Expanded habilitation report for scientific contributions

of Assoc. Prof. Dr. Lyudmila Velkova

regarding application for the academic position "Professor", professional field 4.2 Chemical sciences, scientific specialty "Bioorganic chemistry, chemistry of natural and physiologically active compounds" for the needs of lab. "Chemistry and Biophysics of Proteins and Enzymes (CBPE)"

The present report presents the most significant scientific contributions from the research carried out after acquiring the academic position of "Associate Professor". They are presented in 72 publications, of which 38 are outside the competitions for acquiring the scientific and educational degree of "doctor" and the academic position of "associate professor". In the current competition I participate with 6 publications on indicator V (Q1 - 5, Q2 - 1), 22 publications on indicator G (Q1 - 5, Q2 - 6, Q3 - 8, Q4 - 3), 2 recognized utility models and 265 citations that have not been used in previous competitions, h-index (according to Scopus) 12 (after excluding the self-citations of all co-authors).

All scientific papers submitted for participation in the competition are in the field of bioorganic chemistry, chemistry of natural and physiologically active substances. One of the main trends in the presented research is the discovery of new, more effective and selective drug molecules of natural origin with antimicrobial and antitumor potential.

The main objects of research are the mucus and hemolymph of snails, which are complex multicomponent physiological mixtures, including substances with different biochemical characteristics and properties, with potential pharmacological applications in the fight against microbial pathogens, some oncological diseases, and also neurodegenerative diseases.

My scientific interest is mainly focused on the characterization of biologically active substances from natural sources, such as peptides, proteins and glycoproteins in the mucus and hemolymph of Gastropods with antimicrobial and antitumor activity, with antioxidant and regenerative properties, elucidation of their mechanism of action and the possibilities for their application.

The scientific contributions presented in the competition are the result of interdisciplinary research, which can be summarized in the following thematic areas:

I. Identification and characterization of biocomponents from Gastropods with antimicrobial activity.

II. Antitumor potential of components from the hemolymph and mucus of Gastropods.

III. Mechanism of action of snail biocomponents established by proteomic analysis on 2-dimensional gel electrophoresis (2D-PAGE).

IV. Characterization of other bioactive components from natural sources.

The indicated directions are aimed at important challenges in the public health sector and are priorities for a number of national strategic documents such as the "National Strategy for the Development of Scientific Research in the Republic of Bulgaria 2017-2030", the "Innovation Strategy for Smart Specialization", etc.

Scientific research related to the discovery of the biological activity of biocomponents from natural sources has been conducted in collaboration with teams from national institutes and universities, participants in the National Scientific Program "BioActiveMed" and in projects with the National Science Foundation.

My main contributions concern the development of new approaches for the isolation and characterization of active components in complex multicomponent biological mixtures. Another important aspect of my studies is related to the elucidation of the relationship between structure, function and biological activity, as well as the establishment of the mechanism of action of the active components.

Introduction

Antimicrobial resistance is one of the most serious public health threats in 21 century with important societal and economic consequences. Recently, the World Health Organization identified the rise of multidrug resistance in microorganisms as one of the current global threats to public health and declared the beginning of a "post-antibiotic era".

The study and characterization of natural compounds with antimicrobial activity is a new potential strategy to reduce the spread of antimicrobial resistance. In recent years, antimicrobial peptides (AMPs) have attracted the attention of more and more researchers due to their broad-spectrum antimicrobial activity with high specificity, immunomodulatory properties and their ability to overcome conventional resistance mechanisms. They are found in almost all organisms ranging from prokaryotes to complex eukaryotes, including humans, and show remarkable structural and functional diversity [V1]. AMPs are essential components of the innate immune defence of multicellular organisms. It has been shown that, unlike antibiotics, they interact with multiple targets on the plasma membranes of pathogenic bacteria, as well as intracellular targets, and some of them exhibit potent activity against drug-resistant bacteria. These interactions can lead to membrane permeabilization, depolarization, leakage, or lysis, leading to cell death. In this context, they are a promising therapeutic approach. Besides AMPs, some proteins and glycoproteins in the mucus and hemolymph of gastropods also exhibit antibacterial properties and are an important component of their innate immune response.

Another trend in modern research is the discovery of new selective and more effective antitumor agents. In this regard, copper-containing glycoproteins "hemocyanins", which function as oxygen carriers in the hemolymph of many Molluscs and Arthropods, are of considerable interest, as they combine strong immunostimulatory activity, direct anticancer effect and selectivity. On the other hand, many proteins in the hemolymph of Molluscs and Gastropods are still unknown, which is why their study and characterization causes high scientific interest.

I. Identification and characterization of biocomponents from Gastropods with antimicrobial activity

The focus on the study of biocomponents of snails (invertebrate organisms, class Gastropoda, phylum Mollusca) is justified by the great species diversity and their ability to adapt to different habitats, which make them one of the largest sources of bioactive compounds with antimicrobial activity. These organisms, unlike vertebrates, do not have an adaptive immune response and rely solely on their innate immunity to counteract various pathogens. Their evolutionary success demonstrates the effectiveness of their defense mechanisms.

I.1. Antimicrobial peptides from the mucus of garden snail *Cornu aspersum*

The mucus of land snails is a complex biological substance that not only facilitates movement, reproduction, and assists in copulation, but also protects them from bacterial infections and injuries by stimulating rapid regeneration. Over the past decade, several studies have reported antibacterial and antioxidant properties, as well as the wound healing potential of the mucus of some land snails (*Lissachatina fulica* also called *Achatina fulica*, *Archachatina marginata saturalis*, *Archachatina*

marginata ovum and *Achatina achatina* of the family *Achatinidae, Cryptozona bistrialis, Hemiplecta differenta, Eremina desertorum, Helix lucorum and Helix aspersa* (also called *Cornu aspersum*).

To date, studies on AMPs in the mucus of land snails have been scarce and most of them have focused on the land snail *A. fulica* – mainly homologous forms of the antimicrobial peptide mitimacin-AF with a MW of 9.7 kDa and a MW of 11.45 kDa, and peptides comprising 5 to 25 amino acid residues (AARs) with putative anticancer activity.

My main scientific contributions in the presented multidisciplinary research are related to the isolation and characterization of different fractions of the *C. aspersum* mucus, mass spectrometric analyses and interpretation of the obtained results, development of a methodology based on a bioinformatic approach to clarify the relationship between structure and antimicrobial activity, statement and proof of hypotheses. The potential of the peptide fractions from the *C. aspersum* mucus as a source of new antibacterial agents has been revealed, which is of particular importance in the context of increasing antibiotic resistance for the development of alternative therapies.

