel and OriginLab OriginPro 2021b software.

## Results and Discussion

Bacteria were grown according to the above-mentioned conditions and used for transformation. Almost all explants survived after cocultivation with bacteria for a short time. Roots on leaf explants started forming in 7–14 days after transformation (Fig. 2). The frequency of transformation was 100%. The transformation efficiency was 5 roots per one explant. Twelve "hairy" root lines were subcultivated for more than a year. Two lines (No 3 and No 4) were selected for further research, which were visually characterized by the highest growth rate and had a characteristic "hairy" roots phenotype (significant branching, negative geotropism, the growth on hormone-free medium). However, the selected lines differed slightly in morphology. Thus, the "hairy" roots of the line No 3 were thinner, had a light-green color. The transgenic roots No 4 were more watered, had a light-brown color (Fig. 2).

PCR analysis of *A. rhizogenes* strain, which was used for transformation, indicated the presence of *rol*B, *vir*D, *ifn*-α2b, *nptII* genes in bacterial DNA (Fig. 3). Also, analysis showed the presence of *rol*B and *rol*C genes in both "hairy" root lines. But these genes were not detected in control leaves and roots (Fig. 4, *A*, *B*). The absence of *vir*D (Fig. 4, *C*) and the presence of *rol*B and *rol*C genes confirm that the studied samples were really transformed.

However, the unexpected absence of *ifn*-α2b and *nptII* genes of pCB124 plasmid may be the outcome of growing roots without the use of a selective medium in the absence of kanamycin. Such selection is usually used to screen transgenic samples carrying *nptII* gene, which determines the resistance of plants to specific antibiotic. Therefore, according to the obtained results, it can be concluded that selection in the presence of kanamycin is necessary to select *A. absinthium* root lines that carry not only agrobacterial genes (*rol*B and *rol*C), but also selective *nptII* gene.

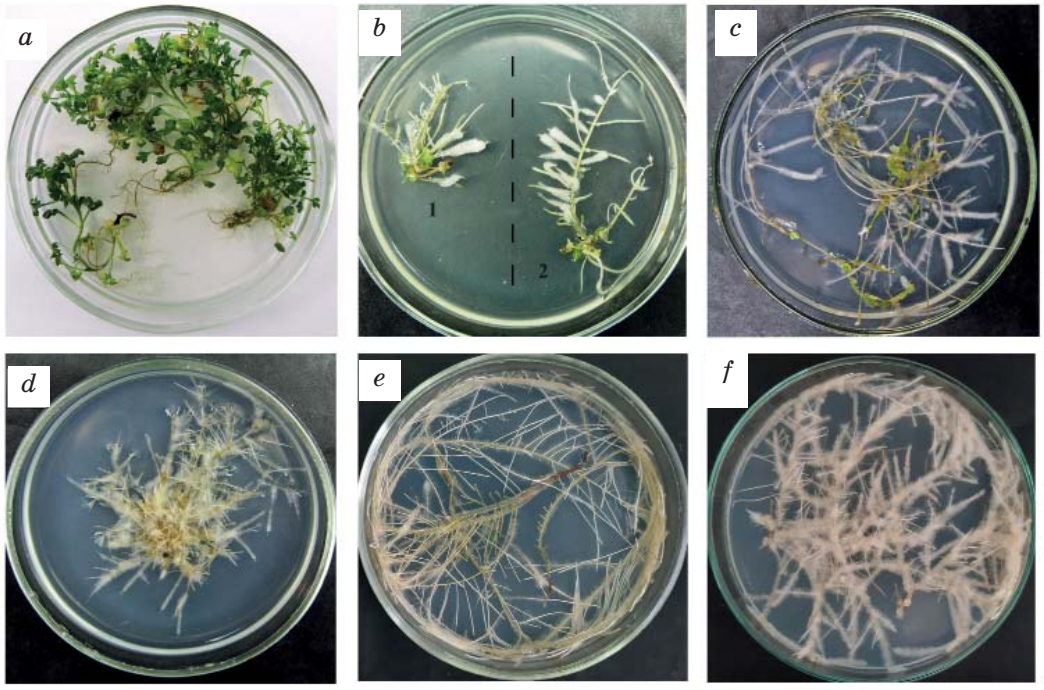


*Fig. 2. A. absinthium "hairy" roots induction*:
*a* — control plant; *b* — the beginning of root formation (1 — line No 3, 2 — line No 4); *c* — transgenic roots No 3 after 6 months of cultivation; *d* — "hairy" roots No 4 after 6 months of cultivation; *e* — roots of line No 3 after a year of cultivation, *f* — line No 4 after a year of cultivation

