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Migalomorphic Spiders Venom: Extraction and Investigation of Biological Activity

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ABSTRACT

A number of researchers have noted that spiders produce a venom consisting of a mixture of potent selective toxins of different nature (from salts to large multi-domain proteins), each of which can have its own specific biological activity. A wide range of similar substances has been presented in venom of spiders which are an admixture of potent and selective toxins, each of which can have a specific biological activity. Considering the action mechanisms of venom, it is logical to assume that among the components of a poison of spiders, the peptides possessing antimicrobial activity can be found. From this point of view, mygalomorph spiders have been the most interesting to be studied. 24 mygalomorph spiders from 12 genera were studied to identify the influence of electrical stimulation parameters on the venom extracting. Electrical stimulation with various parameters allowed determining the spiders with the best ability of venom extracting. The selected venom was studied for insecticide and bactericide activity.

Keywords: *spider, Mygalomorphae, venom, peptides, pharmacology, biological active agents*

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INTRODUCTION

During the evolution, the spiders have reached the greatest biological diversity of the active agents of different chemical nature which are contained in their venom. The composition of venom is unique for each look, and depends on a number of factors, such as the climatic conditions, the gender accessory, the dwelling area, and the nature of the consumed feed. It is worth marking that today, there is information on a hundred spiders' venom, but at the same time, the structure of only several of them has been studied in detail [7-11].

A number of researchers have noted that spiders produce a venom consisting of a mixture of potent selective toxins of different nature (from salts to large multi-domain proteins), each of which can have its own specific biological activity [13-17, 19-22, 36].

Previously, it was found that the venom of most of the studied spiders contains highly specific substances that act on various membrane

transport systems: ion channels, ionotropic receptors, etc [10-12].

These substances are indispensable tools in the study of membrane systems and can find wide application in modern neurobiology.

In total, there are about 42 thousand species of spiders from which 2.5 thousand represent infra unit mygalomorph spiders whose venom has been studied at least in view of a complex proteinaceous and polypeptide structure, and still the mechanism of biologically active agents' biosynthesis has not been discovered [23-26, 36].

In this study, taking into account the fact that the venom of the most species of mygalomorph spiders has not been studied, it was assumed that it is possible to detect the toxin substances

that are modulators of various membrane systems. This is interesting not only for the basic research but also for the applied science, including medicine for treatment of diseases associated with a violation of the function of the membrane transport system.

Mygalomorph spiders are one of the most ancient arachnoid representatives. In an infra unit, there are big differences between the type and intensity of their venom. So, Brachypelma possesses an extremely weak venom, on action reminding bee poison, at the same time, Poecilotheria possesses a venom with paralyzing action [18, 25]. Such variety of mygalomorph spiders' venom action determines a perspective of research.

Thus, on one hand, the study of spiders' venoms is fundamental, and requires the development of new methodologies for their isolation and complete analysis. On the other hand, the venom is a source of substances with great practical importance as drugs or tools for studying the living organisms. It substantiates the relevance and even the need for research on the biodiversity of spider-derived toxins. To realize the practical interest, it is necessary to study how to synthesize the studied venoms artificially, because, even in case of development of a new effective method for venoms' selecting, its quantity will be negligible for the organizations producing medicines [1].

In this connection, it is important to study the venoms of mygalomorph spiders, and also find a method for synthesizing the revealed peptide combinations using (for example) an automated system for solid-phase peptide synthesis.

One more area of potential application of spider venoms could be agriculture. Since the food of spiders mainly consists of insects, venoms of many species have a selective effect on arthropods, and can be used as bioinsecticides for pest control [18]. Moreover, such bioinsecticides will not only have high efficiency, but even less harm to the environment, since they are completely biodegradable.

Rapid growth of stable antibiotics pathogenic microorganisms is also another problem which confirms the necessity of carrying out researches on spider venom.

The antibiotic resistance forms the pathogenic microorganisms leading to increase the mortality from diseases which can be treated earlier. More than one million people in Russian Federation died by resistant infections since 2014 [20]. And one of the most dangerous resistant form of pathogenic has been the strain of tuberculosis.

The last class of antimicrobial agents was found in 80th of the last century that it caused the relevance of the search for the new antimicrobial substances.

Nowadays there is a deficiency of new discoveries and ideas in this field. Modern scientific search for new substances having antibacterial activity is important to devote a special attention to substances of protein or peptide nature. The wide range of similar substances has been presented in spiders' venom which are an admixture of potent and selective toxins, each of which having a specific biological activity [5, 23-26].