- For the first time, the antibacterial potential of 5 peptide fractions (with MW < 3 kDa, MW 3-5 kDa, MW 5-10 kDa, MW < 10 kDa, and MW < 20 kDa), obtained by ultrafiltration of the purified mucus extract from of garden snail *C. aspersum*, has been demonstrated against a number of $Gram^+$ and $Gram^-$ bacteria [V1; V5; G12]. It was found that a fraction with MW < 10 kDa showed promising antibacterial activity against *Escherichia coli* NBIMCC 8785 [V1; G12], and the fraction with a MW below 20 kDa demonstrated the broadest spectrum of antibacterial properties against *Bacillus cereus* 1085, *Propionibacterium acnes* 1897, *Salmonella enterica* 8691, *Enterococcus faecalis* 3915, *Enterococcus faecium* 8754 [V5], *Pseudomonas aureofaciens* [V1], as well as against nystatin- and amphotericin-resistant fungal strains [G22].
- The molecular masses of the compounds in the peptide fraction with MW < 20 kDa were identified via mass spectrometric analyses after purification by high-performance liquid chromatography (RP-HPLC) [B5]. Numerous peptides were detected, represented as molecular protonated ions $[M+H]^+$ at m/z below 3 kDa [V1; V5; G13], as well as several peptides with higher molecular masses preliminary between 4-8 kDa [G12; V5] and polypeptides with MW between 10 and 20 kDa [V5]. The identified components with MW between 10 - 20 kDa are in good agreement with previously identified lectins and polypeptides with antibacterial activity in the mucus of the snails *A. fulica*, *Helix pomatia* and *Helix aspersa* [B5]. In addition to the above peptides, the composition of the fraction with MW < 20 kDa also includes secondary metabolites such as free amino acids, allantoin, glycolic acid, the tripeptide glutathione (γGSH) and antioxidant peptides with MW below 1 kDa, proven by mass spectrometric analysis and ^{1}H-NMR spectroscopy [G1].
- The primary structures of over 30 new peptides with MW below 3 kDa have been identified by *de novo* sequencing and interpretation of the results of MALDI-TOF-MS/MS spectra [V1; V5; G12; G13]. Characterization of the peptides by their physicochemical parameters such as molecular mass, isoelectric points (pIs), grand average hydropathicity (GRAVY) and net charge reveals a rich variety of amphipathic cationic, anionic and neutral peptide structures, mostly with hydrophobic surfaces, which is essential for their selectivity and antibacterial activity. The structural characteristics of the peptides show high levels of amino acid residues – glycine (Gly), leucine (Leu), valine (Val), proline (Pro), tryptophan (Trp), lysine (Lys), aspartic acid (Asp), phenylalanine (Phe) and arginine (Arg), characteristic of AMPs [V1; V5].
- It has been shown that the identified peptides in the mucus of *C. aspersum* belong to the AMP family. Alignment of their amino acid sequences with the AMP database - CAMPSing

(http://www.campsign.bicnirrh.res.in/blast.php) and UniProtKB/Swiss-Prot by the BLAST algorithm (https://blast.ncbi.nlm.nih.gov) revealed high homology (over 70%) with other glycine-rich AMPs, such as acanthoscurrin, ctenidin, procambarin, microcin B; with the Gly/Leu-rich antimicrobial peptide leptoglycin; as well as with defensin-like protein, different forms of gallinacin, shepherin and etc. [V1; V5; G12].

- Prediction of the antimicrobial activity of the newly discovered peptides using the iAMPpred software (http://cabgrid.res.in:8080/amppred) [V5; G12; G13] led to the identification of a number of peptide structures with promising (over 70%) prognostic antibacterial and antifungal activity, as well as several structures with antiviral activity, which can serve as a model for the design of new antimicrobial therapeutics [V1; V5; G12].
- Based on the developed methodology for the characterization of peptides from the *C. aspersum* mucus, important, fundamental information was obtained regarding their potential as new antimicrobial agents for biomedical applications [V5].
- The composition, with promising antibacterial activity against *Pseudomonas aureofaciens* AP9, *Brevibacillus laterosporus* BT271 and *Escherichia coli* NBIMCC 878, comprising a peptide fraction with a MW <10 kDa from the *C. aspersum* mucus and carbon nanoparticles, has been protectedby a utility model [G(1)].

I.1.1. *In silico* and physicochemical characterization of the dynamics of cluster formation in solutions of peptides from *C. aspersum* mucus

In recent years, some researchers have proposed the hypothesis that the phenomenon of self-association and aggregation of cell-penetrating peptides is of great importance for their biological activity. Still, the role of oligomeric peptide structures in the activity and selectivity of AMPs has not been widely studied, and the results of experimental and theoretical studies in recent years on this topic are extremely contradictory.

- The rich diversity of cationic, anionic and neutral peptides with a molecular weight below 3 kDa, identified in the *C. aspersum* mucus, is a prerequisite for possible spontaneous interaction between them and the formation of oligomeric structures before the targeted interaction with the bacterial membrane [V4]. Based on the results *of in silico* simulations of molecular dynamics, a hypothesis was proposed for the spontaneous formation of peptide nanostructures (clusters) in the *C. aspersum* mucus [V4].
- The validation of the stated hypothesis was achieved experimentally through the developed methodology, based on UV-Vis spectroscopic and fluorescence studies and in vivo tests of the antibacterial activity of two peptides p1 (KVKDNQWRP) and p3 (LFGGHQGGGLVGGLWRK) and their two-component mixture [V4 (section 3.4.)]. The focus on these peptides is motivated by their cationic nature, the presence of Trp, which is essential for antibacterial activity, and the significant differences in polypeptide chain length and molecular mass, charge distribution, and hydrophobic content.
- Monitoring the dependence of UV-absorption on the concentration of the two peptides and their mixture, studied under identical conditions in the range from 0.25 mg/ml to 10.0 mg/ml, confirms the process of self-association of the peptides in monocomponent and two-component solutions, and shows the threshold concentrations at which the aggregation processes start. The results of the comparative study of the fluorescence emission of p1, p3 and (p1+p3) also confirm the possibility of self-association of the peptides into oligomeric structures, but with different aggregation dynamics. Thus, it has been experimentally confirmed that the peptide mixture (p1+p3) and p3 has a higher

tendency to form aggregates compared to p1, which is consistent with the distribution of charges and hydrophobic amino acid residues in the polypeptide chains [V4].

- It has been shown that the aggregation process is the result of the balance of different interactions between amino acid residues in the peptide conformations, mainly related to hydrophobic effects, electrostatic interactions and π–π interactions of aromatic residues [V4]. The tracking of the conformational changes under identical conditions in a wide pH range (3.0-12.0) showed different dynamics of aggregation and stability of mono- and bi-component solutions [V4].
- Another confirmation of the hypothesis are the results of the antimicrobial tests against *E. coli* 3458 ($Gram^-$) and *Bacillus subtilis* ($Gram^+$) of one- and two-component peptide mixtures. Over 50% inhibition of the growth of *E. coli* 3458 was found by the two-component mixture (p1+p3) at the lowest tested concentration (0.1 mg/ml), which exceeds by 20%, respectively. 75% the inhibition achieved by the individual components (p3, respectively p1). These results are consistent with an additive or even synergistic effect in this concentration range, which is in accordance with previously stated hypotheses [V1; V5; G12].
- Important information has been obtained about the complex relationship between peptide charge distribution, hydrophobic composition, concentration, aggregation patterns and biological activity, as a result of combining different approaches and methods (such as *in silico* studies, spectroscopic analyses and *in vitro* antibacterial tests) [V4]. The results of the experimentally confirmed self-association processes are in full agreement with the patterns and rates predicted by computational modeling. The observed dependences on the composition and concentration of the samples support the concept of critical concentration thresholds and an extremely complex mechanism of biological activity of natural substances [V4].

