

# NATIONAL CONFERENCE

## "INNOVATIVE LOW TOXIC BIOLOGICALLY ACTIVE AGENTS FOR PRECISION MEDICINE

### National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)"

#### ORGANISING COMMITTEE :

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**Leading organization:** Bulgarian Academy of Sciences

**Partner organizations::** IOHCF-BAS, IMoIB-BAS, IMiKB-BAS, INB-BAS, IEMPAM-BAS, IP-BAS, IIKT-BAS, MU-Sofia, MU-Plovdiv, SU-BF, NSA.



# CONFERENCE PROGRAMME

**11.05.2021**

12:00- 17:00	<b>REGISTRATION</b>
17:00- 17:30	Welcome and Conference Opening Remarks
17:30- 18:00	National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” – Prof. Olya Stoiliva
18:00- 21:00	<b>DINNER</b>

**12.05.2021**

## SESSION : NEW BIOLOGICALLY ACTIVE COMPOUNDS FROM NATURAL ORIGIN WITH ANTI-NEURODEGENERATIVE AND ANTI-TUMOR EFFECT

9:00- 9:30	<b>REGISTRATION</b>
9:30- 10:00	IDENTIFICATION OF PEPTIDES IN THE HEMOLYMPH OF GARDEN SNAIL <i>CORNU ASPERSUM</i> - <b>Aleksandar Dolashki</b> , Lyudmila Velkova, Pavlina Dolashka
10:00- 10:30	PROBING THE MEMBRANE-DESTRUCTION POTENTIAL OF THE NEWLY IDENTIFIED PEPTIDE ASPERSIN DK10 (DLTLNGLSPK) - <b>Nevena Ilieva</b> , Peicho Petkov, Elena Lilkova, Leandar Litov
10:30- 11:00	STUDY OF METABOLITES PROFILING OF <i>HELIX LUCORUM</i> HEMOLYMPH USING $^1H$ NMR AND MASS SPECTROMETRY - <b>Nikolay Vassilev</b> , Svetlana Simova, Miroslav Dangalov, Lyudmila Velkova, V. Atanasov, Aleksandar Dolashki, Pavlina Dolashka
11:00- 11:30	<b>COFFEE BREAK</b>
11:30- 12:30	A POSSIBLE SYNERGISTIC CYTOTOXIC EFFECT BETWEEN CLASSICAL CHEMOTHERAPEUTIC AGENTS AND COMPOUNDS ISOLATED FROM THE SNAIL <i>RAPANA VENOSA</i> ON BREAST CANCER CELL LINES - <b>Alexander Tzintzarov</b> , Alexander Dushkov, Zlatina Vlahova, Lazar Lazarov, Iva Ugrinova
12:30- 13:00	EFFECT OF CANNABIDIOL ON CELL LINES H1299 AND A549 - <b>Lazar Lazarov</b> , Zlatina Vlahova, Iva Ugrinova
13:00- 14:00	<b>LUNCH</b>
14:00- 14:30	<i>IN VITRO</i> ANTINEOPLASTIC ACTIVITY OF HEMOCYANINS - <b>Maria Nikolova</b> , Pavlina Dolashka, Teodora Atanasova, Spiro Konstantinov
14:30- 15:00	AMELIORATING EFFECT OF FRESH EXTRACT OF SNAIL ( <i>HELIX ASPERSA</i> ) IN EXPERIMENTAL MODEL OF ALZHEIMER’S TYPE DEMENTIA - <b>Reni Kalfin</b> , Lyubka Tancheva, Maria Lazarova, Alexander Dolashki, Lyudmila Velkova, Miroslava Stefanova, t Diamara Uzunova, Technician Petya Gavrilova, Elina Tsvetanova, Albena Alexandrova, Pavlina Dolashka

15:00- 15:30	EXTRACT OF SNAIL ( <i>HELIX ASPERSA</i> ) AND ITS NEUTOPROTECTIVE EFFECTS IN 6-OHDA EXPERIMENTAL MODEL OF PARKINSON'S DISEASE - <b>Maria Lazarova</b> , Lyubka Tancheva, Pavlina Dolashka, Alexander Dolashki, Lyudmila Velkova, Albena Alexandrova, Elina Tsvetanova, Reni Kalfin
15:30- 16:00	SAFETY TESTING AND ANTIPROLIFERATIVE ACTIVITY OF HEMOCYANINS FROM <i>HELIX LUCORUM</i> , <i>HELIX ASPERSA</i> AND <i>RAPANA VENOSA</i> - Inna Sulikovska, Ivan Iliev, Katerina Todorova, Ani Georgieva, Valeriya Dilcheva, Ivelin Vladov, Svetlozara Petkova, Reneta Toshkova and Pavlina Dolashka
16:00- 16:30	ANTIVIRAL EFFECTS OF FRACTIONS AND TOTAL EXTRACTS OBTAINED FROM MEDICINAL PLANTS FROM BULGARIAN POPULATIONS - Venelin Tsvetkov, Daniel Todorov, Anton Hinkov, Kalina Shishkova, Stoyan Shishkov, Nadezhda Kostova, Tsvetelina Doncheva Hristo Petkov, Kalina Alipieva, Aleksandar Shkondrov, Ilina Krasteva,
16:30-17:00	NEUROPROTECTIVE PROPERTIES OF SNAIL EXTRACTS FROM <i>HELIX ASPERSA</i> ON SCOPOLAMINE RAT MODEL OF ALZHEIMER'S DISEASE <b>Ventseslav Atanasov</b> , Lyudmila Velkova, Lyubka Tancheva, Aleksander Dolashki, Reni Kalfin, Pavlina Dolashka
17:00-18:00	<b>POSTER SESSION</b>
	PROTECTIVE EFFECT OF MICROPOROUS NATURAL CLINOPTILOLITE ON SUBCHRONIC HEAVY METAL INTOXICATION IN RATS - Julia Karaivanova, Diamara Uzunova, Yozlem Hassanova, Petja Gavrilova, Maria Lazarova, Polina Kirova, Andrej Popatanasov, Albena Alexandrova, Elina Tzvetanova, Miroslava Stefanova, Ayten Solak, Reni Kalfin, Lyubka Tancheva
	POTENTIAL APPLICATION IN MEDICINE OF BIOACTIVE COMPOUNDS FROM MARINE SNAIL <i>RAPANA VENOSA</i> - Lyudmila Velkova, Spiro Konstantinov, Aleksandar Dolashki, Ventseslav Aatanasov, Pavlina Dolashka
	BIOACTIVE COMPOUNDS FROM <i>RAPANA VENOSA</i> AND <i>HELIX ASPERSA</i> WITH ANTITUMOR PROPERTIES AGAINST TWO CANCER CELL LINES Ventseslav Aatanasov, Maria Petrova , Jordana Todorova , Maria Schröder , Aleksandar Dolashki , Lyudmila Velkova , Iva Ugrinova, Pavlina Dolashka.
<b>13.05.2021</b>	
<b>SESSION : ANTIMICROBIAL ACTIVITY AND MECHANISM OF ACTION OF ACTIVE PEPTIDES, GLYCOPEPTIDES AND PROTEINS</b>	
9:00- 9:30	<i>IN VITRO</i> ANTIBACTERIAL EFFECT OF <i>OREGANUM VULGARE</i> OILS WITH POTENTIAL SKIN APPLICATION - Lyudmila Dimitrova, Maya M. Zaharieva, Krassimira Yoncheva, Alexander Kroumov, Vesselin Kussovski, Iva Tsvetkova, Niko Benbassat, Ivanka Spassova, Daniela Kovacheva, <b>Hristo Najdenski</b>
9:30- 10:00	<i>IN VITRO</i> ANTIFUNGAL ACTIVITY OF <i>RAPANA VENOSA</i> HEMOLYMPH AGAINST STRAINS FROM GENERA <i>ASPERGILLUS</i> AND <i>PENICILLIUM</i> Ekaterina Krumova, Pavlina Dolashka, Nedelina Kostadinova, Radoslav Abrashev, Jeny Miteva-Staleva, Aleksander Dolashki, Lyudmila Velkova, Asya Daskalova, Vladislava Dishlijska, Boryana Spasova, Maria Angelova

10:00- 10:30	EFFECT OF TRANSIENT TEMPERATURE DOWNSHIFT ON OXIDATIVE CELL RESPONSE OF PENICILLIUM GRISEOFULVUM ISOLATED FROM ANTARCTICA <u>Ekaterina Krumova</u> , Radoslav Abrashev, Galina Stoyancheva, Nedelina Kostadinova, Jeny Miteva-Staleva, Vladislava Dishliyska, Boryana Spasova, Maria Angelova
10:30- 11:00	CROSSLINKED COPOLYMERS STRUCTURES BASED ON POLY(N-ISOPROPYLACRYLAMIDE) AS POTENTIAL CARRIERS FOR BIOLOGICALLY ACTIVE SUBSTANCES - <b>Darinka Christova</b> , Sijka Ivanova
11:00- 11:30	<b>COFFEE BREAK</b>
11:30- 12:00	<i>PSEUDOMONAS SP.</i> IN THE SNAIL MUCUS - A CARD-FISH STUDY - <b>Mihaela Belouhova</b> , Elmira Daskalova, Yana Topalova, Pavlina Dolashka,
12:30- 13:00	SHORT-TERM METABOLIC ACTIVITY INHIBITION OF E. COLI IN THE PRESENCE OF THREE PEPTIDE FRACTIONS FROM SNAIL MUCUS - <b>Mihaela Belouhova</b> , Elmira Daskalova, Yana Topalova, Pavlina Dolashka,
13:00- 14:30	<b>LUNCH</b>
14:30- 15:00	MODULATION OF NEUTROPHIL FUNCTIONS WITH SIRTUIN 1 ACTIVATOR IN ARTHRITIS - Milena Leseva, Kalin Stoyanov, Lora Simeonova, Luciano Saso <sup>3</sup> , <b>Petya Dimitrova</b>
15:00- 15:30	SNAIL MUCUS PROTECTIVE EFFECT ON ETHANOL INDUCED GASTRIC ULCERS IN MICE – <b>Lubomir Petrov</b> , Mihail Kachaunov, Albena Alexandrova, Elina Tsvetanova, Almira Georgieva, Pavlina Dolashka, Aleksandar Dolashki, Lyudmila Velkova
	<b>POSTER SESSION</b>
16:00-18:00	NEW ADVANCES IN ANALYSIS OF NATURAL EXTRACTS FROM MOLLUSCS - Dimitar Kaynarov, Asya Daskalova, Ventseslav Atanasov, Lyudmila Velkova, Aleksandar Dolashki, Pavlina Dolashka
	IN THE SEARCH FOR ANTIFUNGAL ACTIVITY IN HEMOLYMPH ISOLATED FROM MOLLUSCS AND ARTHROPODS - Ekaterina Krumova , Pavlina Dolashka, Nedelina Kostadinova, Radoslav Abrashev, Jeny Miteva-Staleva, Aleksandar Dolashki, Lyudmila Velkova, Asya Daskalova, Vladislava Dishliyska, Boryana Spasova, Maria Angelova
	COMPARISON OF THE EFFECTS OF NATURAL VANILLIN AND NEUROTENSIN ANALOGUE ON SOME AFFECTIVE SYMPTOMS IN EXPERIMENTAL MODEL OF PARKINSON'S DISEASE IN VIVO - <b>Andrey Popatanasov</b> , Lyubka Tancheva, Tamara Pajpanova, Reni Kalfin
	ANTI-HERPES ACTIVITY OF DIFFERENT HEMOLYMPHATIC COMPOUNDS FROM CLASSES GASTROPODA AND MALACOSTRACA - Venelin Tsvetkov, Daniel Todorov, Anton Hinkov, Kalina Shishkova, Stoyan Shishkov, Aleksandar Dolashki, Lyudmila Velkova, Pavlina Dolashka
	PROAPOPTOTIC ACTIVITY OF MOLLUSCAN HEMOCYANINS ON EHRlich ASCITES CARCINOMA CELL LINE - Elena Ivanova, Inna Sulikovska, Ani Georgieva, Ivan Iliev, Katerina Todorova, Valeria Dilcheva, Ivelin Vladov, Svetlozara Petkova, Pavlina Dolashka, Reneta Toshkova