In recent years, the greatest success in a study of spiders' venom was reached by a group of scientists of Institute of bioorganic chemistry of academicians M. M. Shemyakin and Yu. A. Ovchinnikovof Russian Academy of Science [31]. The experts conducted researches on the molecular variety of the venom of some spiders' species, especially Argiopelobate.

It was detected that the venom of studied spiders has a noticeable structural homology: it is based on a polyamine chain that has primary and quaternary amino and guanidine groups or residue of arginine at one end of the molecule and in most cases – aromatic grouping (different types) – acyl radical – on the other [25,26]. This group gets connected with the polyamine with the amide bond (figure 1).

Mygalomorph spiders were affected to a lesser extent, however, during the execution of researches, the general concepts for spiders' venom were selected. The authors made a conclusion that studying spiders' venom is generally of practical interest and concerns finding new components with the given properties for creation of medicinal prototypes or insecticides.

According to figure 1, the structure of Argiopin (the main substance of Argiopelobate venom) has several general fragments:

1 – acyl radical, 2–betweennes group, 3–polyamine chain, 4– terminal group (arginine residue).

Despite the fact that polyamine toxins are produced by spiders for immobilization of mainly invertebrate by blockade of their glutamate receptors, they affect on the glutamate and acetylcholine receptors of nervous system of vertebrates, too. Moreover, they affect on other ion-tropic receptors and ion channels, and in most cases they are pore blockers that are efficient in micro- and submicromolar concentrations (10^{-8} – 10^{-6} M) [27-30].