I.2. Proteins with antimicrobial activity in the mucus and hemolymph of Gastropods

My main scientific contributions in these interdisciplinary studies are related to the development of a methodology for elucidating the protein profile of fractions from the mucus and hemolymph of Gastropods, based on electrophoretic studies interpreted using the ImageQuant™ TL v8.2.0 software, analysis of the results of mass spectrometric studies, summarization of information from various databases on Gastropods and Molluscs, and hypothesis building.

I.2.1. Antibacterial potential of protein fraction from *C. aspersum* mucus

For the first time, information has been obtained about the promising antibacterial potential of a protein fraction with a MW > 20 kDa from the *C. aspersum* mucus against a number of aerobic bacterial pathogens - *Bacillus cereus* 1085, *Propionibacterium acnes* 1897, *Salmonella enterica* 8691, *Enterococcus faecalis* 3915, *Enterococcus faecium* 8754 [V5], *Pseudomonas aureofaciens* and the anaerobic, spore-forming bacterium *Clostridium perfringens* [V1].

- The presented new approach for characterizing proteins in the *C. aspersum* mucus via electrophoretic analysis (SDS-PAGE), interpreted by the ImageQuant™ TL v8.2.0 software, reveals a complex protein profile dominated by proteins with a MW between 30 - 100 kDa [V5]. A hypothesis for the protein composition of the active fraction was built, based on the correspondence with the UniProt database for proteins in Molluscs and Gastropods, as well as in published data on proteins contained in the mucus of other snails - *A. fulica* and *Helix lucorum* [V5].
- New fundamental information was provided about important bioactive proteins and glycoproteins in the composition of the fraction with a MW > 20 kDa from the *C. aspersum* mucus, based on the electrophoretic profile analysed via ImageQuant™ TL v8.2.0 software and proteomic

analysis, including mass spectrometric analyses and bioinformatics [V5]. The identification of proteins in the active fraction with MW > 20 kDa from the *C. aspersum* mucus was achieved by identifying the primary structures of the extracted peptides from each protein band after trypsin digestion and aligning the obtained amino acid sequences (AASs) into the Non-redundant protein sequences (nr) and UniProtKB/Swiss-Prot databases for extracellular proteins in Gastropods using the BLAST algorithm. Most of the AASs demonstrate identity above 60% and E-values between 1×10^{-13} and 1, suggesting a higher probability that the proteins have a common evolutionary origin. In this way, high homology was established with a number of proteins and glycoproteins, such as NADH dehydrogenase [*Albinaria caerulea*]; glutathione S-transferase [*P. canaliculata*]; H-type lectins; mucus protein with an MW of 39.115 kDa [QEG59312 from *C. aspersum*], which is probably homologous with von Willebrand factor A domain-containing protein; functional unit βc-d of *C. aspersum* hemocyanin; L-amino-acid oxidase like protein (as Achacin from *L. fulica*); FMRFamide-activated amiloride-sensitive sodium channel; zinc finger protein; elastin-like protein; several types of collagen (collagen alpha-1, collagen α-4, and collagen alpha-6); and mucins (mucin-5AC-, mucin-5B-, mucin-2-, and mucin-17-like proteins) [V5 (sections 2.2. and 3.)].

Most of the detected proteins can be associated with antimicrobial and antioxidant properties of this protein mucus fraction. Some of the identified proteins, as proteins with L-amino acid oxidase activity (detected at the range 56.94–59.04 kDa) were found for first time in the *C. aspersum* mucus [V5]. Similar proteins with L-AAO activity have so far been found in the mucus of only a few species land snails of the family Achatinidae [V5].

- The hypothesis of synergy between components identified in the composition of the active fraction with MW > 20 kDa (such as the confirmed antimicrobial protein called "Aspernin", functional units of *N*-glycosylated *C. aspersum* hemocyanin, C- and H-lectins, a protein with L-amino acid oxidase activity and several types of mucins) has been put forward, which are responsible for the high antibacterial effect against a wide spectrum of $Gram^+$ and $Gram^-$ pathogens [V5].
- It has been shown that the antibacterial activity of the protein fraction (at concentrations between 32 and 128 μg/ml) is comparable to the antibiotic activity of vancomycin, but without cytotoxic effects on model eukaryotic cells of *Saccharomyces cerevisiae*. Studies related to the cytotoxicity of a fraction with MW > 20 kDa on *S. cerevisiae* revealed not only a lack of cytotoxicity, but an increase in antioxidant capacity and a decrease in intracellular oxidative damage levels, which is associated with the unique composition of this fraction [V5].
- The developed methodology for identifying proteins in the *C. aspersum* mucus can serve as the basis for other studies to develop multitargeted therapeutics from natural sources capable of overcoming the development of antibiotic resistance [V5].

I.2.2. Antibacterial potential of a protein fraction isolated from the hemolymph of marine snail *Rapana venosa*

A fraction with a molecular weight of 50-100 kDa was isolated from the hemolymph of the marine snail *R. venosa*, which showed high antibacterial activity against *E. coli* NBIMCC 8785 [G19]. The main contributions of this study are:

- The molecular masses of 3 major protein types were determined at 93.088 kDa, 62.100 kDa and 50.230 kDa, after using a new approach for characterizing the components in hemolymph fraction of the marine snail *R. venosa*, based on the electrophoretic profile of the fraction, interpreted with ImageQuant™ TL v8.2.0 software. A hypothesis was built for the probable proteins responsible for the antibacterial activity, based on a summary of the data for extracellular proteins in the hemolymph of

Gastropods in the UniProt database [G19 (sections 2.1.)], three of which were confirmed by proteomic analysis [G19 (sections 2.1.); V3].

- It has been shown that a 50% concentration of the fraction with MW 50–100 kDa was able to eliminate 99% of live bacteria *E. coli* NBIMCC 8785, and the use of only 1% concentration of the active fraction leads to reduced metabolic activity and a 24% reduction in bacterial cell size. The Rv 50-100 kDa fraction has been shown to have a more pronounced effect compared to the peptide fraction with MW <10 kDa from *C. aspersum* mucus, which kills up to 60% of *E. coli* NBIMCC 8785 at 50% concentration, but is not effective at 1% concentration.
- It has been hypothesized that the high antibacterial activity of the hemolymph fraction of *R. venosa* is due to the complex interaction of three main types of proteins, homologous to peroxidase-like protein (detected at 93.088 kDa), Aplycyanin A and L-amino acid oxidase (at 62.100 kDa) and functional units with a MW ~50 kDa of *R. venosa* hemocyanin, which demonstrate different mechanisms of bactericidal and/or bacteriostatic action [G19 (sections 3.)].
- The obtained results demonstrate the high antibacterial potential of the Rv 50–100 kDa fraction as a suitable component of therapeutic agents for the treatment of infections caused by *E. coli*, capable of avoiding the development of antibiotic resistance [G19].