	EFFECT OF TRANSIENT TEMPERATURE DOWNSHIFT ON OXIDATIVE CELL RESPONSE OF <i>PENICILLIUM GRISEOFULVUM</i> ISOLATED FROM ANTARCTICA - Ekaterina Krumova, Radoslav Abrashev, Galina Stoyancheva, Nedelina Kostadinova, Jeny Miteva-Staleva, Vladislava Dishliyska, Boryana Spasova, Maria Angelova
<b>14.05.2021</b>	
<b>SECION : PROTEOMIC PROFILE OF TREATED PATHOGENS FOR THE PURPOSE OF FORECASTING THE MECHANISM OF ACTION OF THE STUDIES</b>	
09:30- 10:00	EVALUATION OF ENZYMES EXPRESSED IN HAPLOID AND DIPLOID SACCHAROMYCES CEREVISIAE CELLS TROUGH SDS PAGE AND 2D SDS PAGE ANALYSIS - <b>Asya Daskalova</b> , Lyudmila Velkova, Ventseslava Petrova, Anna Kujumdzieva, Anna Tomova, Pavlina Dolashka
10:30- 11:00	ANALYSIS OF INTRAVENOUS IMMUNOGLOBULIN OF SDS-PAGE WITH IMAGEQUNATTI SOFTWARE - Aleksandar Dolashki, Lyudmila Velkova, Asya Daskalova, Tchavdar Vassilev, <b>Pavlina Dolashka</b>
11:00- 11:30	PROTEOME ANALYSIS OF IMMUNOGLOBULINS ISOLATED FROM MICE WITH SEVERE INFLAMMATION, INJECTED WITH INTRAVENOUS IMMUNOGLOBULIN (IVIG) - <b>Lyudmila Velkova</b> , Asya Daskalova, Aleksandar Dolashki, Dimitar Kaynarov, Tchavdar Vassilev, Pavlina Dolashka
11:30- 12:00	TREATMENT OF HARD TO HEAL WOUND AND CHRONIC WOUNDS - A NEW APPROACH WITH NATURAL SUBSTANCES - <b>Momchil Kermedchiev</b> , Irina Michael, Evgenia Peneva, Radka Lazarova, Stela Jordanova, Pavlina Dolashka
<b>CLOSING REMARKS FOR THE CONFERENCE</b>	



# **BOOK OF ABSTRACTS**

## **"INNOVATIVE LOW TOXIC BIOLOGICALLY ACTIVE AGENTS FOR PRECISION MEDICINE**



### **PRESENTATION OF NATIONAL RESEARCH PROGRAMME “INNOVATIVE LOW-TOXIC BIOACTIVE SYSTEMS FOR PRECISION MEDICINE (BIOACTIVEMED)”**

Prof. Olya Stoilova, PhD, Bulgarian Academy of Sciences, 1040 Sofia, 15<sup>th</sup> November st. 1

The National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” was approved by DCM No658/14.09.2018 and is performed on the basis of a signed agreement Grant DO1-217/30.11.2018 between Ministry of Education and Science and Bulgarian Academy of Sciences (BAS). The Programme is focused on the development of new low toxic bioactive substances and systems containing extracts of natural sources (of plant or animal origin) from Bulgaria for the prevention and support of the therapy of certain diseases. The first step is the isolation and purification of bioactive substances of plant and animal origin, their appropriate incorporation in suitable innovative systems and development of new methodologies for their characterization and determination of quality and applicability as new products in personalized and preventative medicine. One of the planned long-term applications is the implementation of new innovative healthy and safe food supplements and cosmetics with a preventive potential for personalized medicine. The research has been carried out with the participation of leading scientists with proven scientific capacity, as well as with the active participation of young scientists and PhD students from the Consortium formed.

The leading organization is the Bulgarian Academy of Sciences and the following scientific organizations (seven scientific units from the BAS) and higher education organizations with the highest capacity in the field of the Programme are Partners: Institute of Organic Chemistry with Center of Phytochemistry (Partner 1), Institute of Molecular Biology (Partner 2), Institute of Microbiology (Partner 3), Institute of Neurobiology (Partner 4), Institute of Experimental Morphology, Pathology and Anthropology with Museum (Partner 5), Institute of Polymers (Partner 6), Institute of Information and Communication Technologies (Partner 7), Medical University – Sofia (Partner 8), Sofia University “St. Kliment Ohridski” (Partner 9), Medical University – Plovdiv (Partner 10), Plovdiv University “Paisii Hilendarski” (Partner 11) and National Sports Academy “Vasil Levski” (Partner 12). The research activities are divided in the following 8 work packages (WPs).

## **IDENTIFICATION OF PEPTIDES IN THE HEMOLYMPH OF GARDEN SNAIL *CORNU ASPERSUM***

**Aleksandar Dolashki**, Lyudmila Velkova, Pavlina Dolashka

*Institute of Organic Chemistry with Center of Phytochemistry, 9, Acad. G. Bonchev Str., 1113 Sofia, Bulgaria*

The World Health Organization (WHO) has declared the rise in antimicrobial resistance to conventional antibiotics in recent decades one of the three global threats to human health worldwide and announced the beginning of the “post-antibiotic era”.

The fraction containing components with  $M_w < 3$  kDa isolated from *Cornu aspersum* hemolymph, filtered through a 0.22  $\mu\text{m}$  pore diameter filter, was purified by Reverse phase - High-performance liquid chromatography (RP-HPLC) and analyzed by mass spectrometry.

The molecular weights of the peptides isolated from *C. apersum* hemolymph (fraction with  $MW < 3$  kDa) were determined by MALDI-MS analyzes. Primary peptide structures were identified by MALDI-MS/MS and some of them confirmed by LC-ESI-MS analyzes of LTQ Orbitrap XL. Using this approach, primary structures of 18 novel peptides were characterized by *de novo* sequencing. Most of them contain high level of Val, Pro, Leu/Ile, Lys, Asn, Phe, Met, His, Trp и Tyr residues which are typical for peptides with antioxidant properties and antimicrobial activity. The isoelectric points (pI) and grand average of hydropathicity (GRAVY) of the peptides were predicted by the ExPASyProtParam tool.

The presented results maybe considered as basic information for further investigations on bioactive compounds from *C. apersum* hemolymph for creating new natural products with potential biomedical applications.

**Keywords:** *Cornu aspersum* hemolymph, peptides, mass spectrometry, *de novo* sequencing, MALDI-MS/MS, LC-ESI-MS analyzes of LTQ Orbitrap XL

**Acknowledgements :** This research was carried out with the support of a project under contract No. KP-06-OPR-03-10/20.12.2018, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria and by the Project VS.076.18N (FWO).

## **PROBING THE MEMBRANE-DESTRUCTION POTENTIAL OF THE NEWLY IDENTIFIED PEPTIDE ASPERSIN DK10<sup>1</sup> (DLTLNGLSPK)**

**Nevena Ilieva<sup>1</sup>**, Peicho Petkov<sup>2</sup>, Elena Lilkova<sup>1</sup>, Leandar Litov<sup>2</sup>

<sup>1</sup> *Institute of Information and Communication Technologies, Bulgarian Academy of Sciences*

<sup>2</sup> *Physics Faculty, Sofia University “St. Kl. Ohridski”*

**Antimicrobial peptides** (AMP) play a crucial role as mediators of the primary host defense system against microbial invasion. Their characteristics, the broad-spectrum and largely nonspecific activity qualify them as possible candidates for therapeutic alternatives against multi-drug resistant pathogens. The AMPs’ antibiotic activity is thought to be based on their cationic and amphiphilic nature, which enables them to interact with negatively charged bacterial surfaces and membranes, thus causing membrane disruption or altering metabolic processes. Thus, a relation can be established between cell membrane permeability under the action of AMPs and their antibacterial activity.

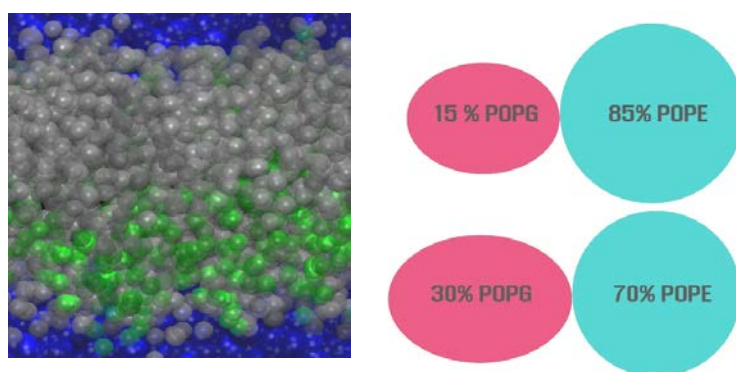
We perform an *in silico* investigation of the antimicrobial potential of the newly identified peptide aspersin DK10 by studying its interaction with a model bacterial membrane. The peptide (with amino acid

sequence DLTNLGLSPK) was isolated from the lightest fraction, below 3 kDA, of the garden snail *Helix aspersa* mucus by P. Dolashka and collaborators. The model membrane was constructed to resemble the *E. Coli* membrane – asymmetric, with POPE (neutral) and POPG (negatively charged) phospholipids in ratios 85/15 and 70/30 in the external, resp. internal layer (Fig. 1). By means of molecular dynamics simulations, the 3D structure of the peptide was modelled and then its membrane-destruction potential was probed. The peptide, most likely due to its specific charge distribution, fully penetrates the membrane (Fig. 2).

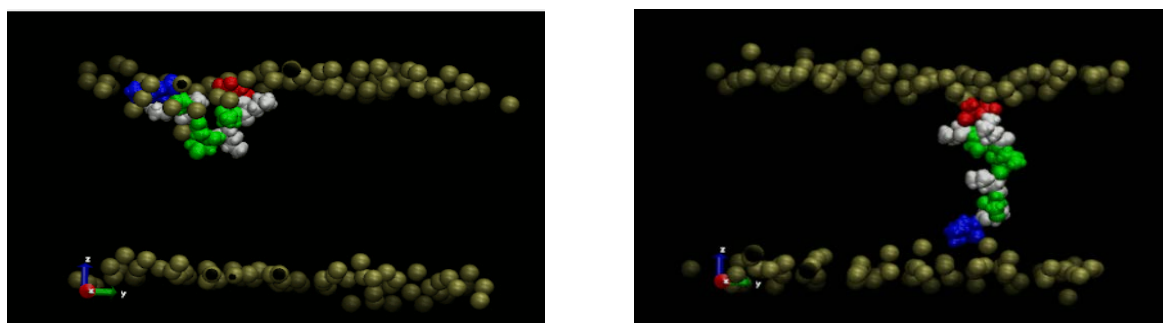
We discuss a metadynamics-based protocol (under development) aiming at the reconstruction of longer periods of the time evolution of the AMP–membrane complex and exploring possibly exhaustively its conformation space. This enhanced sampling technique provides information about the multidimensional free energy surfaces in terms of appropriate collective variables – in this case, the center-of-mass distance.

**Acknowledgements:** This work was partially supported by the Bulgarian Ministry of Education and Science (Grant DOI-358/17.12.2020) under the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM # 658/14.09.2018, and by the Bulgarian Science Fund (Grant KP-06-OPR 03-10/2018). Computational resources were provided by the BioSim HPC Cluster at the Faculty of Physics at Sofia University “St. Kl. Ohridski”, Sofia (Bulgaria) and by CITASK (Centre of Informatics – Tricity Academic Supercomputer & network), Gdansk (Poland).

### Graphical abstract



**Fig. 1** Model asymmetrically charged bacterial membrane containing 85% neutral POPE phospholipids in the outer layer and 70% - in the inner, with 15%, respectively 30%, content of negatively charged POPG



**Fig. 2** Snapshots from the simulation of aspersin DK10 penetrating the model membrane.



## STUDY OF METABOLITES PROFILING OF *HELIX LUCORUM* HEMOLYMPH USING <sup>1</sup>H NMR AND MASS SPECTROMETRY

**Nikolay Vassilev**, Svetlana Simova, Miroslav Dangalov, Lyudmila Velkova, V. Atanasov, Aleksandar Dolashki, Pavlina Dolashka

*Institute of Organic Chemistry with Center of Phytochemistry, 9, Acad. G. Bonchev Str., 1113 Sofia, Bulgaria*

Molluscan hemolymph is a unique kind of body fluid, which in many respects is analogous to human blood, although there are several crucial differences.

The first time, low molecular weight metabolites were identified in the hemolymph of garden snail *Helix lucorum*. Nuclear Magnetic Resonance (NMR) was used

To study the presence and functional role of hemetabolites in hemolymph from land snail *Helix lucorum*. Fourteen metabolites were unambiguously identified by <sup>1</sup>H, 1D TOCSY, 2D J-resolved, 2D COSY, and 2D HSQC NMR spectra with water suppression. The primary structure of 18 novel peptides was identified in low molecular weight fractions (Mw < 1 kDa) by *de novo* sequencing on the COMPACT UHPLC-QqTOF Systems.