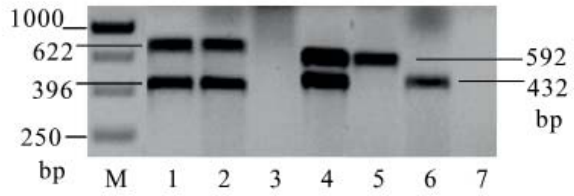


*Fig. 3.* **Electrophoregram of DNA amplification products** with gene-specific primers to *ifn*-α2b and *nptII* (*A. rhizogenes* DNA, 1); control DNA with *ifn*-α2b and *nptII* (2); *rol*B and *vir*D (4); control DNA with *rol*B and *vir*D genes (5, 6); without DNA (3, 7); M — O`GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) marker

The weight gain per one growth point of the "hairy" roots No4 was 0.39 ± 0.07 g after 34 days of cultivation. The growth rate of transgenic roots No 3 was 0.22 ± 0.06 g per one growth point (Fig. 5). The growth of isolated control roots was almost absent.

The "hairy" roots of the line No 3 accumulated 1.2 and 1.3 times more flavonoids than roots and leaves of control plants cultured *in vitro* (4.784 ± 0.10 mg/g, 3.861 ± 0.13 and 3.752 ± 0.12 mg/g FW respectively) (Fig. 6). However, the total flavonoids content in the line No4 was 1.5 times lower than in the control (2.620 ± 0.21 mg/g FW). These results indicate that *A. rhizogenes*-mediated transformation can differently influence not only the growth rate but also the flavonoids synthesis in "hairy" roots. As we can see, genetic transformation can lead to both an increase and a decrease in the total flavonoids content compared to untransformed control. Such features of the influence of genetic transformation can be explained by the fact that in the transformation of plants using agrobacteria, the incorporation of bacterial *rol* genes is indeterminated. In this case, each "hairy" root line is an independent transformational event with its own characteristics of the influence of transferred genes on the functioning of own plant genes and biosynthesis of different compounds including flavonoids.

Total phenolic content analysis showed that the highest concentrations of these compounds were in the extract of transgenic roots No 3 and No 4 (5.605 ± 0.26 and 4.385 ± 0.25 mg/g FW respectively) (Fig. 6). The quantity of phenolic compounds in plant extracts No 3 was 4.1 and 2.8 times higher than in roots and leaves extracts of control plants (1.355 ± 0.15 and 2.023 ± 0.10 mg/g FW respectively). The phenolic content in the "hairy" roots No 4 was 3.2 and 2.2 times higher than in the control roots and leaves. Comparative analysis of the two transgenic lines showed that the total phenolic content in transgenic line No3 was 1.3 times higher than in line No4. Thus, *Agrobacterium*-mediated transformation of *A. absinthium* has led to increased production of phenolic compounds in both "hairy" root lines.

Evaluation of free radical scavenging activity showed that the highest antioxidant activity was demonstrated by the extracts from the "hairy" roots No 3 ($EC_{50}$ = 3.657mg). This activity was 1.8 and 3.6 times higher than roots and leaves extracts from control plants ($EC_{50}$ = 6.716 and 13.226 mg respectively) (Fig. 7). The antioxidant activity of the line No4 extract ($EC_{50}$ = 6.958 mg) was 1.9 times higher than the scavenging activity of the leaf extracts of control plants. Comparative analysis of the two transgenic lines by this parameter indicated that the free radical

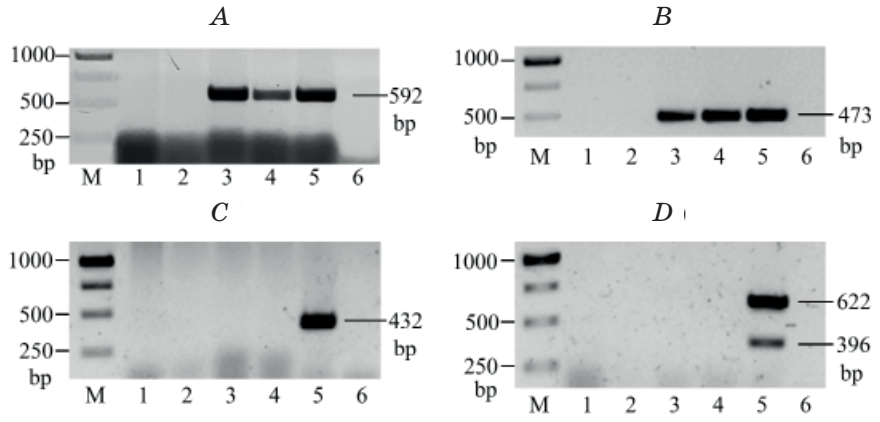


*Fig. 4.* **Electrophoregram of amplification products DNA of leaves (track 1), roots (track 2), No 3 (3) and No 4 (4) "hairy" roots** using specific primers to *rol*B (*A*), *rol*C (*B*), *vir*D (*C*), *ifn*-α2b and *nptII* (*D*) genes; 5 — plasmid DNA, 6 — without DNA; M — O`GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) marker