I.1.3. Antifungal activity of fractions from the *R. venosa* hemolymph and the *C. aspersum* mucus

The antifungal activity of two fractions of gastropods against fungal strains resistant to nystatin and amphotericin was evaluated for the first time. The results demonstrate a significant fungicidal effect of both fractions against *Aspergillus niger* and *Penicillium griseofulvum*, which correlated with increased levels of oxidative stress biomarkers [G22].

- The peptide fraction (MCa/1-20) from the *C. aspersum* mucus and the protein fraction (HLRv/3-100) from the *R. venosa* hemolymph were obtained by a non-invasive method based on ultrafiltration of purified native extracts. Although the two fractions showed similar antifungal activity, they are completely different in composition [G22].
- A hypothesis for the composition of the components in the active fraction HLRv/3-100 is presented, based on the interpreted electrophoretic profile of HLRv/3-100, using the ImageQuant™ TL v8.2.0 software and the summarized results of the search conducted in the UniProt database for proteins localized extracellularly in Gastropods and Molluscs and previous studies [G22]. The proteins with MW 37.851 kDa are in good agreement with the extracellular protein cathepsin-L endopeptidase and/or cathepsin L-like cysteine proteinase (identified in the hemolymph of several species abalones of the genus Haliotis), which play an important role in the innate immune defense of invertebrate organisms; proteins with MW between 17–30 kDa most likely include C- and H-type lectins and galectins found in the hemolymph of various gastropods, molluscs, and marine arthropods; and proteins at 93.765 kDa, 62.601 kDa, and 49.950 kDa have been identified in previous studies by proteomic analysis [G22 (section 3.1)]. It is likely that the synergy between the bioactive compounds in the hemolymph is responsible for the observed antifungal activity.

Most of the proposed proteins are multifunctional and play an extremely important role in the innate immune system of Gastropods and Molluscs to protect against invading microorganisms, through various mechanisms of action such as phagocytosis, cellular encapsulation, and agglutination phagocytosis, etc. [G22 (section 3.1.)]. A hypothesis was built that synergy between bioactive compounds in the hemolymph is responsible for the observed antifungal activity [G22 (section 3.1.)].

II. Antitumor potential of components from the hemolymph and mucus of Gastropods

- The high potential of different isoforms and some functional units of hemocyanins from *H. lucorum* (HlH), *H. aspersa* (HaH) and *R. venosa* (RvH), as well as fractions of the *R. venosa* hemolymph with MW 10-50 kDa and MW 50-100 kDa and fractions of the *C. aspersum* mucus with MW above 20 kDa and above 50 kDa, against cancer cell lines of diverse origin and different genetic profiles (human colorectal carcinoma cell line HT-29, bladder cancer cell lines (CAL-29 and T-24), breast cancer cell lines, lung cancer A549 and H1299, etc.) has been shown [G2; V2; G14; V3; G18]. It was found that the antiproliferative activity of the tested hemocyanins and fractions is mainly associated with the induction of apoptotic and to a lesser extent late apoptotic or necrotic changes in tumor cells, and in some cases with the induction of autophagy [G2; V2; G14; V3; G18)], which emphasizes their advantages for influencing resistant neoplastic processes.
- New approaches have been applied, including combinations of different methods and techniques to optimize the methods of isolation and purification of native hemocyanins and their isoforms [G2; V2; G14; G18]. The isolated hemocyanins and fractions from mucus and hemolymph have been characterized by electrophoretic and MALDI-TOF/MS analyses.
- It has been hypothesized that the antiproliferative changes observed in tumor cells after treatment with different forms of hemocyanins are primarily related to the specific oligosaccharide structures located on the surface of the functional and structural subunits of hemocyanins [G; G14].
- The antitumor activity of the *C. aspersum* mucus fractions and *R. venosa* hemolymph fractions is likely due to a complex interaction between proteins with different functions that can induce cell death through different mechanisms of action [V3; G18].

II.1. Antitumor potential of components from the *Rapana venosa* hemolymph

Breast cancer is the most common cancer in women and the second most common cancer overall with high morbidity and mortality. The main reasons for this alarming statistic are related to its high molecular heterogeneity with multiple subtypes, leading to significant variations during the course of the disease, high risk of metastasis, recurrence and progression. On the other hand, the use of conventional therapies is very often accompanied by undesirable side effects, ineffective induction of cell death in tumor tissues and development of drug resistance.

For the first time, the antitumor potential of bioactive components from the *R. venosa* hemolymph has been investigated in comparison with cisplatin and tamoxifen against a panel of breast cancer cell lines [V3].

- Promising antitumor activity of biologically active compounds isolated from the *R. venosa* hemolymph – a fraction with MW between 50-100 kDa (HRv 50–100 kDa) and both hemocyanin isoforms (RvH1 and RvH2) – was found against six lines of different molecular subtypes of breast cancer MDA-MB-231, MDA-MB-468, BT-474, BT-549, SK-BR-3 and MCF-7 and one non-cancerous MCF-10A line. It was shown that the HRv 50–100 kDa fraction has the highest antitumor potential, even higher than the hemocyanin isoforms (RvH1 and RvH2), affecting cell viability, cancer cell morphology and autophagy activation [V3].
- Protein glycosylation is of great importance in the development of new anticancer therapies. The glycosylated nature of proteins in the HRv 50–100 kDa fraction has been demonstrated and the higher degree of glycosylation of RvH1 compared to RvH2 has been confirmed by the orcinol/H_2SO_4 screening test [V3].
- Based on the combination of three modern approaches - proteomic analysis of 1D-PAGE, *de novo* sequencing (MALDI-MS/MS) and bioinformatics, proteins in the active fraction of the *R. venosa*

hemolymph have been identified for the first time [V3]. After excision of the protein bands from the gel, followed by decolorization, reduction and alkylation, and trypsin digestion, the extracted peptides were analysed by mass spectrometry. Two approaches were used:

- The first approach involved determining the molecular masses of the peptides extracted from each protein band by MALDI-MS and interpreting them with the Mascot Peptide Mass Fingerprint in the SwissProt database. This approach, however, did not lead to the identification of the proteins, since the information in the Molluscs extracellular protein database is still insufficient [V3].