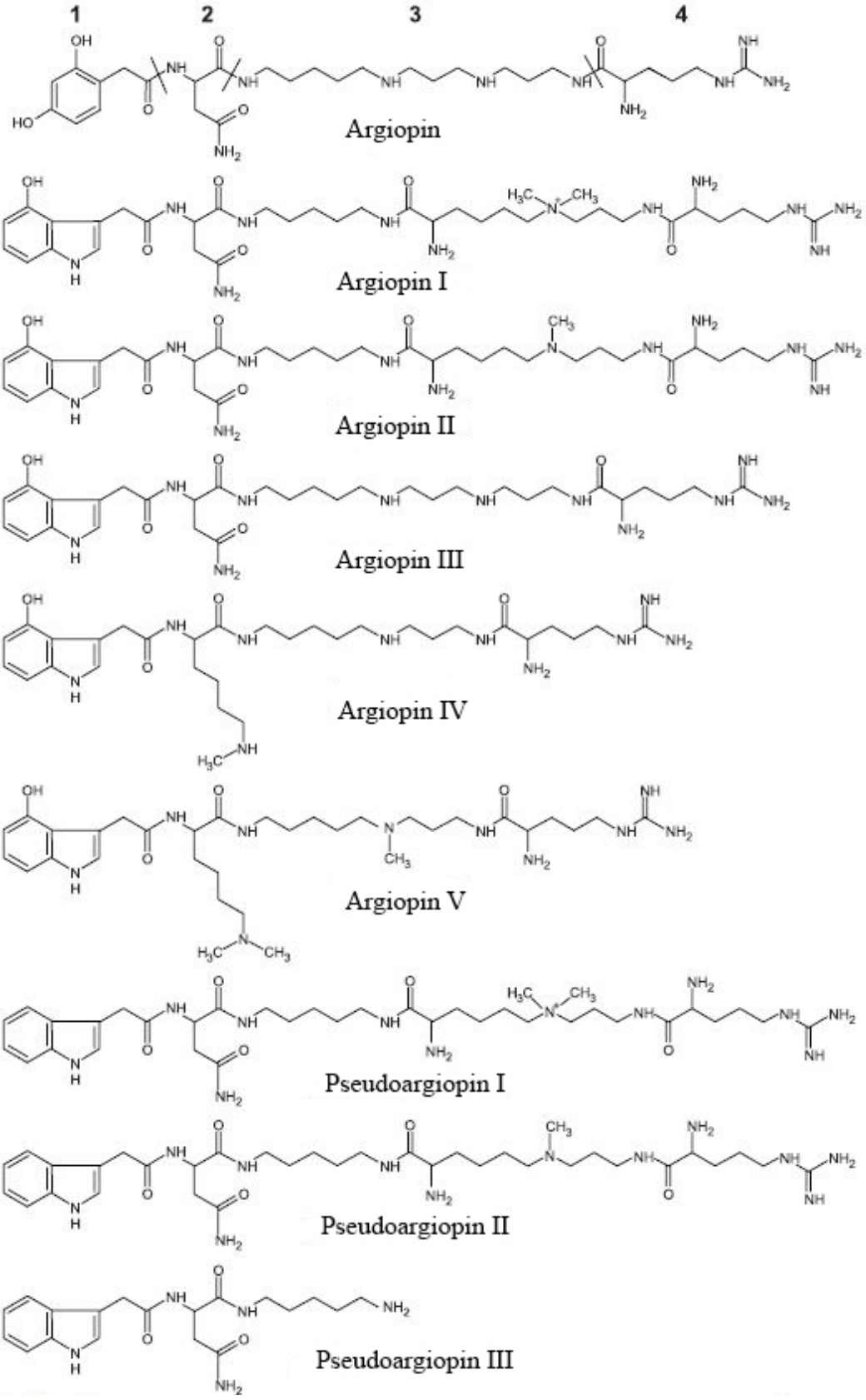

Figure 1. Combinatory of acylpolyamine toxins in venom of Argiopelobate (Described by [31])

Scientific search also identified several highly specialized researches in this field:

1) Studying two-domain toxins of spiders venoms. [31-33] studied new two-domain spiders' toxins; the structures and biological activities of modular toxins; the structures of the

genes coding two-domain toxins of spiders; the mechanisms of molecular evolution of modular toxins.

2) Studying cytolytic and antimicrobic peptides from spiders' venom of Lachesanatarabaevi. [31] produced identification and allocation of the CPU and AMP from the whole venom; established the primary structure of the active peptides; carried out the functional characteristic of new substances; studied the amino-acid sequence of the predecessors of the CPU and AMP; developed the system of aheterological expression of the genes of new peptides) [28,29]. The studied peptides have been visualized in figure 2.

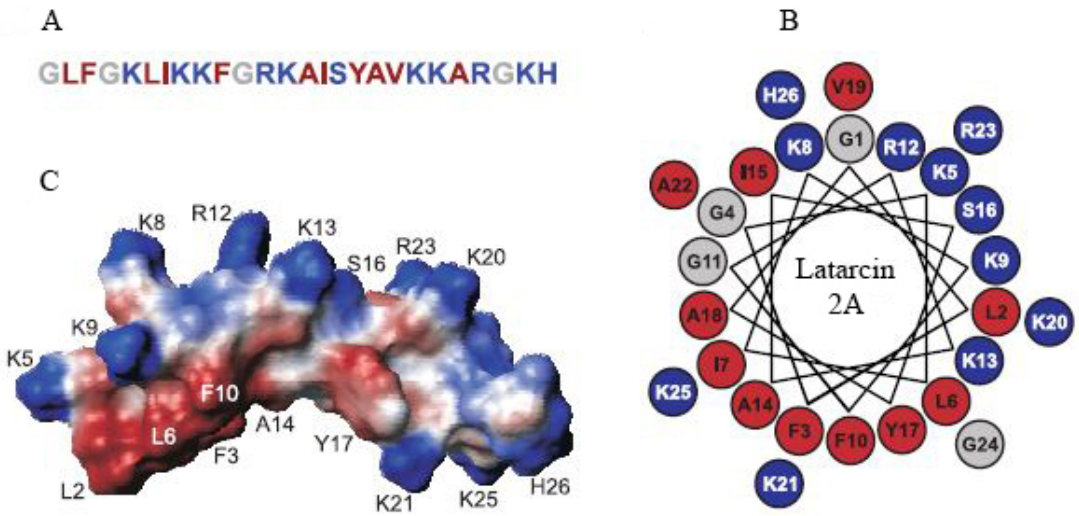

Figure 2. Cytolytic peptide latarcin 2A from the venom of Lachesanatarabaevi

A – amino acid sequence (code from Uni Prot database Q1ELU1) [4]

B - projection of the "spiral wheel". Hydrophobic residues marked by

red, hydrophilic – by blue, the residues of the glycine – by grey.

C – space structure of peptide in combination with detergent micelles (code in Uni Prot database PDB 2G9P).

An imaginary surfaces of peptide molecules were colored in accordance with the hydrophobic potential (hydrophobic areas are red, hydrophilic are blue) using the MOLMOL program [23,24].