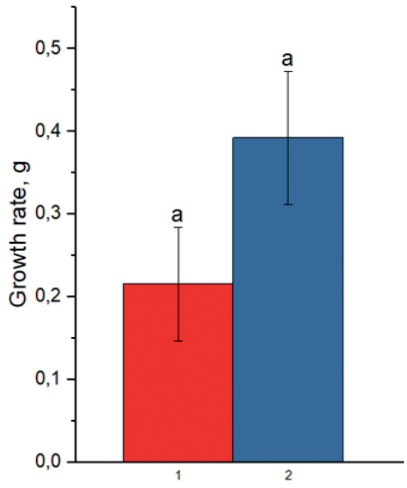


***Fig. 5*. Growth of "hairy" roots No 3 (1) and No 4 (2) for 34 days**
Bars "a" letter denote absence of significant differences in growth rate between two values at $P < 0.05$

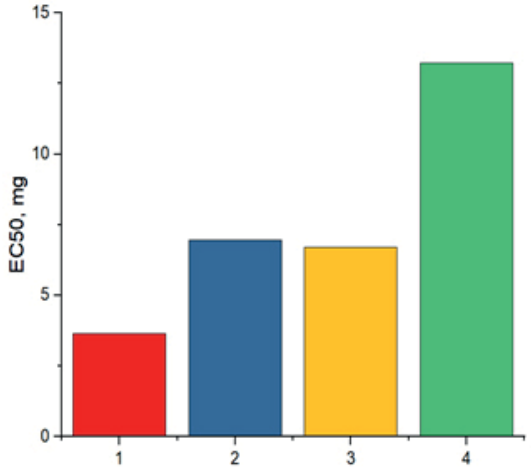


***Fig. 7*. Antioxidant activity of water-ethanol extracts of "hairy" roots No 3 (1) and No 4 (2), roots (3) and leaves (4) of control plants**

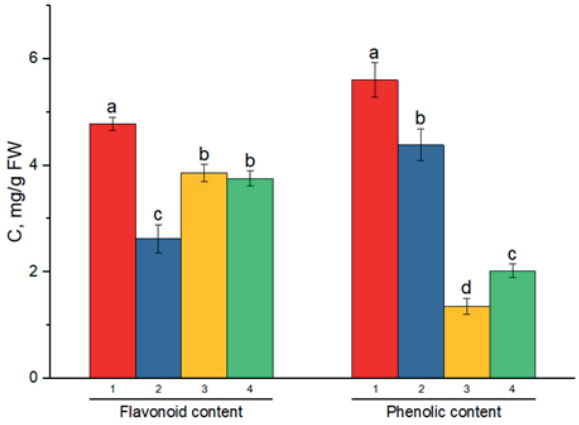


*Fig. 6*. **Total flavonoids and phenolic content** in ethanol extracts of transgenic roots No 3 (1) and No 4 (2), and roots (3) and leaves (4) of control plants. Bars with different small letters denote significant differences in values among four extracts at $P < 0.05$

scavenging activity of "hairy" roots No 3 extract is 1.9 times higher than the transgenic roots No 4 extract.

As it can be seen from the above-mentioned data, there is a correlation between the flavonoids content and antioxidant activity of transgenic roots No 3 extract. Thus, the extract of these "hairy" roots had the highest flavonoids concentration and the highest antioxidant activity. However, the correlation between these two parameters for other samples is weak (Pearson's coefficient is 0.4). For example, although the line No4 extract had the lowest concentration of flavonoids, its free radical scavenging was not the lowest. This may be related to the accumulation of other antioxidant compounds, such as phenols. Its content in "hairy" roots No4 were higher than in the control (Fig. 6), and the correlation between this parameter and antioxidant activity was average (Pearson's coefficient was 0.63).