- Second approach. The identification of the target proteins was achieved by *de novo* sequencing, determination of the primary structure of the extracted peptides from each protein band and comparing AASs with UniProtKB/SwissProt database for Molluscs using the BLAST algorithm. High homology was found with peroxidase-like proteins in the hemolymph of *Lottia gigantea* (at ~97 kDa), Aplysianin-A and L-amino acid oxidase found in *Aplysia kurodai* and *Aplysia califonica* (at ~62 kDa), as well as functional units of hemocyanin at ~50 kDa. Most of the presented hits demonstrate identities above 60% and E-values between 1×10^{-4} and 1, suggesting a higher probability that the sequences share a common evolutionary origin [V3].

- The discovery of a peroxidase-like protein at ~97 kDa, Aplysianin-A and L-amino acid oxidase at ~62 kDa are of fundamental importance, since so far only some functional units of *R. venosa* hemocyanin, actin and proline-rich antimicrobial peptides with MW below 10 kDa have been identified in the *R. venosa* hemolymph. The biological functions of the identified proteins may explain not only the antitumor effect against a panel of human breast cancer cell lines [V3], but also the established high antibacterial activity against *E. coli* NBIMCC 8785 [G19] and the antioxidant properties of this fraction [G21].

- It is hypothesized that the antitumor effect is due to the synergy between *N*-glycosylated proteins in this fraction, with proven high antitumor potential, such as proteins with L-AAO activity (L-amino acid oxidase and Aplysianin-A) and functional units of RvH at ~50 kDa, which can induce cell death via different mechanisms of action [V3]. The observed selective cytotoxicity and the potential involvement of apoptosis and autophagy pathways highlight the therapeutic potential of this fraction in the treatment of breast cancer [V3].

- For the first time, the combination of HRv 50–100 with cisplatin or tamoxifen has been shown to demonstrate significant synergistic and additive effects on all tested cell lines. The combined effect of Rv50–100 kDa fraction and cisplatin has been shown to be three times more effective than treatment with the classical chemotherapeutic agent alone on two cell lines, MDA-MB-231 and BT-549, which have high metastatic potential and are at risk of developing drug resistance [V3].

- The used approaches reveal new perspectives for the application of natural bioactive compounds as antitumor agents, used alone used alone or as a booster in combination with different chemotherapies [V3].

II.2. Study of the antitumor potential of hemocyanins from *Rapana venosa* and *Helix lucorum* on the human bladder carcinoma T-24 cell line

The high molecular heterogeneity of bladder tumors is responsible for significant variations during the course of the disease, as well as for the high rates of relapse and progression, which makes it difficult to introduce new therapeutic agents. The discovery of selective and more effective therapeutics for this disease is one of the greatest challenges for pharmacology and oncology. Some hemocyanins

from Gastropods have significant immunological and selective antitumor potential, which is a prerequisite for their application in new drug development.

- The antitumor activity of different forms of *R. venosa* and *H. lucorum* hemocyanins was studied in comparison with KLH and doxorubicin. A high selective cytotoxic effect of the structural subunit RvHII and two functional units βc-HlH-h and RvHII-e on the proliferation of human bladder carcinoma T-24 cell line compared with the normal urothelial cell line HL 10/29 was established [V2].
- It has been shown that treatment with the *N*-glycosylated functional unit βc-HlH-h of *H. lucorum* hemocyanin demonstrates the highest antiproliferative effect (similar to doxorubicin) and leads primarily to apoptotic and to a lesser extent to late apoptotic or necrotic changes in tumor cells [V2]. It has been hypothesized that specific glycan epitopes of *N*-oligosaccharide structures located on the surface of βc-HlH-h are responsible for the observed antitumor effect [V2].

III. Mechanism of action of snail biocomponents established by proteomic analysis on 2-dimensional gel electrophoresis (2D-PAGE)

III. 1. Study of a mechanism of action of hemocyanin βc-HlH-h on human bladder carcinoma T-24 cell line by proteomic analysis

- The mechanism of antiproliferative action of functional unit of hemocyanin βc-HlH-h on the human bladder carcinoma T-24 cell line was investigated for the first time by proteomic analysis [V2].
- Based on a comprehensive approach including proteomic analysis of 2D-PAGE and bioinformatics, 40 proteins were identified in the T-24 cell line of bladder carcinoma that changed their expression after treatment with βc-HlH-h [V2]. Most of them play an important role in the glycolytic pathway, lysosomal and proteasome degradation pathways, as well as proteins that regulate cytoskeletal and extracellular matrix [V2].
- Protein identification was achieved based on MALDI-TOF/MS analyses of peptides (extracted from the protein spots after trypsin digestion) and interpreted with Mascot Peptide Mass Fingerprint (http://www.matrixscience.com/). Nine proteins associated with the induction of apoptosis of tumor cells were confirmed by their AASs (identified by MALDI-TOF/MS/MS analyses) [V2].
- Downregulation of a number of key proteins for the proliferation of the T-24 bladder urothelial carcinoma cell line has been shown, such as heat shock protein heat Hsp27, glyceraldehyde-3-phosphate dehydrogenase (G3P), annexin A1 (ANXA1), triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase M2 (PKM2), etc. [V2]. The inhibition of anti-apoptotic heat shock proteins, including HSP27, as well as GAPDH and PKM2 (related to energy metabolism and signalling pathways in cancer metabolism) has been shown as a novel strategy for bladder cancer therapy. The change in TPI levels in urine or tissues can be used as a marker to monitor the progression of the disease [V2].
- The results shown confirm and complement published data on the significant pro-apoptotic activity of molluscan hemocyanins against various tumor cell lines, which is a prerequisite for their potential application in anticancer therapy [V2].

III. 2. Study of the mechanism of action of *C. aspersum* mucus extract on an animal model of scopolamine-induced Alzheimer's-type dementia

In the search for new therapeutic strategies to reduce symptoms and slow the progression of neurodegenerative diseases, a multi-target approach plays a leading role. The basis of this approach is

the concept of using substances that affect different molecular structures as pharmacological targets. Natural components from various sources are the main source of such multifunctional agents.

For the first time, information has been provided on the capacity of a standardized extract of the *C. aspersum* mucus to improve memory and cognitive abilities in scopolamine-induced Alzheimer's-type dementia in rats [G16]. The mucus extract has been found to exhibit moderate antioxidant properties and modulate the content of monoamines in brain structures related to memory [G16]. It has been shown that some secondary metabolites, osmolites, betaine, choline, allantoin, glutathione and some peptides and proteins related to the antioxidant and antimicrobial properties of mucus are responsible for the established effect. The composition with a beneficial effect on Alzheimer's-type dementia, comprising an extract of the mucus of the garden snail as the main component, has been protected by a utility model [G(2)].