3) Developing a strategy to use genes of anti-microbic peptides from arthropods' venom in gene therapy agents ([25] – recombinant plasmid vectors, expressing genes of anti-microbic peptides – Melittin from venom of a bee and peptides (latartsin and oksiopinin)from venom of two species of spiders were designed; the system of tetracycline-dependent regulation of an expression of genes was adapted to the received recombinant plasmid vectors; infectious models of mycoplasmoses and clamidioses at laboratory animals were created; the received recombinant plasmid vectors in culture of cages and on the models of mycoplasmoses and clamidiosis were tested on laboratory animals; the main mechanisms of the effect of antimicrobic medicines on the development of mikoplazmenny and chlamydial infections were installed) [25].

4) A research was done on the biological activity of antimicrobic peptides from the venom of a spider of Lachesanatarabaevion Chlamydia trachomatis using an infection model ([25] studied the antimicrobic activity of chemically synthesized latartsin of Ltc1, Ltc2, Ltc3a, Ltc4b, Ltc5 and cyto-insectotoxin CIT1a from the spider venom of L. tarabaevi concerning C. Trachomatis;the efficiency of the suppression of the development of a chlamydial infection at an expression of the genes coding of latartsin and cyto-insectotoxin CIT1a in HEK293 line cages was evaluated; the inhibiting action of cyto-insectotoxin CIT1a was studied at an expression of the gene coding in HEK293 line cages on the development of C. trachomatis in different phases of life cycle of a pathogen) [27].

[5] marked that the questions and tasks of "distant perspective" require the new level of understanding of a problem. The mechanism of venom biosynthesis, its biodiversity and practical application were switched on in the main problem. Scientists pointed that many spiders are characterized by small sizes and venom quantity which can be limited to microliters. That is one of the main reasons of extreme scarcity or even absence of information about the venom of representatives of many spiders families. Standard methods of

fractionation and establishment of structure of venoms' active components have not been optimum for wide-ranging studies. Therefore, developing new methodologies for collection of venom and using essentially new technologies are required.

Thus, at the modern level of scientific development, the structure of spider's venom and the mechanism of its synthesis has not been studied. In view of the high potential of the application of mygalomorph spiders' venom in pharmacology and the possibility of creation of new medicines on that basis, scientific society have got a need of studying toxins as it is possible by big representatives of mygalomorphs and finding possible ways for artificial synthesis of venoms.

MATERIALS AND METHODS

Objects of research:

24 mygalomorph spiders belonging to the family of tarantulas were used in this research. Among them were the representatives of the following genera: Avicularia, Brachypelma, Chromatopelma, Lasiodora, Nhandu, Haplopelma, Psalmopoeus, Tapinauchenius, Poecilotheria, Cyriopagopus, Pterinochilus, Theraphosa.

Method of venom bleeding:

A technique was developed for taking venom from mygalamorph spiders. The spider was fixed in an inverted state, and the clear slide was placed under the chelicera. The electrodes were delivered to the cephalothorax (figure 3). The discharge treatments were done by using 12-48 volts and a constant current of 1 ampere (an optimal value for miorelaxation and venom bleeding without physiological damage according to [2,3]) and the frequency of 50-60 hertz (general industrial current parameter) with the duration from 5 to 20 seconds.

Figure 3 – Method of bleeding of the spider's venom by using the discharge treatment.

The extracted venom of each spider was collected in graduated capillary tubes to determine the volume of the venom. The collected venom was also used in physiological assays to determine their functional activities. The venom after bleeding was stored in a vialsin refrigerator at -8°C.

All the experiments were conducted in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigations of the experimental pain in conscious animals. The number of spiders was the minimum necessary to get statistically significant results. During the research, no specimen was injured or died.

The method of investigating peptides concentration:

The concentration of the peptides was determined spectrophotometrically. The spectrums of the optical absorption were recorded using Shimadzu UV-1800 spectrometer. For the calculation of the

peptides' concentration, the following formula was used:

$OD_{280} = \varepsilon \times c \times l$ (1);

Where:

OD_{280}– optical density of the solution at λ = 280 nm,

ε–molar extinction coefficient of the peptide,

c– peptide concentration,

l –optical pathway length [32].

The method of determining the insecticide activity:

Insecticidal activity was studied on the larvae of CulexpipiensandZophobasmorio. The peptides were dissolved in saline, and 5 µl were injected into the fourth segment of the larvae. Saline was used as a control. Lethal and paralytic effects were recorded within 24 hours after the injection [33].