The type and concentration of metabolites in the two low molecular weight fractions (<1kD and <3kD) are very similar. Metabolites with known antioxidant, antibacterial, and antimicrobial activities have been detected by NMR metabolic analysis and tandem mass spectrometry of hemolymph samples from *H. lucorum*.

**Keywords:** hemolymph, *Helix lucorum*, <sup>1</sup>H NMR, mass spectrometry, *de novo* sequencing, peptides, metabolites.

**Acknowledgements:** This research was funded by the Bulgarian Ministry of Education and Science through the National Scientific Program “Innovative Low-Toxic Biologically Active Means for Precision Medicine” BioActiveMed, grant number DOI-217/30.11.2018 and Project VS.076.18N (FWO).

## A POSSIBLE SYNERGISTIC CYTOTOXIC EFFECT BETWEEN CLASSICAL CHEMOTHERAPEUTIC AGENTS AND COMPOUNDS ISOLATED FROM THE SNAIL *RAPANA VENOSA* ON BREAST CANCER CELL LINES

**Alexander Tzintzarov**, Alexander Dushkov, Zlatina Vlahova, Lazar Lazarov, Iva Ugrinova

*Institute of Molecular Biology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str, bl. 21 Sofia 1113 Bulgaria*

Cancer is a problem that has been persistently faced by humanity; in the latest decades, considerable research in the direction of finding new drugs that tackle it without being toxic to the patient's organism has been done. Natural compounds (compounds extracted from either plants, fungi, or animals) have proven to be of interest, since some of them exhibit anti-tumor activity *in vitro* without being toxic to healthy cells. Many compounds with an effect on human cells have been discovered in marine organisms; their metabolites have diverse structures and modes of action. Hemolymph and hemocyanins from the snail *Rapana venosa*, an invasive species found in the Bulgarian waters of the Black Sea, have been proven to have a cytotoxic effect on certain cancer cell cultures *in vitro*; at the same time, they have a relatively low cytotoxicity compared to traditional chemotherapeutics. The aim of this study is to evaluate a possible synergistic effect between hemolymph and hemocyanins isolated from *R. venosa* and the chemotherapeutic agent tamoxifen, with the goal of establishing a therapeutic approach where the addition of *R. venosa* – derived compounds can aid in reducing the dose of agents such as tamoxifen, which is toxic to the patient. In the study we used two different human breast cancer cell lines – MCF7,

hormone dependent and MDA-MB-231, triple negative, to conduct an MTT cytotoxicity assay. The assay was performed for compounds extracted from *R. venosa* and for Tamoxifen separately and for a combination of the two and we obtained the half-maximal inhibitory concentrations (IC<sub>50</sub>). In addition we performed statistical analysis for quantification of the synergistic effect between the natural compounds and the chemotherapeutic agent using the Chou-Talalay.

**Keywords:** natural products; hemocyanins; anti-tumor activity; combination index (CI); synergistic effect

**Acknowledgments:** *This work was fully supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM # 658/14.09.2018.*

## EFFECT OF CANNABIDIOL ON CELL LINES H1299, A549

**Lazar Lazarov**, Zlatina Vlahova, Iva Ugrinova

*Institute of Molecular Biology “Roumen Tsanev”, Bulgarian Academy of Sciences*

Cannabidiol is one of over 100 cannabinoids extracted from the plant *Cannabis Sativa* and is characterized with complex and contradictory biological function. This cannabinoid affects many cellular processes in a multitude of ways. Through the CB-2 receptors cannabidiol can affect the immune system, but compared to most of the other cannabinoids it has lower affinity to the receptors of the endocannabinoid system. There are intensive studies on its antitumor effects in the last 10 years. However only a fraction of these tumor suppressive mechanisms of action of cannabidiol are explained.

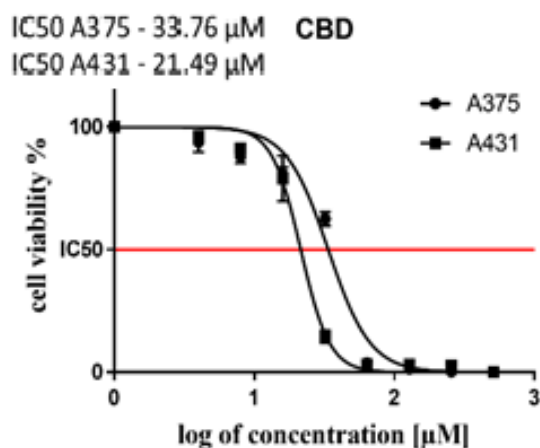
The aim of the study is to further investigate the unknown factors which dictate the antitumor activity of cannabidiol and its effect on various types of cancer cell lines. Based on our previous data on lung cancer cells we decided to investigate the effect of this substance on their metastatic potential. We also evaluated the cytotoxicity of cannabidiol on two types of skin cancer with different origin in *in vitro* cell-based assays.

In this study were used human cancer cell lines - two lung cell lines A549 (p53 positive), H1299 (p53 negative) and two skin cancer cell lines - A375 (malignant melanoma) and A431 (epidermoid carcinoma). A standard protocol for MTT assay was used to determine the cytotoxic effect of cannabidiol on the cell lines described above. Analysis of the dose-response curve was done using the Software GraphPad PRISM. After this a wound healing assay was performed to determine if CBD has effect on the cell migration. Following the treatment with identical CBD concentrations for both lung cancer cell lines (5 μM, 10 μM, 15 μM) the area of the wound was photographed (at 0h; 4h, 24h, 48h, 72h), subsequently the cells in each well were collected, stained with trypan blue and counted with Corning® Cell Counter. This cell counting was performed in order to validate the potential cause for the suppression of the cell migration. As we have shown in our previous study, the main difference in the effect of the cannabidiol treatment in these two cell lines is the triggering of apoptosis in the p53<sup>+/+</sup> A549, in contrast to the necrosis in the p53<sup>-/-</sup> H1299. The counted cells showed a significant difference in the total cell number for the two lines. The total number of p53<sup>+/+</sup> cells was significantly higher compared to the p53<sup>-/-</sup> cells. This hints directly at our previous data obtained from the FACS apoptotic population analysis. The area of the wound was used to quantify the cell migration, using Image J software. Our results show almost identical tendency of a dose dependant inhibition of the cell migration.

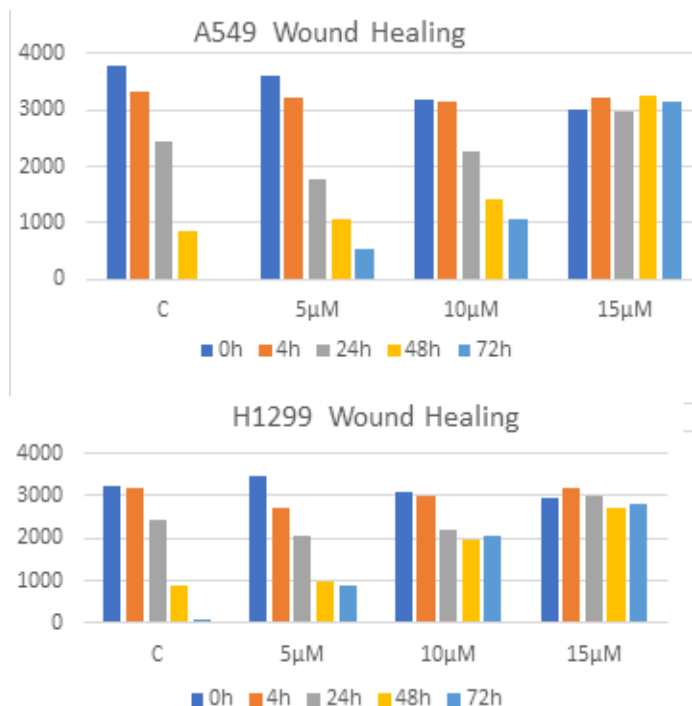
**Keywords:** cannabidiol, metastatic potential, lung cancer, skin cancer, *in vitro* cell based assays

**Acknowledgments:** *The author acknowledges the support of the National Scientific Program BioActivMed (D01-217) by the Bulgarian Ministry of Education.*

## Graphical abstract



**Fig 1.** Half-maximal inhibitory dose  $IC_{50}$  values of cannabidiol treated cells. The two skin cancer cell lines A431 and A375 were treated with different concentrations of CBD in a range from 16 to 512  $\mu M$  for 72 h exposure and were analyzed by MTT test. The data are normalized to the corresponding non-treated control cells. The mean values of three independent experiments performed in quadruplicates are presented with standard deviation ( $n=3$ )  $\pm SD$ .



**Fig 2.** Graphic representation of the cell migration (wound healing) in cancer lung cancer cell lines A549 and H1299 over the period of 72 hours. The cells were treated with the following concentrations of cannabidiol 5 $\mu M$ , 10 $\mu M$ , 15 $\mu M$ .

## IN VITRO ANTINEOPLASTIC ACTIVITY OF HEMOCYANINS

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In the modern world, malignant diseases are one of the major healthcare problems. Traditional therapies such as surgery, chemotherapy and radiotherapy are still not effective enough against cancer. In recent years, scientists are searching for new substances with natural origin and strong antineoplastic activity. Hemocyanins are huge glycoproteins found out freely dissolved in the hemolymph of molluscs and their main function is to transport oxygen to the cells. Last decades, it became clear that hemocyanins have immuno-modulating antineoplastic activity. Because of their complex structure, hemocyanins are used as vaccine carriers and they find out place in anticancer immunotherapy.

In our study, we demonstrate *in vitro* antineoplastic properties of hemocyanins. The snail and *Rapana venosa* (Rv) extracts were kindly provided by Prof. Dr. Pavlina Dolashka, Institute of Organic

Chemistry with Centre of Phytochemistry, BAS. The antineoplastic effect was measured by the colorimetric MTT assay and the cell lines that were used were: T-24 (urinary bladder cells), CAL-29 (urinary bladder transitional cell carcinoma) and MJ cells (cutaneous T cell lymphoma; mycosis fungoides). Significant antineoplastic activity occurs at 1000 µg/ml concentrations. Notable cytotoxic effect appeared on T-24 cells treated with the subunit βc of *Helix aspersa* hemocyanin (βc-HaH) and the subunit I of native *Rapana venosa* hemocyanin (RvH I). MJ cell viability was decreased by βc-HaH, functional unit 5 of the subunit βc *Helix lucorum* hemocyanin (βc – HlH), βc – HlH, active components isolated of Rv hemolymph and RvH I. Also, extract of hemolymph *Rapana venosa* 50-100 kDa demonstrated 80% inhibition of cell viability to CAL-29 cell line. Additionally, fluorescence microscopy of MJ, CAL-29 and T-24 cells was performed, after treatment with snails and Rv extracts at concentration 500 µg/ml. The cells were stained with propidium iodide and as a result, chromatin condensation and DNA fragmentation were observed (indicating apoptosis).

Based on our results, we propose that one of the reasons for hemocyanins' antineoplastic activity *in vitro* is their ability to activate apoptosis in cancer cells. This, give us a reason to continue our future experimental work related to the cytotoxic activity of hemocyanins.

**Keywords** MJ cells, subunit βc *Helix lucorum*, CAL-29 cell, hemolymph of *Rapana venosa*

**Acknowledgements** The author acknowledges the support of the National Scientific Program BioActivMed (DOI-217) by the Bulgarian Ministry of Education.

## AMELIORATING EFFECT OF FRESH EXTRACT OF SNAIL (*HELIX ASPERSA*) IN EXPERIMENTAL MODEL OF ALZHEIMER'S TYPE DEMENTIA

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Alzheimer's disease is the most common cause of dementia, which accounts for 60-80% of dementia cases. At present Alzheimer's has no current cure, but worldwide efforts are made to find better ways to treat this disease, delay its onset, and prevent it from developing. In this regard there are many data about preventive and therapeutic effect of Snail extract(SE) against some diseases because of its rich biological activities (antioxidant, anti-inflammatory, membrane stabilizing etc.). However, so far there are no available data in the literature concerning the effect of snail products on neurodegenerative disorders. Hence, the aim of the current research was to evaluate the neuroprotective effects of fresh snail *Helix aspersa* mucus by its oral administration on the memory and neurotransmitter dysfunction, accompanied by the increased oxidative stress, induced by scopolamine (Sco) in male Wistar rats with experimental model of Alzheimer's type dementia. The complexity of the neuroprotective potential of SE was evaluated behaviorally via passive avoidance and hole board tests, and biochemically by measuring the acetylcholinesterase (AChE) activity, the content of monoamines noradrenaline, serotonin and main oxidative stress parameters in the two brain structures mostly related to memory- cortex and hippocampus.