- For the first time, the mechanism of the preventive effect of a standardized mucus extract from *C. aspersum* snail (SE), enriched in a fraction wit MW above 20 kDa, on Alzheimer's-type dementia in rats was investigated by proteomic analysis on 2D-gel electrophoresis (2D-PAGE). The protein expression in the cortex homogenate of the two groups of animals was compared: the first treated intraperitoneally with scopolamine (Sco, 2 mg/kg, 11 days), and the second group (Sco + SE), also treated intraperitoneally with Sco (Sco, 2 mg/kg) and protected with snail mucus extract (0.5 ml/100 g body weight), administered daily orally for 11 days [V6].
- New information was obtained on the changes in protein expression in the rat cortex based on 2D-PAGE, analysed by Melanie^{TM} Coverage 9 software [V6]. A good agreement was found between the experimentally determined pI and MW of proteins on 2D-PAGE and the information in the UniProt database for rat brain proteins [V6].
- Identification a number of dysregulated proteins was achieved by peptide mass fingerprinting based on MALDI-TOF/MS analyses of peptides (extracted from the gel after trypsin digestion of the excised protein spots), compared by MASCOT software with theoretical molecular masses generated in the Mascot database-Fingerprint (http://www.matrixscience.com/). Eight key proteins were further confirmed by amino acid sequences determined by MALDI-MS/MS analysis, after comparison with the Swiss-Prot proteins database for rats [V6].
- Using this comprehensive approach, a number of proteins related to memory and cognitive functions have been identified. Summary information has been obtained on the biological functions (structural, antioxidant, metabolic, signaling, and etc.) of the proteins identified in rat cortex homogenate and their involvement in various aspects of Alzheimer's disease progression [V6].
- Based on proteomic analysis, a significant change in protein expression of Ubiquitin carboxyl-terminal hydrolase isoenzyme L1, Calbindin, Vacuolar ATP synthase subunit A, Tropomyosin, 14-3-3 zeta/delta, Kinesin and Stathmin was shown for the first time in the cortex of rats preventively treated with snail mucus extract compared to demented animals treated only by Sco, that outlines these proteins as potential therapeutic targets for Alzheimer's-type dementia treatment [V6].

IV. Characterization of other bioactive components from natural sources

IV.1. Antioxidant properties of peptides and secondary metabolites from the hemolymph of the garden snail *H. lucorum*

- The primary structures of 17 novel peptides in the fraction with MW below 1 kDa from the hemolymph of the garden snail *H. lucorum* with potential antioxidant activity were determined based on *de novo* sequencing [G4; G9]. Characterization of the peptides revealed predominantly cationic

amphipathic structures with hydrophobic surfaces, which differ significantly from the peptides with potential antioxidant properties found in the *C. aspersum* mucus [G7; G1; G15].

- The established antioxidant potential of the fraction with MW below 1 kDa, from the *H. lucorum* hemolymph, is mainly due to peptides with low molecular weight and specific amino acid sequences, primarily including Leu, Val, Phe, His, Pro, Lys and Tyr residues. The results obtained confirm that the higher proportion of hydrophobic AARs compared to hydrophilic ones is a key factor for the high ability to capture hydroxyl radicals in the fraction with MW below 1 kDa of the *H. lucorum* hemolymph [G4; G9].

IV.2. Isolation and characterization of cyclolipopeptides from *Bacillus velezensis* R22

My main contributions are related to:

- A methodology has been developed for isolation, purification and characterization of cyclopeptides from cell-free supernatants of *B. velezensis* R22. Using mass spectrometric analyses (LC-MS and LC-MS/MS) on a UHPLC-Q-TOF system, the main active compounds in the cell-free extract from *B. velezensis* R22 were determined, such as surfactins with molecular masses of 1035.698 Da and 1057.7 Da, and fengycins with molecular masses of 1462.8 Da, 1476.8 Da, 1490.8 Da and 1504.8 Da, and their primary structures were characterized [G21].

Research prospects in the next 5 years

My research work will continue in the above-described directions, and will be expanded with new objects and development of new approaches for the isolation and characterization of proteins, glycoproteins and AMPs. The prospects for my research work are closely related to my participation in the following projects:

1. Project funded by BNSF: KP-06-N-81/6 from 04.12.2024: "New biocomponents and biogenic nanoparticles from the hemolymph of the sea snail Rapana venosa against skin cancer".

2. Project funded by BNSF: KP-06-N-61/8 from 2022: "Development of new biological nanoparticles from the mucus of the garden snail Cornu aspersum, as antimicrobial agents".

3. Project funded by RRP: BG-RRP-2.017-0009-C01: "Obtaining a biofungicidal preparation from waste biomass: biotechnology for sustainable organic agriculture" (under procedure BG-RRP-2.017: Funding of scientific research projects in the field of green and digital technologies - 2).

4. Project funded by RRP: BG-RRP-2.017-0006-C01: "Environmental technology for conversion of waste biomass to an innovative product (activated carbon) with wide application (EcoTechProduct)" (under procedure BG-RRP-2.017: Financing of scientific research projects in the field of green and digital technologies - 2).

5. Project BG05M2OP001-1.002-0019-C02, Center of Competence "Clean technologies for a sustainable environment - water, waste, energy for a circular economy" (Second stage 2025-2029).

The main focuses of the work will be directed towards:

I. Research of bioactive compounds from natural sources, as new natural therapeutics with antimicrobial and antitumor activity:

- Isolation, identification and characterization of biocomponents - secondary metabolites, peptides, proteins and glycoproteins in the hemolymph of Gastropods using modern methods and

techniques, including chromatographic and electrophoretic analyses, preparation of a proteome map and mass spectrometric studies.

II. Research of the possibilities for developing new antimicrobial and antitumor agents based on biomolecules of natural origin in combination with nanotechnologies.

- Green synthesis and characterization of biogenic nanoparticles with antitumor properties, mediated by biocomponents in the hemolymph of the sea snail R. venosa.

III. Investigation of the mechanism of antitumor and antimicrobial action of various biocomponents and biogenic nanoparticles by proteomic analysis of 1D- or 2D- electrophoresis, followed by mass spectrometric analyses and bioinformatics. Identification of key proteins as potential therapeutic targets.

IV. Development of a methodology for purification and mass spectrometric identification of lipopeptides produced from *Bacillus* spp. for the needs of organic agriculture.