The method of evaluating bactericide effects:

The determination of antimicrobial activity of polypeptides was carried out by double serial dilutions. Cell lines of E. coli, B. subtilisand S. aureus were grown on an Endo culture medium. In the logarithmic growth phase, the culture was diluted with medium to a concentration of 1×10^5CFU/ml. The peptides were dissolved in 10 µl of deionized water and mixed with 90 µl of bacterial culture. The peptides, the control samples containing no peptides, and the samples of sterility control were tested in two replications. The microbiological tablets with the samples were incubated overnight at 37°C. The inhibition of the bacterial growth was determined by measuring the optical absorption at λ=620 nm. The concentration of the peptides, completely suppressing the growth of bacterial culture was accepted as the minimum inhibiting concentration (MIC). The bactericidal effect of the peptide against E. coli B. subtilis and S. aureuswas was estimated by sowing bacteria on Agarised medium after 3 h of incubation with the peptide at the concentrations equal to MIC, 2×MIC and 4×MIC, and the evaluation of the number of colonies which were formed was done after 24 h [31].

RESULTS AND DISCUSSION

The results of research on venom extracted by electrical stimulation have been shown on graphics in figure 4-5.

The quantity of venom was increased by the growth of the current parameters (till 30V) and the duration of the treatment. The venom recovery was poor when the current was below the 18-24V which was applied for 5-10 seconds. The maximum amount of venom for the majority of spiders was recovered at 30V (24V for Lasiodora and Pterinochilus).

The results of studies done by [34] suggested that current intensity of more than 30V may be harmful to arthropod species. As it has been shown in figure 4-5, the voltage more than 30V is not efficient enough, too, so it provided the safety procedure for spiders. No documented data addressing the physical damages caused by the extraction of venom or harmful effects of electric stimulation were found , however, few publications , marked by [34], have been available related to the metabolic costs of envenomation in terms of the induced oxygen consumption [35].

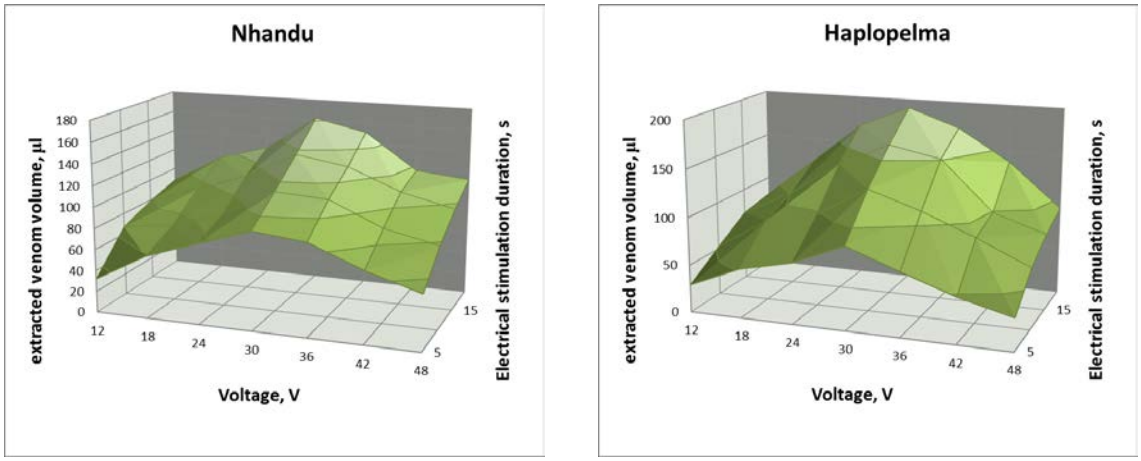

Figure 4. The graphics of the influence of electrical stimulation parameters on venom yield of spiders from genus Avicularia, Brachypelma,Chromatopelma, Lasiodora, Nhandu, Haplopelma