We detected that SE (0.5 mL/100 g, applied orally for 16 consecutive days: 5 days before and 11 days simultaneously with Sco (2 mg/kg, intraperitoneally for 11 days) significantly compensated the memory deficits (Figure 1), observed in dement rats with positive effect on short- and long- term memory processes (increase by 32% and by 37%, respectively).

This effect of snail extract was accompanied by inhibition of AChE activity (by 32% in hippocampus), presented on Figure 2.

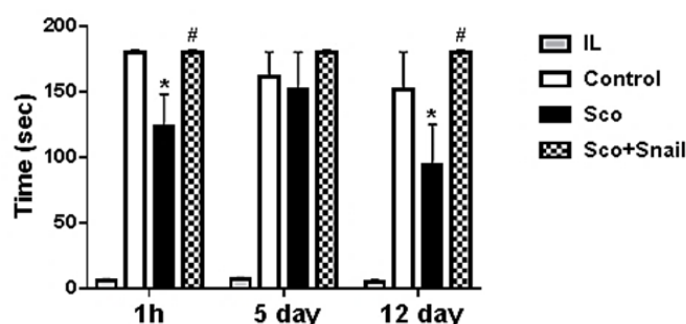
Snail extract also reduced significantly the oxidative stress (both in cortex and hippocampus). It decreased lipid peroxidation levels (in cortex by 38% and in hippocampus by 46%) vs Sco-treated group. Beneficial effect of SE on the total glutathione concentrations (increase by 75%) was observed in the hippocampus. Catalase activity was restored by SE in both structures, near to control levels and the activity of superoxide dismutase was elevated significantly by 62% in hippocampus vs Sco-treated group. The noradrenaline and serotonin levels were enhanced by 133% and by 82 %, respectively (in cortex) and by 67% and by 56% (in hippocampus) as compared to dement rats. The present results demonstrate strong neuromodulatory capacity of fresh Snail extract to elevateneuromediator levels of noradrenaline and serotonin, changed by scopolamine.

Established memory improving effect on rats with AD type dementia is a result of complex ability of snail extract from *Helix aspersa* to inhibit AChE activity (especially in hippocampus) as well as of its antioxidant and neuromodulatory effects on the brain structures related to memory.

**Keywords:** Snail extract, Alzheimer's disease, neuroprotection, neuromodulation

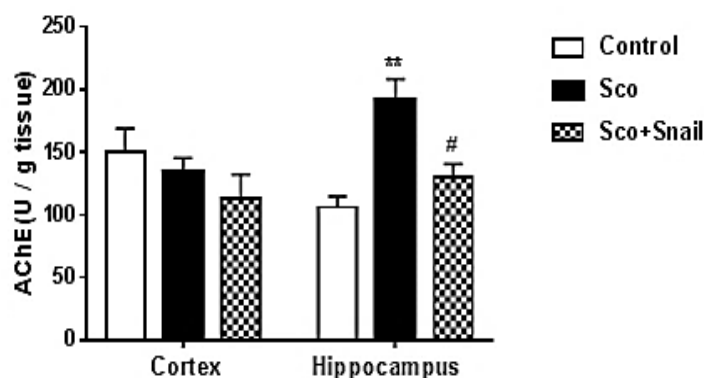
**Acknowledgements:** This work was supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)" approved by DCM # 658/ 14.09.2018.

### Graphical abstract



**Figure 1.** Effect of fresh water snail extract on the step-through latency (STL) in a single-trial passive avoidance test of scopolamine (Sco) induced model of dementia in rats. Snail extract (0.5 ml/100g, b.w; p.o) was administered for 16 days (5 days before and 11 days of simultaneously with Sco). STL was measured at the 1<sup>st</sup> hour, on the 5<sup>th</sup> and 12<sup>th</sup> day after the first Sco (2 mg/kg, i.p) treatment. Results are

expressed as mean values  $\pm$  SEM (n = 6 animals per group). Asterisks above bars indicate a significant difference in STL between the Sco and Control group at  $*P < 0.05$ . Hashtags above bars indicate a significant difference in STL between the Sco+Snail and Sco groups at  $\#P < 0.05$ . Statistical analysis was performed by Student's *t*-test.



**Figure 2.** Effects of fresh water snail extract on acetylcholinesterase (AChE) activity in cortex and hippocampus of rats with Scopolamine (2 mg/kg, i.p) induced model of dementia. Snail extract (0.5 ml/100g, b.w; p.o) was administered for 16 days (5 days before, and 11 days simultaneously with Sco). AChE activity was measured in brain homogenates. Results are expressed as a mean value  $\pm$  SEM (n = 6 animals per group). Asterisk above bars indicate a significant difference in AChE

activity between the Sco and Control group at  $**P < 0.01$ . Hashtags above bars indicate a significant difference in AChE activity between the Sco+Snail and Sco groups at  $\#P < 0.05$  Statistical analysis was performed by Student's *t*-test.



## EXTRACT OF SNAIL (HELIX ASPERSA) AND ITS NEUTOPROTECTIVE EFFECTS IN 6-OHDA EXPERIMENTAL MODEL OF PARKINSON'S DISEASE

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Inflammation is associated with the pathogenesis of many acute and chronic diseases, including neurodegenerative disorders. As a rich source of biologically active substances with antibacterial, antiviral, immunostimulating and antitumor activity, molluscs, including snail have been used as a cure of variety of medical states for centuries. One of the most common therapeutic applications is for treatment of inflammatory conditions. Hence, the aim of the present study was to check potential neuroprotective and therapeutic effect of snail extract on 6-OHDA experimental model of Parkinson's disease (PD) in rat *in vivo*.

Snail extract was collected and purified from *Helix aspersa* snails, grown in Bulgarian farms using patented technology without suffering any snail. Experimental rat PD model was induced via 6-OHDA unilateral striatal injection (10 µg/2 µl) as previously described. Male Wistar rats were divided into following groups: sham-operated, striatal 6-OHDA-lesioned control group and 6-OHDA-lesioned rats treated for 13 days with fresh water snail extract orally (0.5 ml/100 g body weight, 6 days before and 7 days after striatal lesion). The evaluation of its neuroprotective effect was made behaviorally and biochemically. On the 2<sup>nd</sup> and 3<sup>rd</sup> week post lesion rats were subjected to apomorphine-induced rotation, rotarod and step-through behavioral tests. One hour after last test rat's brains were removed and two main oxidative stress markers were determined: lipid peroxidation level and superoxide dismutase (SOD) activity. Our results demonstrated that in PD group apomorphine-induced rotations, number of falls and memory impairment increased significantly as compared to sham-operated group (Figure 1). These changes were accompanied by significant decrease in brain lipid peroxidation level and recovery of brain superoxide dismutase activities, increased by 6-OHDA-treatment. In 6-OHDA+Snail group the superoxide dismutase activity was reduced by 28 % ( $P < 0.05$ ) in ipsilateral side and by 25 % ( $P < 0.05$ ) in contralateral intact side as compared to 6-OHDA group (Figure 2).

In conclusion, our studies demonstrated the ameliorating effect of snail extract on Parkinson's disease rat model *in vivo*.

**Keywords:** Snail extract, Parkinson's disease, neuroprotection

**Acknowledgements:** This work was supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)" approved by DCM # 658/ 14.09.2018.

## Graphical abstract

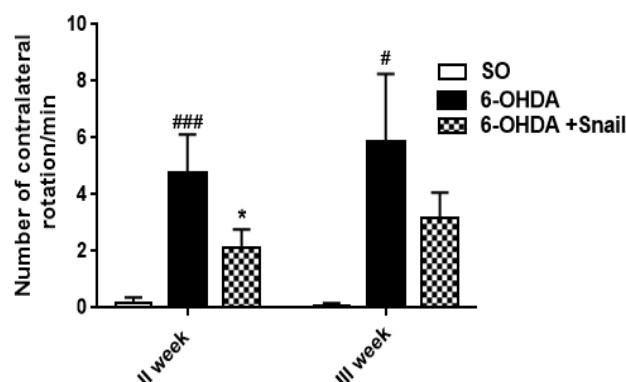
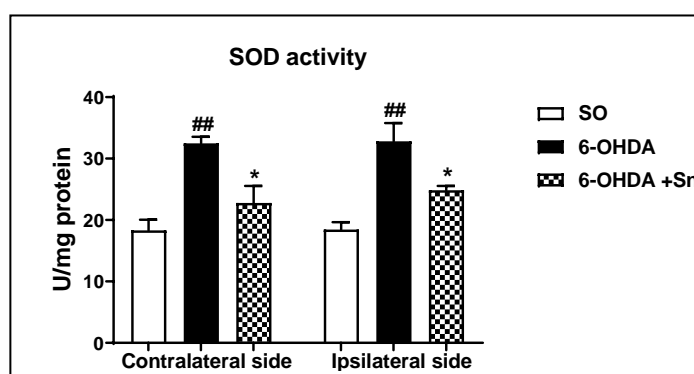


Figure 1. Effect of fresh water snail extract on apomorphine-induced rotational behaviour in rats with unilateral 6-OHDA striatal lesion. Snail extract (0.5 ml/100g, b.w; p.o) was administered for 13 days (6 days before and 7 days after striatallesion). Data are expressed as mean $\pm$ SEM from 6 animals per group. (\*)  $P < 0.05$  vs 6-OHDA and (#)  $P < 0.05$ ; (###)  $P < 0.001$  vs SO (one-way ANOVA and Dunnett's as the post hoc comparison test).



**Figure 2.** Effect of fresh water snail extract on brain superoxide dismutase activities in rats with unilateral 6-OHDA striatal lesion. Snail extract (0.5 ml/100g, b.w; p.o) was administered for 13 days (6 days before and 7 days after striatal lesion). Data are expressed as mean $\pm$ SEM from 6 animals per group. (\*)  $P < 0.05$  vs 6-OHDA and (##)  $P < 0.01$  vs SO (one-way ANOVA and Dunnett's as the post hoc comparison test).

## SAFETY TESTING AND ANTIPROLIFERATIVE ACTIVITY OF HEMOCYANINS FROM *HELIX LUCORUM*, *HELIX ASPERSA* AND *RAPANA VENOSA*

Inna Sulikovska<sup>1</sup>, Ivan Iliev<sup>1</sup>, Katerina Todorova<sup>1</sup>, Ani Georgieva<sup>1</sup>, Valeriya Dilcheva<sup>1</sup>, Ivelin Vladov<sup>1</sup>, Svetlozara Petkova<sup>1</sup>, Reneta Toshkova<sup>1</sup> and Pavlina Dolashka<sup>2</sup>

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Hemocyanins are copper-containing glycoproteins, localized in the haemolymph of invertebrate gastropods belonging to Mollusca family. They are often used for direct application in bladder cancer. There is data mainly about Keyhole Lympet Haemocyanin (KLH), isolated from *Megatura crenulata*. However, the restricted geographical areal of *M. crenulata* does not allow the extraction of huge quantity hemocyanin.

As an alternative of KLH we propose haemocyanins, isolated from *Helix lucorum*, *Helix aspersa* and *Rapana venosa*, which are widespread, including in Bulgaria.

The aim of the present study was to determine the phototoxicity and antiproliferative activity of hemocyanins isolated from *Rapana venosa*, *Helix lucorum* and *Helix aspersa*. The biological activity of the hemocyanins was studied under *in vitro* conditions. For safety testing, 3T3 Neutral Red Uptake Cytotoxicity / Phototoxicity assay was used. Experiments for antiproliferative activity were performed on a human normal fibroblasts (BJ) and human skin melanoma (SH-4). The effects of the hemocyanins on the cell viability were analyzed by MTT Dye Reduction assay.

The evaluation of *in vitro* cytotoxicity / phototoxicity showed that the tested hemocyanins are not cytotoxic / phototoxic. The results from the *in vitro* antitumor activity testing on SH-4 melanoma cells indicated that the subunit  $\beta$ c-HIH and HaH mucus are the most active compounds among the tested samples.

**Keywords:** hemocyanins, phototoxicity, antiproliferative activity

**Acknowledgement:** This study was supported by contract ДО1-358/17.12.2020 г., Program "Innovative Low-Toxic and Biologically Active Means for Precision Medicine" – BioActiveMed. Ministry of Education and Science of the Republic of Bulgaria and Grant Nr KP-06-N31/1.