V. Development of a methodology for the determination of pesticides or traces of pesticides in environmental samples.

List of publications participating in the competition for the academic position of "Professor"on indicator "V":

V1.	Dolashki, A., **Velkova, L.*,** Daskalova, E, Zheleva, N., Topalova, Y., Atanasov, V., Voelter, W., Dolashka, P. Antimicrobial Activities of Different Fractions from Mucus of the Garden Snail *Cornu aspersum*. *Biomedicine*s **2020**, *8*(9), 315-332. https://doi.org/10.3390/biomedicines8090315	IF 4.717	Q1
V2.	Dolashki, A., Antonova O., **Velkova L.*,** Kaynarov D., Voelter W., Dolashka P. Selective cytotoxicity and changes in protein expression of T24 bladder carcinoma permanent cell line after treatment with hemocyanins. *Current Medicinal Chemistry* **2022**, *29*(42), 6479-6498. https://www.eurekaselect.com/article/125674	IF 4.46	Q1
V3.	Petrova, M., Vlahova, Z., Schröder, M., Todorova, J., Tzintzarov, A., Gospodinov, A., **Velkova, L.*,** Kaynarov, D., Dolashki, A., Dolashka, P., Ugrinova, I. Antitumor Activity of Bioactive Compounds from *Rapana venosa* against Human Breast Cell Lines. *Pharmaceuticals* **2023**, *16*(2), 181. https://doi.org/10.3390/ph16020181	IF 4.67	Q1
V4.	Kaynarov D., Marinova, K. Marinova, R., Petkov, P., **Velkova, L.*,** Dolashki, A., Petrov, P., Litov, L., Lilkova, E., Dolashka, P., Ilieva, N. In silico and physico-chemical characterization of cluster formation dynamics in peptide solutions. *Biochemistry and Biophysics Reports* **2024**, *39*, 101753. https://doi.org/10.1016/j.bbrep.2024.101753	IF 2.79	Q2
V5.	**Velkova L.*,** Dolashki A, Petrova V, Pisareva E, Kaynarov D, Kermedchiev M, Todorova M, Dolashka P. Antibacterial Properties of Peptide and Protein Fractions from Cornu aspersum Mucus. *Molecules* **2024**, *29(*12), 2886. https://doi.org/10.3390/molecules29122886	IF 4.79	Q1
V6.	Atanasov, V., **Velkova, L.*,** Tancheva, L., Dolashki, A., Kalfin, R., Dolashka, P. Key proteins in rat cerebral cortex: application of *Cornu aspersum* extract as neuroprotective agent in Alzheimer's type dementia. *Molecules* **2024**, *29*(22), 5375. https://doi.org/10.3390/molecules29225375	IF 4.2	Q1

List of publications participating in the competition for the academic position of "Professor" on indicator "G":

G1.	Vassilev, N. G., Simova, S., Dangalov, M., **Velkova, L.**, Atanasov, V., Dolashki, A., Dolashka, P. An ^{1}H NMR and MS based study of metabolomics profiling of garden snail *Helix aspersa* Mucus. *Metabolites* **2020**, *10*(9), 360-374. https://doi.org/10.3390/metabo10090360	IF 4.097	Q2
G2.	Georgieva, A., Todorova, K., Iliev, I., Dilcheva, V., Vladov, I., Petkova, S., Toshkova, R., **Velkova, L.**, Dolashki, A., Dolashka, P. Hemocyanins from *Helix* and *Rapana* snails exhibit *in vitro* antitumor effects in human colorectal adenocarcinoma. *Biomedicines* **2020**, *8*(7), 194-207. https://doi.org/10.3390/biomedicines8070194	IF 4.717	Q1
G3.	Ilieva, N., Petkov, P., Lilkova, E., Lazarova, T., Dolashki, A., **Velkova, L.**, Dolashka, P., Litov, L. In Silico Study on the Structure of Novel Natural Bioactive Peptides. *Lecture Notes in Computer Science (LNCS), Springer Verlag* **2020,** *11958*, 332-339. https://link.springer.com/chapter/10.1007/978-3-030-41032-2_38	IF 1.053	Q2
G4.	Alexandrova, A., Petrov, L., **Velkova, L.**, Dolashki, A.**,** Tsvetanova, E., Georgieva, A., Dolashka, P**.** Antioxidant activity of fractions isolated from hemolymph of garden snail *Helix lucorum*. *Journal of Pharmacy & Pharmacognosy Research* **2021**, *9*(2), 143-152. https://jppres.com/jppres/pdf/vol9/jppres20.935_9.2.143.pdf	IF 1.37	Q2
G5.	Idakieva, K., Todinova**,** S., Dolashki, A., **Velkova, L.**, Raynova, Y., Dolashka, P**.** Biophysical characterization of the structural stability of *Helix lucorum* hemocyanin. *Biotechnology & Biotechnological Equipment* **2021**, *35*(1), 18-28. https://doi.org/10.1080/13102818.2020.1837010	IF 1.762	Q3
G6.	Daskalova, A**.,** Petrova, V., **Velkova, L.**, Kujumdzieva, A., Tomova, A., Voelter, W., Dolashka, P. Investigation of protein expression of *Saccharomyces cerevisiae* cells in quiescent and proliferating state before and after toxic stress. *Biotechnology & Biotechnological Equipment* **2021**, *35*(1), 366-376. https://doi.org/10.1080/13102818.2021.1879677	IF 1.762	Q3
G7.	Daskalova, A.V.**,** Tomova, A.A., Kujumdzieva, A.V., **Velkova, L.G.**, Dolashka, P. A.**,** Petrova, V.Y. Menadione and hydrogen peroxide trigger specific alterations in RNA polymerases profiles in quiescent Saccharomyces cerevisiae cells. *Biotechnology and Biotechnological Equipment* **2021**, *35*(1), 1190-1199. https://doi.org/10.1080/13102818.2021.1941255	IF 1.762	Q3
G8.	**Velkova, L.**, Daskalova, A**.,** Dolashki, A., Dolashka, P**.,** Vassilev, T. Immunomodulating potential of IgG antibodies with induced polyspecificity. *Comptes Rendus de L'Academie Bulgare des Sciences* **2021**, *74*(10), 1488-1492. http://www.proceedings.bas.bg/DOI/doi2021_a_08.html	IF 0.378	Q3
G9	Vassilev, N**.,** Simova, S., Dangalov, M., **Velkova, L.***, Atanassov, V., Dolashki, A., Dolashka, P. An 1H NMR and MS Based Study of Metabolomics Profiling of Garden Snail *Helix lucorum* Hemolymph. *Bulgarian Chemical Communications* **2021**, *53*A, 49-56. http://www.bcc.bas.bg/BCC_Volumes/Volume_53_Special_A_2021/BCC2021-53-SI-A-049-056.pdf	IF 0.398	Q4
G10.	Aleksova, M., **Velkova, L.**, Dolashka, P**.,** Radeva, G. Antibacterial activity of bioactive fractions from mucus and hemolymph of different snails species and crab. *Bulgarian Chemical Communications* **2021**, *53*A, 022-026. http://bcc.bas.bg/BCC_Volumes/Volume_53_Special_A_2021/BCC2021-53-SI-A-022-026.pdf	IF 0.98	Q4

G11. Krumova, E., Dolashka, P**.**, Abrashev, R., **Velkova, L.**, Dolashki, A., Daskalova, A**.**, Dishliyska, V., Atanasov, V., Kaynarov, D**.**, Angelova, M. Antifungal activity of separated fractions from the hemolymph of marine snail *Rapana venosa*. *Bulgarian Chemical Communications* **2021**, *53*A, 042-048. http://www.bcc.bas.bg/BCC_Volumes/Volume_53_Special_A_2021/BCC2021-53-SI-A-042-048.pdf — IF 0.398 — Q4