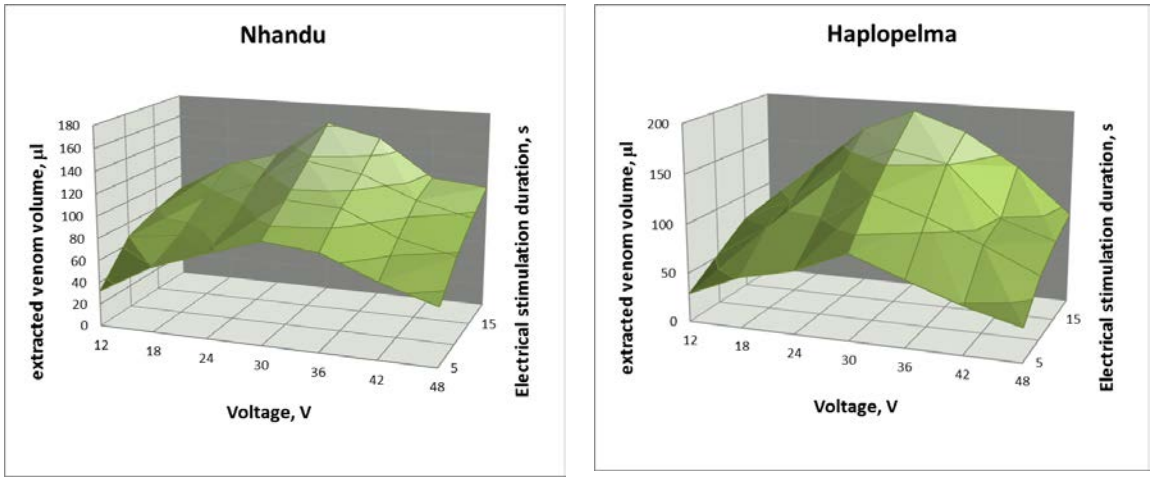

Figure 4. The graphics of the influence of electrical stimulation parameters on venom yield of spiders from genus Avicularia, Brachypelma,Chromatopelma, Lasiodora, Nhandu, Haplopelma
X-axis – Voltage, volts
Y-axis – bleeded venom volume, in microliters
Z-axis – Electrical stimulation duration, in seconds

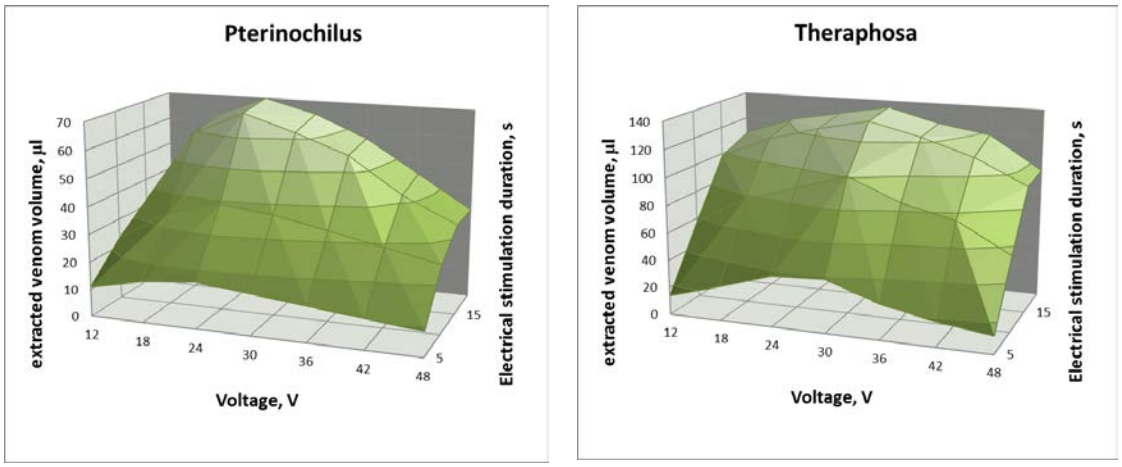

Figure 5. The graphics of the influence of the electrical stimulation parameters on the venom yield of spiders from genus Psalmopoeus, Tapinauchenius, Poecilotheria, Cyriopagopus, Pterinochilus, Theraphosa
X-axis – Voltage, volts
Y-axis – Bleeded venom volume, in micro liters
Z-axis – Electrical stimulation duration, in seconds

After 20 seconds, the current of 24V was applied to the spiders of different species. The maximum volume was obtained from Lasiodora (73±5 µl) and Pterinochilus (70±3µl). 30 seconds of 30V current treatment gave the maximum volume from other spiders. The most intense venom release due to the electrical stimulation was fixed on Haplopelma spider – 191±7µl, which exceeded the second and the third "venomous" spiders (Tapinauchenius and Nhandu) on 29µl.
Based on the described results, it was decided to study insecticide and bactericide activity of Haplopelma spider venom as a general model which was better extracted by the electrical stimulation.
It was recorded that Haplopelma toxin exhibited the insecticidal and cytolysis activity. In the tests on the larvae of Culexpipiens, LD50 was 83 mg/g (6.7 nmol/g). The venom showed the same poison activity on Zophobas morio: LD50 on the larvae of the darkling beetle was 95 µg/g (7.4 nmol/g).
It was previously found that spider venom was able to induce the lysis of red blood cells (figure 6). Its effective concentration causing 50% hemoglobin yield was ~10 µm. On cell lines, 50% cell death was observed at the concentrations of ~20 µm and ~15 µm,

respectively, which confirmed the cytolyses activity of the studied venom.