## ANTIVIRAL EFFECTS OF FRACTIONS AND TOTAL EXTRACTS OBTAINED FROM MEDICINAL PLANTS FROM BULGARIAN POPULATIONS

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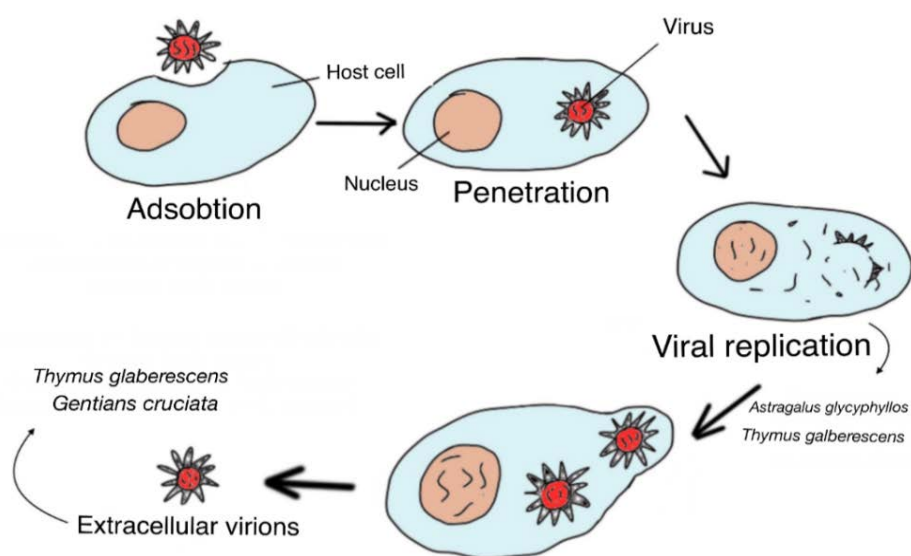
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"Herpes" is a medical condition caused by members of the genus Simplexvirus (Human Alphaherpesviruses (HHV types 1 and 2). According to the WHO, worldwide 3.7 billion people under age 50 (67%) and 491 million people aged 15-49 (13%) are infected with HHV-1 and HHV-2, respectively. Infection with both types of HHV may be asymptomatic (but the virus can still be transmitted to others). In the presence of symptoms, painful sores and blisters around the mouth and lips (when a person is infected with HHV-1) and on the genitals and anus (when a person is infected with HHV-2) are observed. The risk group is immunocompromised patients and neonates in whom the lack of an adequate immune response is the cause of encephalitis, meningitis, neonatal herpes and even death. All Herpesviridae family representatives establish a latent infection that results in recurrence of symptoms. As the latent virus cannot be affected, modern therapy is aimed at controlling the symptoms of the primary and recurrent HHV infections. Nucleoside analogues such as acyclovir (ACV) and its derivatives are most commonly used for treatment. Although this group of substances are very effective selective inhibitors, their improper use leads to the development of resistant viral mutants and toxicity, limiting the effect of therapy. An effective alternative is therefore needed. Such are, for example, substances of natural origin, including traditional medicinal plants.

In the present study six extract from three plant species of *Thymus* genus, seven extracts from the plant *Gentiana cruciata*, two extracts from the plant *Astragalus glycyphyllos* and one extract and two isolated flavonoids from the plant *A. monspesulanus* subsp. *monspesulanus* was tested against replication of ACV sensitive F and BA strain of Human alphaherpesvirus type 1 and 2 respectively *in vitro*. Cytotoxicity against MDBK (Madin-Darby Bovine Kidney) cell line was determined by colorimetric MTT assay. According to MTT-based colorimetric assay for detection of HHV replication inhibition the most potent of all tested extracts was the one named AGO from *Astragalus glycyphyllos*. In maximum nontoxic concentration it reaches ~84% and ~94% of cell protection against HHV-1(F) and HHV-2(BA) respectively. Both flavonoids and all other extracts showed very weak or no antiherpes virus activity. No virucidal activity was measured against both used virus models for tested substances and extracts.

**Acknowledgments:** This work was supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)".

## Graphical abstract



## NEUROPROTECTIVE PROPERTIES OF SNAIL EXTRACTS FROM *HELIX ASPERSA* ON SCOPOLAMINE RAT MODEL OF ALZHEIMER'S DISEASE

**Ventseslav Atanasov<sup>1</sup>**, Lyudmila Velkova<sup>1</sup>, Lubka Tancheva<sup>2</sup>, Aleksander Dolashki<sup>1</sup>, Reni Kalin<sup>2</sup>, Pavlina Dolashka<sup>1</sup>

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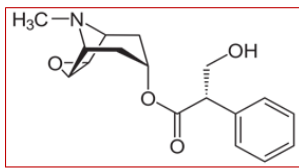
Alzheimer's disease (AD) is the most widespread neurodegenerative disorder. The scopolamine is frequently used agent for induction of Alzheimer in experimental animals. We used scopolamine model for assessment of potential neuroprotective effect of extract from garden snail *Helix aspersa* on neurodegenerative processes *in vivo*. We used male sexually mature experimental rats. They are divided on three groups: a control group of healthy rats, a scopolamine group (treated with scopolamine) and an experimental group treated with scopolamine and snail extract together. Two major memory-related brain structures (hippocampus and prefrontal cortex) are isolated. The obtained proteins were separated by SDS – PAGE and analyzed with MALDI-MS. Using MASCOT Peptide Mass Fingerprint and IQTL software the cortex and hippocampal proteins have been identified and compared.

**Keywords:** Alzheimer's disease (AD), scopolamine, snail extract, neuroprotective effect, rat brain proteins

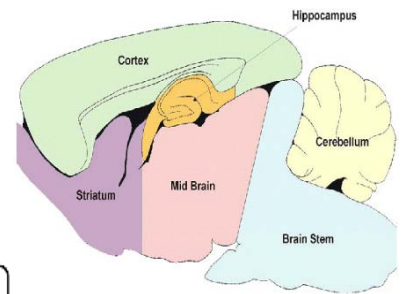
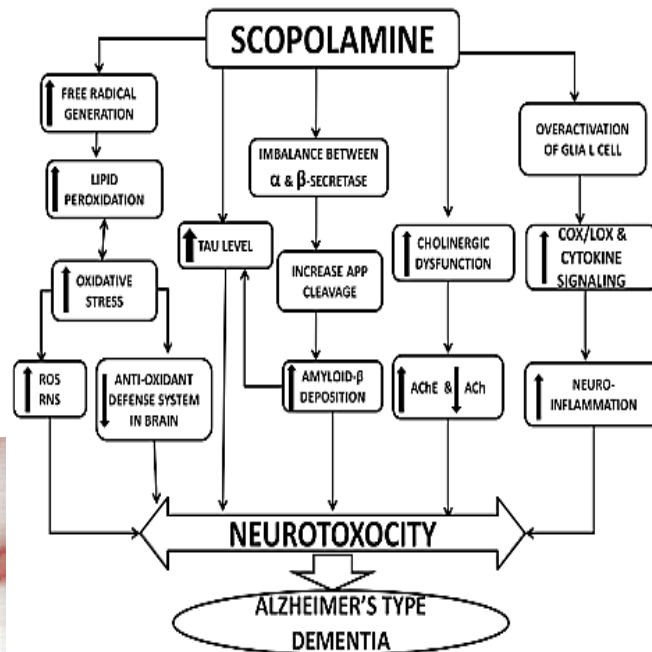
**Acknowledgement:** This work was supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)" approved by DCM № 658/14.09.2018, funded by the SRF of the Ministry of Education and Science, of Republic Bulgaria.

## Graphical abstract

### Scopolamine Model of AD in rats



*Scopolamine*



Schematic representation of gross anatomical brain areas of rat brain homogenized for analysis

The model of scopolamine induced dementia in rodents is one of the well established animal models of memory dysfunction and is widely used as a primary screening test for anti-Alzheimer drugs.



## SESSION : ANTIMICROBIAL ACTIVITY AND MECHANISMS OF ACTION OF BIOACTIVE PEPTIDES, GLYCOPEPTIDES AND PROTEINS

### IN VITRO ANTIBACTERIAL EFFECT OF *OREGANUM VULGARE* OILS WITH POTENTIAL SKIN APPLICATION

Lyudmila Dimitrova<sup>1</sup>, Maya M. Zaharieva<sup>1</sup>, Krassimira Yoncheva<sup>2</sup>, Alexander Kroumov<sup>3</sup>, Vesselin Kussovski<sup>1</sup>, Iva Tsvetkova<sup>1</sup>, Niko Benbassat<sup>2</sup>, Ivanka Spassova<sup>4</sup>, Daniela Kovacheva<sup>5</sup>, **Hristo Najdenski<sup>1</sup>**

<sup>1</sup>Department of Infectious Microbiology and <sup>3</sup>Laboratory of Microbial Biosynthesis and Ecology, The Stephan Angeloff Institute of Microbiology, BAS, Acad. G. Bonchev Str., Bl. 26, 1113 Sofia, Bulgaria;

<sup>2</sup>Faculty of Pharmacy, Medical University of Sofia, Sofia 1000, Dunav 2 Str.;

<sup>4</sup>Department of Adsorption and Catalysis and <sup>5</sup>Department of Inorganic Materials Science, Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 11, 1113 Sofia, Bulgaria

Antimicrobial resistance is a global public health challenge mainly caused by the widespread use of antibiotics in human and veterinary medicine for over 60 years. Therefore, many researchers focused their attention to discovery of new natural active substances. Our studies were conducted with *Oreganum vulgare* oils from Bulgarian (alone and included in a chitosan nano-delivery system), Turkish and Greek origin. We investigated the *O. vulgare* oils minimal inhibitory and bactericidal concentrations (MIC/MBC) [ISO 20776-1:2006(E)] and determined the dehydrogenase activity (DEHA) by MTT-test of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pyogenes*, *Yersinia enterocolitica* and *Candida albicans*. The biofilm reduction was evaluated by using the modified protocol of Stepanovich<sup>1</sup>. The *in vitro* cytotoxicity of nano-encapsulated Bulgarian oregano oil was compared to that of pure oregano oil on normal mice fibroblasts (CCL-1) and normal human keratinocytes (HaCaT) using the MTT dye reduction assay [ISO 10993-5/2009]. The bacterial DEHA and the median inhibitory concentrations (IC<sub>50</sub>) were calculated with the mathematical software MAPLE. The most perspective oils were tested in H-albino guinea pigs to their ability to cause skin sensitization, according to ISO 10993-10:2013. The Greek oregano oil showed less antimicrobial activity than Bulgarian and Turkish ones. The pure Bulgarian oregano oil and encapsulated oregano oil inhibited the DEHA and the biofilm formation of the treated bacterial strains in concentrations significantly lower than the determined MICs. The IC<sub>50</sub> calculated for the encapsulated oregano oils were significantly lower than the values of the pure oil. The latter inhibited to a greater extent the biofilm formation of all tested strains. Both formulations were not toxic to the cell lines in concentrations exerting strong antimicrobial activity. The pure Bulgarian oregano oil and encapsulated oregano oil did not cause skin sensitization paving the way for their future pharmacological development.

**Key words:** antimicrobial activity, *Oreganum vulgare* oils, chitosan nano-delivery system, inhibition of biofilms, *in vitro* cytotoxicity, skin sensitization test

**Acknowledgments:** This study is financed by the Bulgarian Ministry of Education and Science, Grant DOI-217 from 30 November 2018.