G12. Topalova, Y., Belouhova, M., **Velkova, L.**, Dolashki, A., Zheleva, N., Daskalova, E., Kaynarov, D**.,** Dolashka, P. Effect and mechanisms of antibacterial peptide fraction from mucus of *Cornu aspersum* against *Escherichia coli* NBIMCC 8785. *Biomedicines* **2022**, *10*(3), 672. https://doi.org/10.3390/biomedicines10030672 — IF 4.65 — Q1

G13. Belouhova, M., Daskalova, E., Yotinov, I., Topalova, Y., **Velkova, L.**, Dolashki, A., Dolashka, P. Microbial diversity of garden snail mucus. *MicrobiologyOpen* **2022**, *11*(1), e1263.https://doi.org/10.1002/mbo3.1263 — IF 3.904 — Q2

G14. Nikolova, M., Konstantinov, S., **Velkova, L.**, Kaynarov, D**.**, Dolashki, A., Dolashka, P. Antitumour activity of different bioactive compounds from hemolymph and mucus of mollusca against human urinary bladder cancer cell lines. *Comptes Rendus de L'Academie Bulgare des Sciences* **2022**, *75*(5), 726-736. https://doi.org/10.7546/CRABS.2022.05.13 — IF 0.373 — Q3;

G15. Petrov, L., Kachaunov, M., Alexandrova, A., Tsvetanova, E., Georgieva, A., Dolashki, A., **Velkova, L.,** Dolashka, P. Snail Mucus Protective Effect on Ethanol-Induced Gastric Ulcers in Mice. *Life (MDPI)* **2022**, *12*(8), 1106. https://doi.org/10.3390/life12081106 — IF 3.269 — Q2

G16. Tancheva, L., Lazarova, M., **Velkova, L.**, Dolashki, A., Uzunova, D., Minchev, B., Petkova-Kirova, P., Hassanova, Y., Gavrilova, P., Tasheva, K., Taseva, T., Hodzhev, Y., Atanasov, A. G., Stefanova, M., Alexandrova, A., Tzvetanova, E., Atanasov, V**.**, Kalfin, R., Dolashka, P**.** Beneficial Effects of Snail *Helix aspersa* Extract in an Experimental Model of Alzheimer's Type Dementia. *Journal of Alzheimer's Disease* **2022**, *88*(1), 155-175. https://journals.sagepub.com/doi/full/10.3233/JAD-215693 — IF 4.819 — Q1

G17. Daskalova, E., Zheleva, N., Belouhova M., Topalova Y., **Velkova, L.,** Dolashki, A., Dolashka P. Antibacterial activity of combined nanodiamonds and snail fractions with biocompounds with mw below 10 kDa and above 30 kDa. *Comptes Rendus de L'Academie Bulgare des Sciences* **2023**, *76*(1), 35–43. https://doi.org/10.7546/CRABS.2023.01.04 — IF 0.3 — Q3

G18. Petrova, M., Vlahova, Z., Schröder, M., Tzintzarov, A., **Velkova L.,** Kaynarov, D., Dolashki, A., Dolashka, P., Ugrinova, I. Anti-tumour activity of bioactive compounds isolated from the hemolymph and mucus of the garden snail *Helix aspersa* against a panel of human cancer cell lines. *Comptes Rendus de L'Academie Bulgare des Sciences* **2023**, *76*(9), 1350–1359. https://doi.org/10.7546/CRABS.2023.09.05 — IF 0.3 — Q3

G19. Kirilova M, Topalova Y, **Velkova L.*,** Dolashki A, Kaynarov D, Daskalova E, Zheleva N. Antibacterial Action of Protein Fraction Isolated from *Rapana venosa* Hemolymph against *Escherichia coli* NBIMCC 8785. *Pharmaceuticals (Basel)* **2024**, *17*(1), 68. https://doi.org/10.3390/ph17010068 — IF 4.3 — Q1

G20. Armenova, N., Petrova, P., Gerginova, M., Krumova, E., Kaynarov, D., **Velkova, L**., Dolashka, P., Petrov, K. *Bacillus velezensis* R22 inhibits the growth of multiple fungal phytopathogens by producing surfactin and four fengycin homologues. *Biotechnology and Biotechnological Equipment* **2024**, *38*, art.no 2313072. https://doi.org/10.1 080/13102818.2024.2313072 — IF 1.5 — Q3

G21. Alexandrova, A., Petrov, L., Tsvetanova, E., Georgieva, A., **Velkova, L.**, Atanasov, V., Dolashki, A., Dolashka, P., Mileva, M. Isolation, identification and redox-modulation capacity of hemolymph's subunits from *Rapana venosa* inhabiting the Bulgarian Black sea. *Farmacia* **2024**, *72*(4), 917-923. https://doi.org/10.31925/farmacia.2024.4.20 — IF 1.2 — Q2

G22. **Velkova, L.**, Abrashev, R., Miteva-Staleva, J., Dishliyska, V., Dolashki, A., Spasova, B., Dolashka, P., Angelova, M., Krumova, E. The Role of Oxidative Stress in Antifungal Activity of Two Mollusc Fractions on Resistant Fungal Strains. *Int. J. Mol. Sci*. **2025**, *26*(3), 985. https://doi.org/10.3390/ijms26030985 — IF 4.9 — Q1

List of patents and utility models participating in the competition for the academic position of "professor":

G(1). Invention Utility model "Composition with antibacterial action", Application number №5317 of 07.06.2021, Protection number №4101, date of issue 11.08.2021 (Inventors: Pavlinka Aleksandrova DOLASHKA, Alexander Konstantinov DOLASHKI, **Lyudmila Georgieva VELKOVA**), applicant: Institute of Organic Chemistry with Center for Phytochemistry - BAS. Term of protection 07.06.2025, Status - Active PM. https://portal.bpo.bg/bpo_online/-/bpo/utility-model-detail

G(2). Invention Utility model "A means for beneficially influencing Alzheimer's type dementia", Application number №5699 from 15.03.2023, Protection number 4426, date of issue/registration 26.04.2023 (Inventors: Pavlinka Aleksandrova DOLASHKA, **Lyudmila Georgieva VELKOVA**, Alexander Konstantinov DOLASHKI, Lyubka Pavlova Tancheva, Reni Emil Kalfin), applicants: Institute of Organic Chemistry with the Center for Phytochemistry - BAS, Institute of Neurobiology - BAS). Term of protection - 15.03.2027, Status - Active PM. https://portal.bpo.bg/bpo_online/-/bpo/utility-model-detail

Information on the distribution of the scientific works of Assoc. Prof. Dr. L. Velkova, with which she is applying in this competition:

Total number of publications: 28 with impact factor (Total impact factor – 64.853).

Distribution by quartiles according to SJR (www.scimagojr.com) as follows:

Q1 10 pcs.
Q2 7 pcs.
Q3 8 pcs.
Q4 3 pcs.

Utility models: 2.

Corresponding author and/or first author in 10 publications.