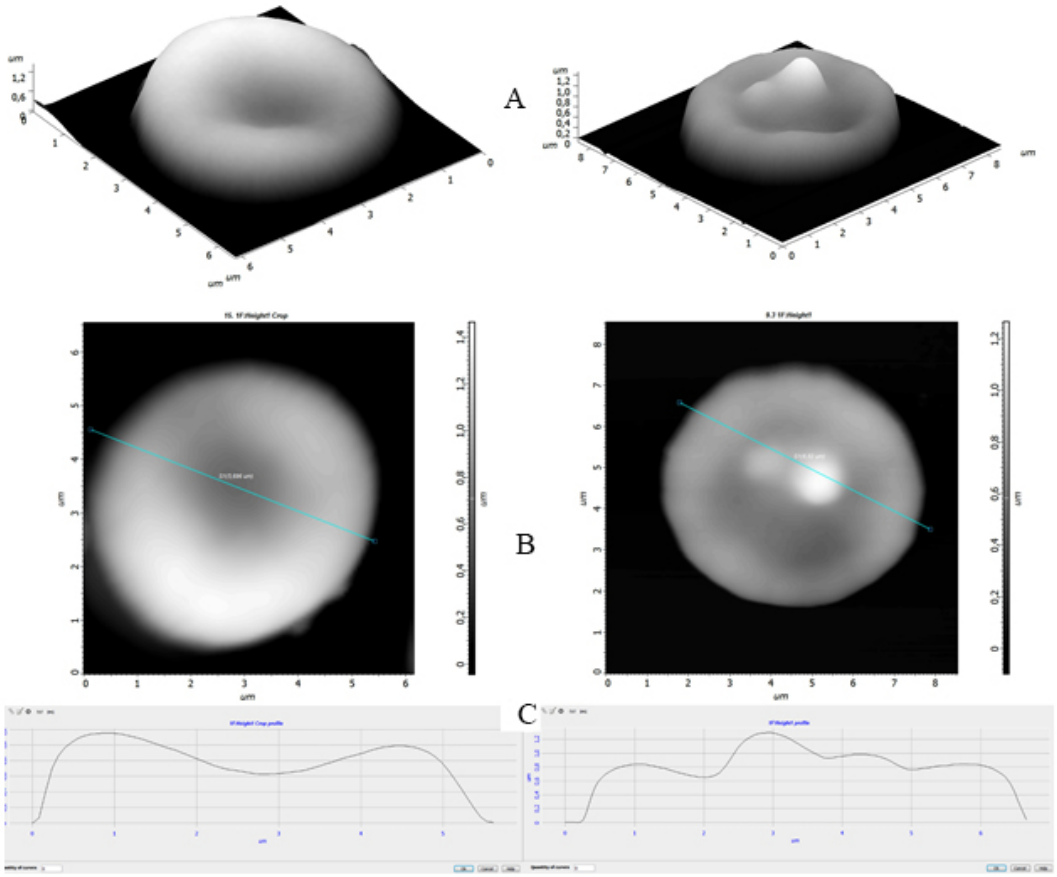

Figure 6. Scan of an abnormal-shaped RBC under spider venom peptide effect obtained by AFM (scanned in air).
A) 3d image of single cells of normal and abnormal erythrocyte shape;
B) 2-dimensional image of normal and abnormal cells;
C) Normal and abnormal cell cross-section diagram;

The image was previously published in "Features of the study of morphology of the abnormal forms of red blood cells by atomic force microscopy" [6].
In further tests, it was found that the venom of studied spiders not only inhibits the bacterial growth, but also has bactericidal activity. For E. coliand B. subtilis, the values of the minimum bactericidal concentration (MBC) and MIC did not have a statistical difference (table 1), the bactericidal effect developed rapidly. The incubation of S. aureus for 15 minutes in a medium containing a peptide at a concentration equal to twice the MBC, led to the complete death of the bacterial cells. After the incubation of S. aureus for 15-30 minutes in a medium containing 1.15 μM and sown on agar medium, no more than 2-3 colonies per Petri dish grew. After a longer incubation (more than 1 h), the viable bacteria died.