### IN VITRO ANTIFUNGAL ACTIVITY OF *RAPANA VENOSA* HEMOLYMPH AGAINST STRAINS FROM GENERA *ASPERGILLUS* AND *PENICILLIUM*

**Ekaterina Krumova<sup>1</sup>**, Pavlina Dolashka<sup>2</sup>, Nedelina Kostadinova<sup>1</sup>, Radoslav Abrashev<sup>1</sup>, Jeny Miteva-Staleva<sup>1</sup>, Aleksander Dolashki<sup>2</sup>, Lyudmila Velkova<sup>2</sup>, Asya Daskalova<sup>2</sup>, Vladislava Dishlijska<sup>1</sup>, Boryana Spasova<sup>1</sup>, Maria Angelova<sup>1</sup>

<sup>1</sup>The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia

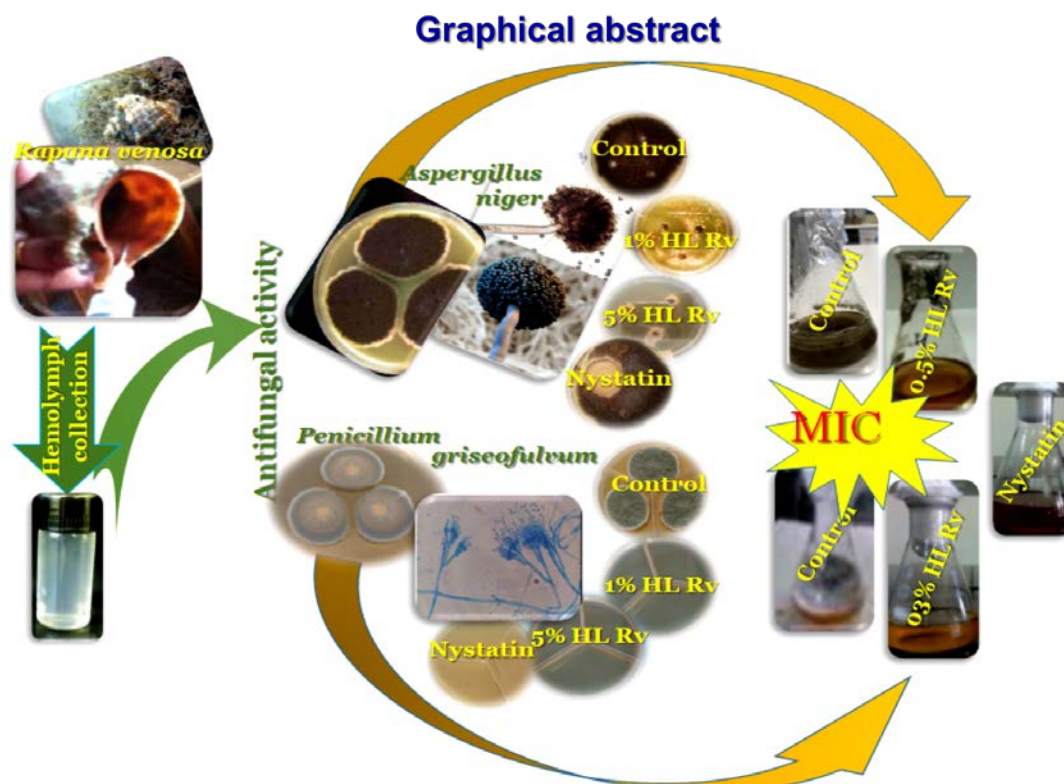
In the past few decades, the data show a serious increase in the incidence of fungal infections. Moreover, a rise in the resistance of some fungi to different fungicides used in practice has been observed. Hence, there is a great demand for novel antifungal agents. Mollusks could be a pharmacologically significant outlet. Molluscan hemolymph has been investigated predominantly for its anti-tumor, anti-inflammatory, cytotoxic, and antibacterial effect. However, the reports on antifungal properties could be very seldom found. This study was designed to investigate the antifungal potential of *Rapana venosa* hemolymph. The aim of the study was to evaluate the inhibitory effect on fungal strains belonging to the genera *Aspergillus* and *Penicillium* as well as to compare this effect to that obtained after treatment with nystatin.

The hemolymph from *R. venosa* was collected and purified using different membrane techniques. Then it was tested for a fungicidal effect against two fungal strains, *Aspergillus niger* 26 and *Penicillium griseofulvum* P29. Antifungal activity was assayed through the cultivation of conidiospores on agar or liquid medium. Nystatin was used as a positive control.

Tested *R. venosa* hemolymph demonstrated a strong fungicidal effect against both the strains during surface and submerged cultivation. Concentration of 1% completely inhibited fungal growth for 96 h, and delayed mycelia formation compared to the control for 14 days. At the same time, the use of 5% *R. venosa* hemolymph led to 100% growth inhibition. The minimal inhibitory concentration (MIC) was 0.3% and 0.5% for *P. griseofulvum* and *A. niger*, respectively. The results obtained are comparable to those of the nystatin experiments, indicating a similar antifungal effect between both agents. In conclusion, the hemolymph of marine snail *R. venosa* were found to be promising antifungal agents.

**Key words:** marine snail, *R. venosa* hemolymph, antifungal activity, *Aspergillus niger* 26 and *Penicillium griseofulvum* P29

**Acknowledgement:** This research was carried out with the support of D01-217/30.11.2018 National scientific program “Innovative low-toxic biologically active precise medicine (BioActiveMed) and a project under contract No. DN 01-14/19.12.2016, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.



# EFFECT OF TRANSIENT TEMPERATURE DOWNSHIFT ON OXIDATIVE CELL RESPONSE OF *PENICILLIUM GRISEOFULVUM* ISOLATED FROM ANTARCTICA

**Ekaterina Krumova**, Radoslav Abrashev, Galina Stoyancheva, Nedelina Kostadinova, Jeny Miteva-Staleva, Vladislava Dishliyska, Boryana Spasova, Maria Angelova

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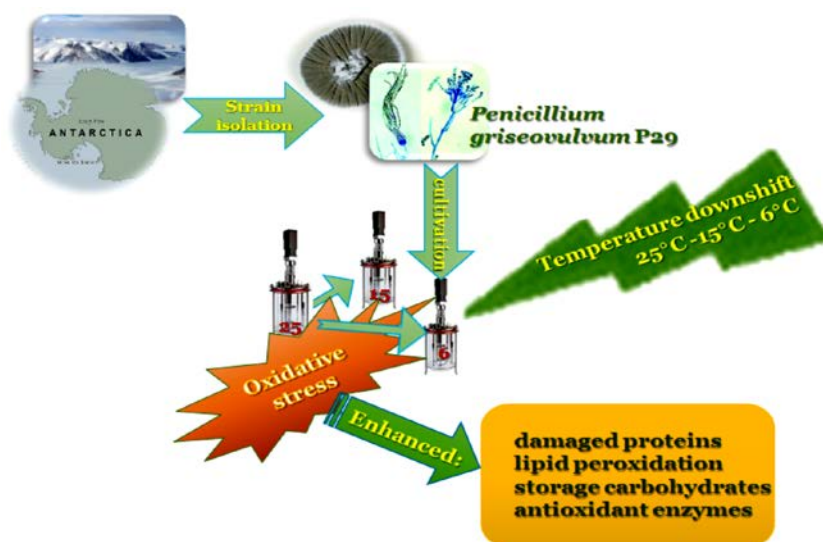
Antarctic fungi are capable of developing at extreme low temperatures using specific adaptive strategies. The increasing antioxidant defense is one such strategy. There are experimental evidences to conclude that the protection to low temperature includes changes in oxidative stress biomarkers and antioxidant enzyme activity. However, there is a lack of studies on the relationship between the transient temperature downshift and oxidative cell response in low eukaryotes such as filamentous fungi.

Here we compared the effect of short-term temperature downshift from optimal temperature to 15 or 6°C on antioxidant cell response in Antarctic fungus *Penicillium griseofulvum*. The results indicated typical oxidative stress reactions: significant reduction of biomass production; increase in the levels of oxidative damaged proteins, lipid peroxidation and accumulation of storage carbohydrates (glycogen and trehalose) in comparison to growth at optimal temperature. In addition, significantly enhanced superoxide dismutase and catalase activity was evaluated. This response is cold-shock degree-dependent. During the phase of temperature recovery, cells resumed growth and protein synthesis, but maintained high level of reserve carbohydrates, damaged proteins, and antioxidant enzyme activity compared to the control.

**Keywords:** oxidative cell response, temperature downshift, Antarctic fungi

**Acknowledgments:** This work was supported by the Bulgarian National Science Fund (grant DN-01/1-2016) to which we owe our sincere thanks.

## Graphical abstract



## CROSSLINKED COPOLYMERS STRUCTURES BASED ON POLY(N-ISOPROPYLACRYLAMIDE) AS POTENTIAL CARRIERS FOR BIOLOGICALLY ACTIVE SUBSTANCES

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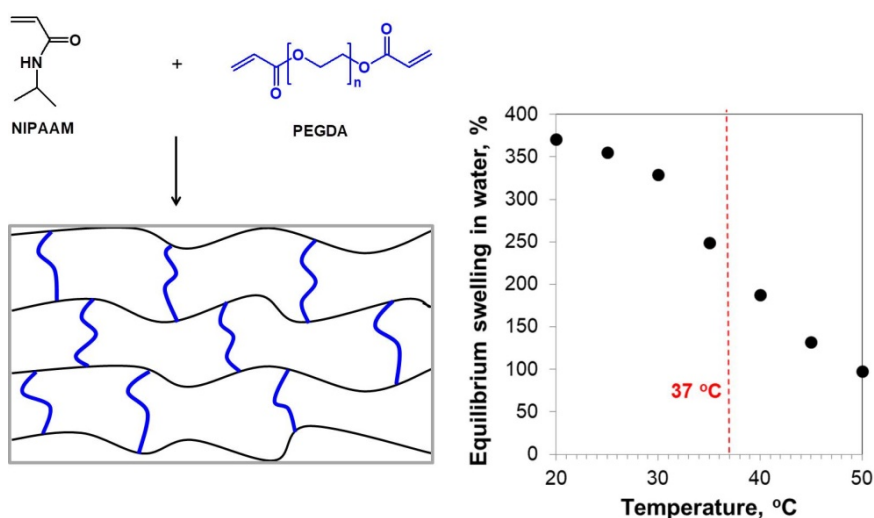
Smart polymers capable to respond reversibly to the changes in external temperature are becoming increasingly important with significant contribution to biomedical research. New polymeric structures are being designed as useful tools for drug delivery, tissue engineering, biosensor design, wound healing and other metabolic control mechanisms. In this context, poly(N-isopropylacrylamide) (PNIPAAm) is among the most widely studied temperature-responsive polymers due to its specific structure and unique properties in aqueous media. PNIPAAm macromolecules contain both hydrophilic amide groups –C(=O)–NH– and hydrophobic isopropyl side groups –CH(CH<sub>3</sub>)<sub>2</sub> and the delicate hydrophilic-hydrophobic balance governs the unique properties of this polymer in aqueous solution. At temperatures below ~ 32 °C PNIPAAm macromolecules are hydrated and dissolve well in an aqueous medium, while at elevated temperatures their solubility decreases, the polymer chains undergo a phase transition and aggregate. Accordingly, PNIPAAm-based copolymer structures reversibly change their properties in the temperature range 20-40°C and these phenomena, along with the proven biocompatibility are pointing out for application as carriers of biologically active substances.

In this paper, different synthetic approaches for the design of PNIPAAm-based functional networks will be presented that allow preparation of copolymer structures composed of blocks and segments with varied chemical composition and properties. It will be shown that through a precise macromolecular design of the copolymer architecture, the properties of the networks and corresponding hydrogel derivatives can be controlled and adjusted for specific biomedical application. Phase morphology, main physicochemical characteristics and swelling properties of the developed copolymer networks will be presented and their potential as new platforms for sustainable delivery of bioactive molecules with different solubility will be discussed. The possibilities for using the "intelligent" properties of the studied hydrogels in the process of loading a drug substance as well as for modulating its release will be demonstrated.

**Keywords:** Smart polymer materials; poly(N-isopropylacrylamide); copolymer networks; drug carriers

**Acknowledgements:** This work was supported/(partially supported) by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM # 658 / 14.09.2018.

### Graphical abstract



**Synthesis of a PNIPAAm copolymer network with poly(ethylene oxide) crosslink segments and its temperature-responsive swelling in water**



## PSEUDOMONAS SP. IN THE SNAIL MUCUS - A CARD-FISH STUDY

Mihaela Belouhova<sup>1</sup>, Elmira Daskalova<sup>1</sup>, Yana Topalova<sup>1</sup>, Pavlina Dolashka<sup>2</sup>

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The snail mucus is known for its healing and stimulating properties. It is used in the traditional medicine and it is a source for isolation of different biologically active compounds. However, its microbiome remains poorly studied. The present investigation was focused on a microbiological study of *Cornu aspersum* mucus. Two types of techniques were used – cultivation technique for determination of the quantity of key bacterial groups (aerobic heterotrophs, anaerobic heterotrophs, *Pseudomonas* sp., *Aeromonas* sp., *Lactobacillus* sp., *Enterococcus* sp.) and the highly specialized molecular technique catalyzed reporter deposition – fluorescence *in-situ* hybridization (CARD-FISH) for *Pseudomonas* spp.