Thus, *Agrobacterium*-mediated transformation of *A. absinthium* plants has led to changes in "hairy" roots secondary metabolism. Particularly, it has resulted in significant increase in the phenolic content. Also, an increased flavonoids content in transgenic roots No 3 correlated with enhanced antioxidant activity. Similar influence of genetic transformation on the biosynthetic activity of "hairy" roots cells has been described in plants of other species. For example, the total flavonoids and phenolic content in the *Althaea officinalis* L. transgenic roots was higher than in control plants [36]. *Cucumis anguria* L. "hairy" roots had a higher concentration of phenolic compounds and flavonoids as well as higher antioxidant activity compared to untransformed plants [37].

Such changes may be caused by transferring bacterial *rol*-genes to the plant genome and its activity. In particular, the results of a study of *Agrobacterium*-mediated transformation of *Lactuca serriola* L. roots indicate that the *rol*B genes integration into the plant genome increased the expression of *CHI, PAL, FLS* and *CHS* genes, which are responsible for

flavonoid biosynthesis. According to the authors, these changes in plant genome led to increase in phenolic and flavonoids content in "hairy" roots extract and increased antioxidant activity compared to the control [35].

## Conclusions

This research is the first study of the effect of *Agrobacterium rhizogenes*-mediated transformation on flavonoids and phenolic compounds synthesis and antioxidant activity of *A. absinthium* "hairy" roots. It showed that wormwood transformation using *A. rhizogenes* agropin strain A4 was possible. The transformation frequency was 100%. The transfer of bacterial *rol*B and *rol*C genes has been confirmed. However, the expected transfer of neomycin phosphotransferase II and human interferon-α2b genes did not occur. Probably, it happened due to the lack of a selection step on kanamycin medium.

The analyzed root lines differed in growth rate, flavonoids and phenolic content, and antioxidant activity. Moreover, the high flavonoids content (4.784 ± 0.10 mg/g FW) in transgenic roots No 3 correlated with increased antioxidant activity rate ($EC_{50}$ = 3.657mg compared to 6.716 and 13.226 mg for control roots and leaves). Therefore, the obtained results indicate that the *A. absinthium* transformation using *A. rhizogenes* can be applied to obtain lines with a high phenolic content and a high antioxidant activity.

The work was done within the framework of the departmental fundamental theme of the National Academy of Sciences of Ukraine "Targeted genome changes and pleiotropic effects in genetically transformed plant systems", State registration 0120 U 100849.

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## *Agrobacterium rhizogenes* — ОПОСЕРЕДКОВАНА ТРАНСФОРМАЦІЯ ЯК СПОСІБ СТИМУЛЮВАННЯ СИНТЕЗУ АНТИОКСИДАНТНИХ СПОЛУК У *Artemisia absinthium* L.

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Рослини *Artemisia absinthium* L. відомі як продуценти речовин з антиоксидантними властивостями. Зокрема, в них виявлено поліфеноли та флавоноїди. Активізувати синтез цих сполук можна шляхом генетичної трансформації навіть без перенесення специфічних генів, які беруть участь у біосинтезі. Так, «бородаті» корені, одержані після *Agrobacterium rhizogenes*-опосередкованої трансформації, можуть бути продуцентами комплексу цінних метаболітів.

*Метою* роботи було одержати «бородаті» корені *A. absinthium* як продуценти поліфенольних сполук.

*Методи.* «Бородаті» корені одержували шляхом культивування листків з суспензією *A. rhizogenes* з вектором *pCB124*. Наявність перенесених генів підтверджували методом ПЛР. Для визначення вмісту флавоноїдів та поліфенолів використовували реакції з $AlCl_3$ та реактивом Фоліна-Чокальте. Антиоксидантну активність оцінювали за здатністю екстрактів відновлювати DPPH радикал.

*Результати.* ПЛР аналіз виявив наявність бактеріальних *rol* генів та відсутність генів плазміди pCB124. Лінії коренів відрізнялися між собою за швидкістю росту. «Бородаті» корені характеризувалися більшим вмістом поліфенолів, зокрема, флавоноїдів (до 4.784±0.10 мг/г ВМ) та вищим рівнем антиоксидантної активності ($EC_{50}$ = 3.657 мг) у порівнянні з контролем (3.861±0,13 мг/г СМ та $EC_{50}$ = 6.716 мг відповідно).

*Висновки.* Трансформацію *A. absinthium* із застосуванням *A. rhizogenes* може бути використано для одержання ліній з підвищеним вмістом поліфенольних сполук та більшою антиоксидантною активністю.

*Ключові слова: Artemisia absinthium* L., *Agrobacterium rhizogenes*-опосередкована трансформація, «бородаті» корені, флавоноїди, поліфенольні сполуки, антиоксидантна активність.