The bactericidal effect of venom peptides was most likely associated with the formation of defects in the cytoplasmic membrane, since the optical density of the solutions grew, which could be explained by the leakage of the cytoplasm content.

Table 1. Antimicrobial activity of venoms' peptides

Bacterial strain	Indexes	
	MIC, μM	MBC, μM
E. coli	0,16±0,02	0,25±0,02
B. subtilis	0,15±0,02	0,22±0,02
S. aureus	0,8±0,04	1,15±0,02

During the discharge, the spiders were given an amount of venom, approximately equal to 100-200 μl, which was an extremely small amount even for the research purposes, and the industrial usage of venoms produced in this way was impossible, since they would have an

extremely high price. Thus, the next stage of research should be directed to synthesis of the venom peptides after the determination of peptides' combination by chromatography.

The synthesis of peptides can be carried out with the usage of automatic system for solid-phase synthesis "Libertypeptides". The comparison between the native and artificially synthesized peptides' activity should be done, too. In case of the successful results, the influence of the external factors of the environment on the stability and activity of the chosen peptides would be established. The environmental factors: pH of the mixture with peptide, temperature and also the influences of the existence of collateral inorganic components in the environment should be considered.

The synthesized peptides should be studied on the antimicrobial activity at each allocated component concerning the culture of E. Coli, and in case of detecting antimicrobial effects – further study should be done on the activity of the allocated antimicrobial peptides on clinically significant microorganisms making the so-called ESKAPE group: Enterococcus faecalis (E), Staphylococcus aureus (S), Klebsiellapneumoniae (K), Acinetobacter baumannii(A), Pseudomonas aeruginosa (P), Enterobacter iaceae, including Enterobacter (E). Choosing this group of microorganisms can be explained by their high resistance to modern antibacterial drugs, and also a high mortality among the patients. It is also very important to study the effects of interaction between the modern antibiotics and antimicrobial peptides with simultaneous impact on the above-mentioned microorganisms.

CONCLUSION

The electrical stimulation with various parameters was used to determine the spiders with the best ability of venom extracting. After 20 seconds, the current of 24V was applied to the spiders of different species, and the maximum volumes were obtained from Lasiodora (73±5 μl) and Pterinochilus (70±3 μl). 30 seconds of 30V current treatment gave the maximum volume from other spiders. The most intense venom release due to the electrical stimulation was fixed on Haplopelma spider – 191±7 μl, which exceeded the second and the third "venomous" spiders (Tapinauchenius and Nhandu) on 29 μl.

It was recorded that spider venom has a powerful antimicrobial, insecticidal and cytolytic activity, due to the presence of active peptides and polyamines.

In the tests on the larvae of Culex pipiens, LD50 was 83 mg/g (6.7 nmol/g). Venom showed the same poison activity on Zophobas morio: LD50 on the larvae of the darkling beetle was 95 μg/g (7.4 nmol/g).

Microbiological analysis showed MIC for E. coli (0.16±0.02μM), B. subtilis (0.15±0.02μM), S. aureus (0,8±0,04μM). It was found that the venom of studied spiders not only inhibited the bacterial growth, but also had the bactericidal activity. MBC of E. coliand B. subtilisis was the same and four-times lower than MBC of S. aureus.

In conclusion, it is important to note that 100-200 μl of the extracted venom from each spider is an extremely small amount even for the research purposes, and the industrial usage of venoms produced in this way is impossible, since they will have an extremely high price. Thus, the next stage of research should be directed to the synthesis of venom peptides after the determination of peptides' combination by chromatography.

In addition, venom samples may represent the starting point of functional assays, which determine the biomedical and pharmacological potential of their constituent molecules. Nearly all the spiders produce venom, and the wide diversity of venom components synthesized by individual species suggests a vast diversity of venom molecules which are yet to be discovered.

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Author's contribution:

The article was written at the expense of the authors.

Conflict of interest:

In the article, there was no information capable of provoking conflicts of interest, with the exception of the information contained in previously published articles by Nagdalian[1], Andrey Ashotovich[1], Pushkin[1], Sergey Viktorovich[1], Povetkin[1], Sergey Nikolaevich[1], Kopchekchi[2], Marina Egorovna[2], Marinicheva[2], Marina Petrovna[2], Lopteva[3], Maria Sergeevna[3].

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