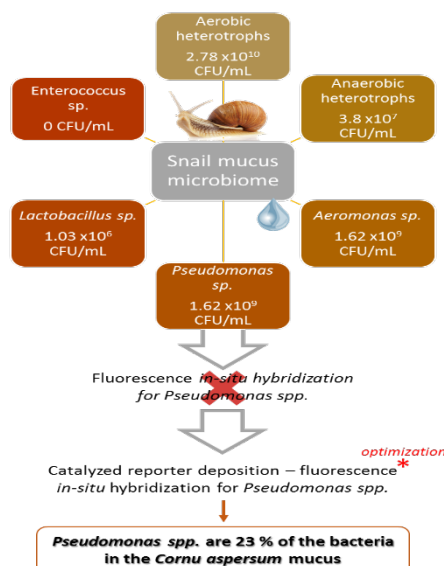
The cultivation analyses showed that the *C. aspersum* mucus is highly populated with bacteria. The aerobic heterotrophs were  $2.78 \times 10^{10}$  CFU/mL. The anaerobic heterotrophs were presented in considerably lower numbers ( $3.8 \times 10^7$  CFU/mL) but their quantity was still very high. The bacteria from the genus *Aeromonas* were  $6.2 \times 10^7$  CFU/mL. The *Lactobacillus* sp. were  $1.03 \times 10^6$  CFU/mL in the snail mucus. *Pseudomonas* sp. were found in a very high concentration –  $1.62 \times 10^9$  CFU/mL. The considerable share of these microorganisms in the snail microbiome was indirect prove for their importance. That is why a molecular technique (FISH) was used for their examination directly in the samples. The obtained fluorescence signals were too weak for proper determination, probably because of the insufficient metabolic activity. In order to study the target bacteria in the mucus an advanced FISH technique was used (CARD-FISH). Significant amplification of the signal was obtained. The quantity of the *Pseudomonas* spp. was 23 %, while according to the cultivation technique it was 6 %.

The results from the present study demonstrated that the snail mucus is rich in bacteria and among them the ones belonging to genus *Pseudomonas* have a major part. Most probably they contribute to the production of the biologically active compounds. The obtained data also highlights the fact that in a complex matrix such as the *C. aspersum* mucus advanced molecular techniques need to be used in order to obtain relevant information.

**Keywords:** *Cornu aspersum*, mucus, *Pseudomonas*, CARD-FISH, microbiome

**Acknowledgments:** This study was supported financially by the project DH 01/14/19.12.2016 "Proteomic analysis of new natural peptides, isolated from snail "*Cornu aspersum*" – NSF, Ministry of Education and Science, Bulgaria as well as well as was supported by funding for materials by the National Scientific Program "Innovative Low-Toxic Biologically Active Means for Precision Medicine" (BioActiveMed).

### Graphical abstract





# SHORT-TERM METABOLIC ACTIVITY INHIBITION OF *E. COLI* IN THE PRESENCE OF THREE PEPTIDE FRACTIONS FROM SNAIL MUCUS

Mihaela Belouhova<sup>1</sup>, Elmira Daskalova<sup>1</sup>, Yana Topalova<sup>1</sup>, Pavlina Dolashka<sup>2</sup>

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The antibiotic resistance is a major problem nowadays. Considerable efforts are put into tracking the resistant microorganisms, development of new generations of antibiotics and exploration of the applicability of natural substances. The molluscs are organisms whose defense mechanisms against different types of bacteria could be used in human benefit. The present study is focused on three peptide/protein fractions: 1/ peptides with MW below 10 kDa (from *Cornu aspersum*); 2/ proteins with MW above 30 kDa (from *C. aspersum*); 3/ proteins with MW 50-100 kDa (from *Rapana venosa*). Their effect on the viability and the metabolic activity of *Escherichia coli* was studied after 1 hour of incubation in the presence of 50 % of the peptides/proteins fractions. The analyses were based on CTC (5-Cyano-2,3-ditolyl tetrazolium chloride) and DAPI (4',6-Diamidino-2-Phenylindole) staining combined with digital analysis of the fluorescence images.

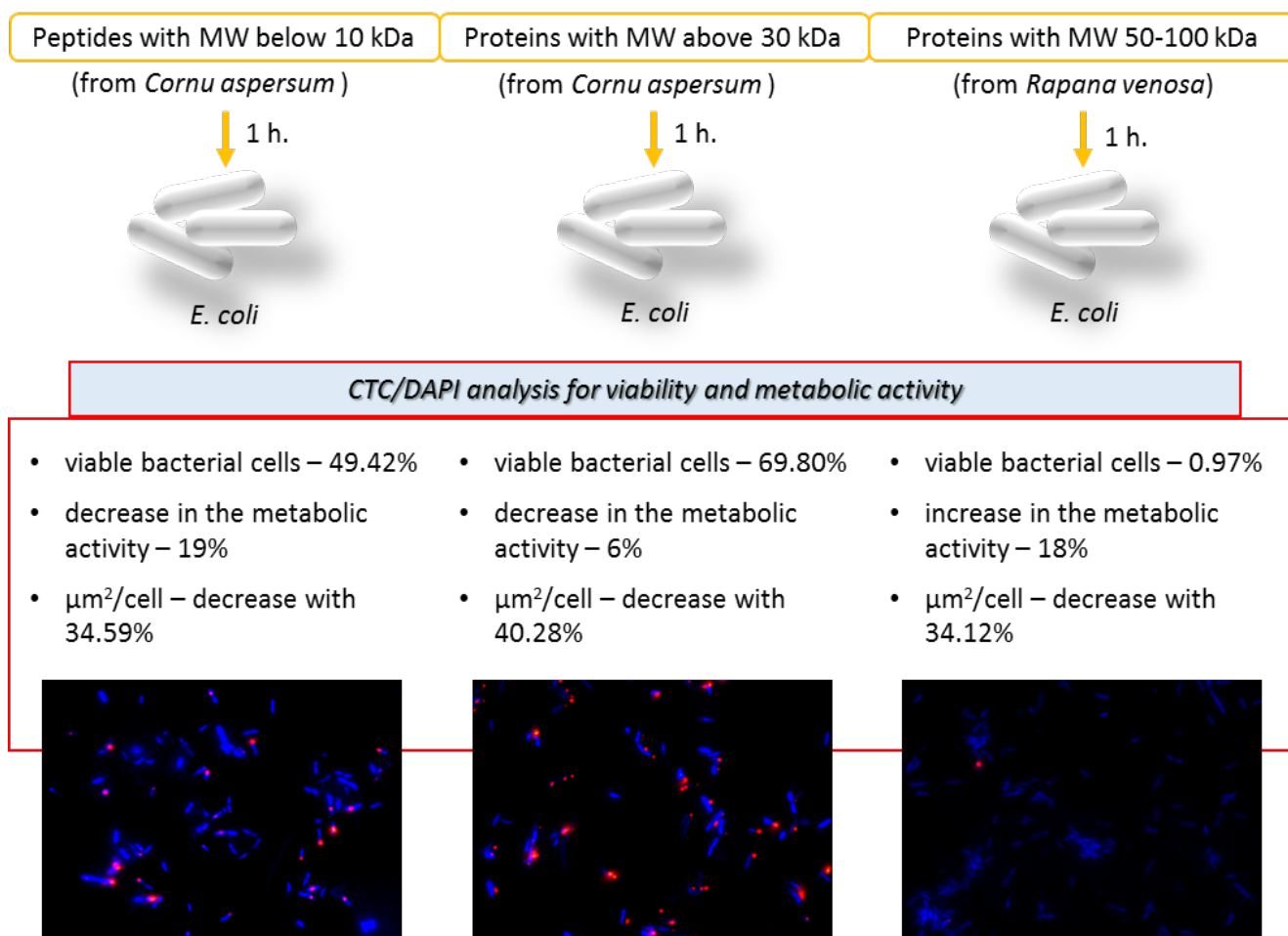
The obtained results showed that the viable bacterial cells were 69.80 % when the proteins with MW above 30 kDa was present in the media. Their part was 49.42 % when the peptides fraction with MW below 10 kDa was used. In the analyses with the proteins from *R. venosa* the quantity of the live bacteria was very low consisting only 0.97 % of the cells in the samples. In the absence of the peptides/proteins the viable (CTC stained) *E. coli* cells were 120.88 % of the DAPI stained cells highlighting the strong viability of the bacterial culture in normal conditions. The intensity of the CTC signal corresponds to the metabolic activity of the cells. A decrease in it with 19 % was found for the samples with peptides with MW below 10 kDa. For the samples with the proteins with MW above 30 kDa the value was with 6 % lower while in the presence of the proteins from *R. venosa* the live cells were with 18 % more active than the control. The digital analysis of the CTC/DAPI images permitted evaluation of the changes in the morphology of the cells of *E. coli* in presence of the biologically active compounds. The mean area per cell decreased with 36 % indicating that the bacterial cells had shrunk in presence of the peptides/proteins.

The registered changes in the *E. coli* cells in presence of the three peptide/protein fractions after 1 hour consisted of considerable decrease of the number of the live bacteria, their metabolic activity and morphological changes of the cells. All the effects demonstrated the potential of the three peptides fraction as antibacterial agents. They could have significance related to inhibition of the growth of the bacteria in critical health problems with persistent infections.

**Keywords:** *E. coli* viability, *Cornu aspersum*, peptides/proteins fraction, metabolic activity, fluorescence analysis

**Acknowledgments:** This study was supported financially by the project DH01/14/19.12.2016 "Proteomic analysis of new natural peptides, isolated from snail "Cornu aspersum" – NSF, Ministry of Education and Science, Bulgaria as well as well as was supported by funding for materials by the National Scientific Program "Innovative Low-Toxic Biologically Active Means for Precision Medicine" (BioActiveMed).

## Graphical abstract



## MODULATION OF NEUTROPHIL FUNCTIONS WITH SIRTUIN 1 ACTIVATOR IN ARTHRITIS

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<sup>2</sup>Department of Virology, TheStephanAngeloff Institute of Microbiology, BAS, 1113Sofia, Bulgaria

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Sirtuins are a family of NAD<sup>+</sup> dependent enzymes with protein deacylase activity. Sirtuin 1 (SIRT1) is a protein that can be located in the cytosol and nucleus and mitigates cellular stress through transcriptional regulation and interference with cellular metabolism. Modulation of SIRT1 activity has been proposed as a therapeutic strategy for a variety of diseases. Here, we investigated if the pharmacological activator of SIRT1 normalized the altered neutrophil functions associated with arthritis.

Arthritis was induced by i.p. injection of a cocktail of monoclonal antibodies against collagen type II in Balb/c mice (CAIA). Synthetic compound which specifically activate Sirt1 activity - SRT2183 was applied subcutaneously for 5 days at a dose of 10 mg/kg/mouse. At day 7 of CAIA we assessed the effect of Sirt1 activity modulation on clinical scores in arthritis mice. We used flow-cytometry to determine neutrophils population in circulation, bone marrow (BM) and spleen of mice with CAIA. We evaluated the *in vivo* and *in vitro* effect of SIRT2183 on several neutrophils functions characteristic for

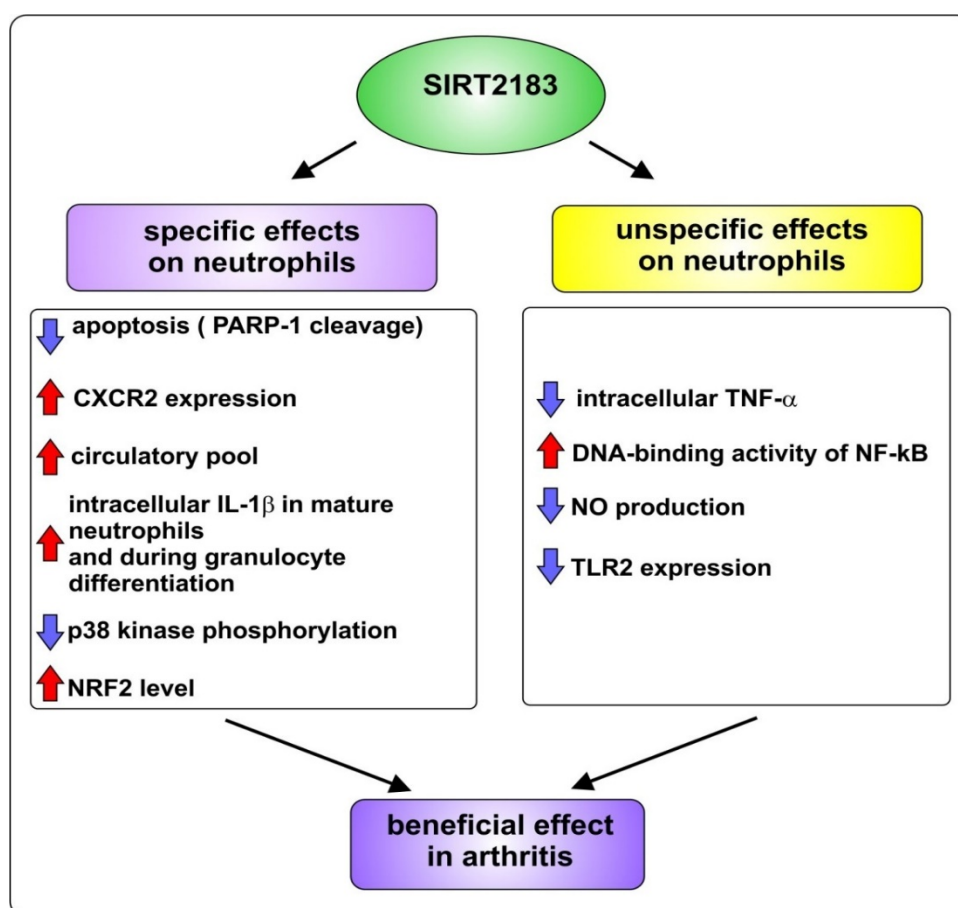
mature BM-derived neutrophil cells - expression of integrin and chemokine receptors, production of cytokines, activation of transcription factors.

SIRT2183 down-regulated CD11b and up-regulated CXCR2 in healthy blood and BM neutrophils, suggestive of neutrophil mobilization from the BM to the periphery following treatment. When arthritis was induced, the compound was beneficial for CAIA, exerting a prolonged effect after cessation of treatment. SIRT2183 was able to up-regulate IL-1b in healthy and CAIA mice, which we also observed in un-stimulated neutrophils following treatment *in vitro* but inhibited the production of TNF- $\alpha$ . We determined that Parp1 cleavage, a marker for cell death, was reduced in SIRT2183 treated BM neutrophils. The binding activity of transcription factor NF-kB was increased in neutrophils from arthritic mice was normalized.

In conclusion, Sirt1 activation appears to have beneficial effects on CAIA outcome. The potential therapeutic effect might be related with restoration of altered neutrophil homeostasis and functional state in arthritis.

**Acknowledgements** This study was supported financially by the National Scientific Program "Innovative Low-Toxic Biologically Active Means for Precision Medicine" (BioActiveMed).

### Graphical abstract



# SNAIL MUCUS PROTECTIVE EFFECT ON ETHANOL INDUCED GASTRIC ULCERS IN MICE

**Lubomir Petrov<sup>1</sup>**, Mihail Kachaunov<sup>1</sup>, Albena Alexandrova<sup>1,2</sup>, Elina Tsvetanova<sup>2</sup>, Almira Georgieva<sup>2</sup>, Pavlina Dolashka<sup>3</sup>, Aleksandar Dolashki<sup>3</sup>, Lyudmila Velkova<sup>3</sup>

<sup>1</sup>*Department of Physiology and Biochemistry, National Sports Academy, 21, Acad. Stefan Mladenov str., 1700 Sofia, Bulgaria*

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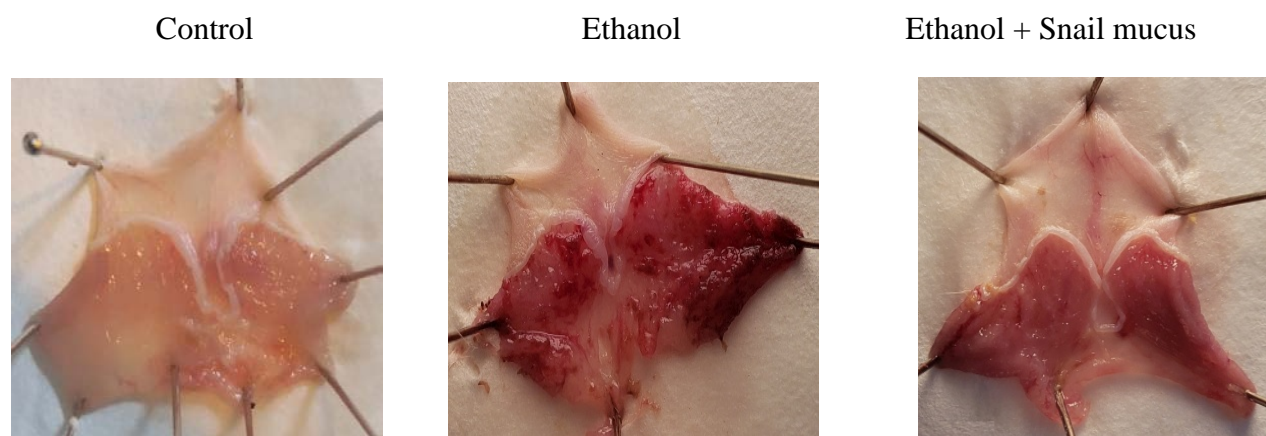
<sup>3</sup>*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 9, Georgi Bonchev str., 1113 Sofia, Bulgaria*

Nowadays, an increased interest in research into the biological effects of natural compounds with preventive or therapeutic potential for various pathological conditions was observed. Given the involvement of oxidative stress in the pathogenesis of gastric ulcer (GU), as well as the wide range of bioactive compounds isolated from snails, our study aimed to investigate the possible protective effect of *Helix aspersa* mucus on ethanol induced gastric ulcers in mice and elucidate its efficacy mechanism in terms of oxidative stress measures. Male Albino mice were divided into Control, Ethanol and Mucus+Ethanol treated groups. The GUs were induced by administration of 96% ethanol (10 ml/kg, *per os*). The mice of Mucus+Ethanol group were pre-treated with snail mucus (20 mg/kg, *per os*) one hour before ulcer induction. The administration of ethanol to mice caused grave lesions with large hemorrhagic necrosis of gastric mucosa and significant decrease of GSH levels and activities of superoxide dismutase, catalase and glutathion peroxidase. In the animals with pre-administration of snail mucus were observed: only a small number of hemorrhagic fields; significantly reduced GU index compared to ethanol group (4.7% vs 17.3% respectively) ( $P < 0.001$ ); protection percentage calculated - 73%; significant recovery of mucosal GSH level and activity of glutathione peroxidase; rise in the activities of the other tested enzymes. Thus, the protective effect of *Helix aspersa* snail mucus on both gastric mucosa and on gastric antioxidant potential in ethanol induced GU model suggests that it may serve as a good tool for treatment or concomitant treatment of this disease.

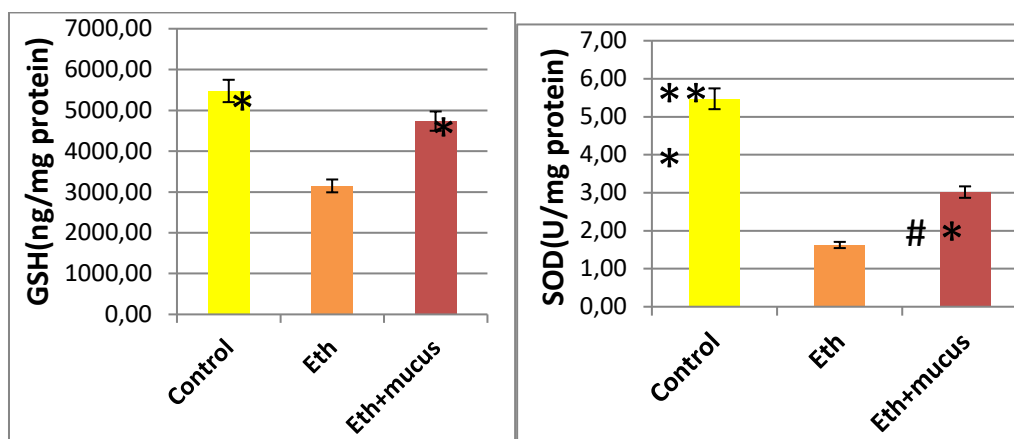
**Key words:** *Helix aspersa* mucus, oxidative stress, gastric ulcers

**Acknowledgements:** This work was supported by the Bulgarian Ministry of Education and Science [Grant D01-217/30.11.2018] under the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM#658/14.09.2018.

## Graphical abstract



Effect of Snail mucus on the macroscopic morphology in ethanol-induced GU mice: Contro - control group; Ethanol - ethanol group; Snail mucus - group pretreated with snail mucus.



GSH concentration and antioxidant enzymes activity of SOD in stomach homogenate from the three experimental groups: Control, Ethanol, Ethanol+Mucus \* -  $p < 0.05$  vs. Ethanol group; \*\* -  $p < 0.01$  vs. Ethanol group; \*\*\* -  $p < 0.001$  vs. Ethanol group, # -  $p < 0.05$  vs. Control group

### SESSION : PROTEOMIC PROFILE OF TREATED PATHOGENS USED FOR PROGNOSIS OF MECHANISMS OF ACTION OF BIOLOGICALLY ACTIVE COMPOUNDS

#### EVALUATION OF ENZYMES EXPRESSED IN HAPLOID AND DIPLOID *SACCHAROMYCES CEREVISIAE* CELLS THROUGH SDS PAGE AND 2D SDS PAGE ANALYSIS

**Asya Daskalova<sup>1</sup>**, Lyudmila Velkova<sup>1</sup>, Ventseslava Petrova<sup>2</sup>, Anna Kujumdzieva<sup>2</sup>, Anna Tomova<sup>2</sup>, Pavlina Dolashka<sup>1</sup>

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<sup>2</sup>*The Sofia University "St. Kliment Ohridski", Faculty of Biology, Bulgaria*

It is a well-known fact that the majority of all eukaryotic organisms spent their natural lives in quiescent state. *Saccharomyces cerevisiae*, being a single-celled fungus microorganism, is a well-studied eukaryote with conserved basic cell processes. That is why *S. cerevisiae* draws more specific attention to investigating stationary phase.

The main scope of the research is to identify essential enzymes expressed in *S. cerevisiae* involved in the response to oxidative stress with hydrogen peroxide ( $H_2O_2$ ) and menadione (M) during stationary phase.

Comparative analyses by SDS PAGE technique coupled with MALDI-TOF/MS was designed to search for changes in the haploid (strain BY4741) and diploid (strain 584) *S. cerevisiae* cells in quiescent state. Putative enzymes were determined through MS and MS/MS data aligned with Mascot database-Fingerprint analysis. Key enzymes were manifested by hydrogen peroxide treated diploid cells. An intensively expressed band at around 46 kDa resulted for the metallo-enzyme enolase 2 (ENO 2) included in the glycolytic pathway. Alcohol dehydrogenase 1 (ADH 1) enzyme was dominantly represented at around 36 kDa. In comparison to the diploid cells, those enzymes were down-regulated in the menadione or hydrogen peroxide treated haploid cells.

Additional analyses, by two-dimensional gel polyacrilamide gel electrophoresis (2 D SDS PAGE), were implemented for the treated and non-treated haploid *S. cerevisiae* cells. The resulting gel spots were visualized and analyzed with the image software Melanie 9™. Concerning haploid cells in quiescent state, upregulation of enzymes characterized the *S. cerevisiae* cells undergoing oxidative stress. The preliminary spot examination confirmed the presence of ADH 1 and ENO 2 in the treated with hydrogen peroxide



haploid cells in comparison to the non-treated cells (control). Further experiments will be carried out with diploid strain 584 through 2 D SDS PAGE analysis coupled with ESI Q TOF mass spectrometry.

Yeast system shows prospect for investigating changes in cell oxidant status as well as in energy metabolic activity in response to different environmental stressors.

**Keywords:** haploid and diploid cells, *Saccharomyces cerevisiae*, 2D SDS PAGE, quiescent state, “dormant stage”, MALDI-TOF/MS analysis, oxidative stress, enzymes

**Acknowledgements:** *This work was supported by the research project ДН 11/10 from 15.12.2017, funded by the Scientific Research Fund of the Bulgarian Ministry of Education and Science.*

## ANALYSIS OF INTRAVENOUS IMMUNOGLOBULIN OF SDS-PAGE WITH IMAGE QUNAT TL SOFTWARE

Aleksandar Dolashki<sup>1</sup>, Lyudmila Velkova<sup>1</sup>, Asya Daskalova<sup>1</sup>, Tchavdar Vassilev<sup>2</sup>, **Pavlina Dolashka<sup>1</sup>**

<sup>1</sup>*Institute of Organic Chemistry with Center of Phytochemistry, 9, Acad. G. Bonchev Str., 1113 Sofia, Bulgaria*

<sup>2</sup>*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

Immunoglobulins (antibodies) are glycoprotein molecules that play a key role in adaptive immunity. They protect using the hostile environment of bacteria, viruses, and parasites. Intravenous immunoglobulin (IvIg) is purified IgG preparations made from the pooled plasma from thousands of healthy donors and are being tested in preclinical mouse models. In the last years it has found that the polyspecificity could be also the result of the contact of aggressive molecules with circulating IgG *in vivo*-a modification referred to as “induced polyspecificity”. Our previous study has proved that a single intravenous administration of pooled IgG with additionally induced polyspecificity significantly improved survival with experimental sepsis and with aseptic severe inflammation in mice.

The aim of the present research is to find out if the *in vivo* newly-generated polyspecific antibodies can bind to and block at least some products of the “genomic storm” in severe generalized inflammation regardless of its causes.

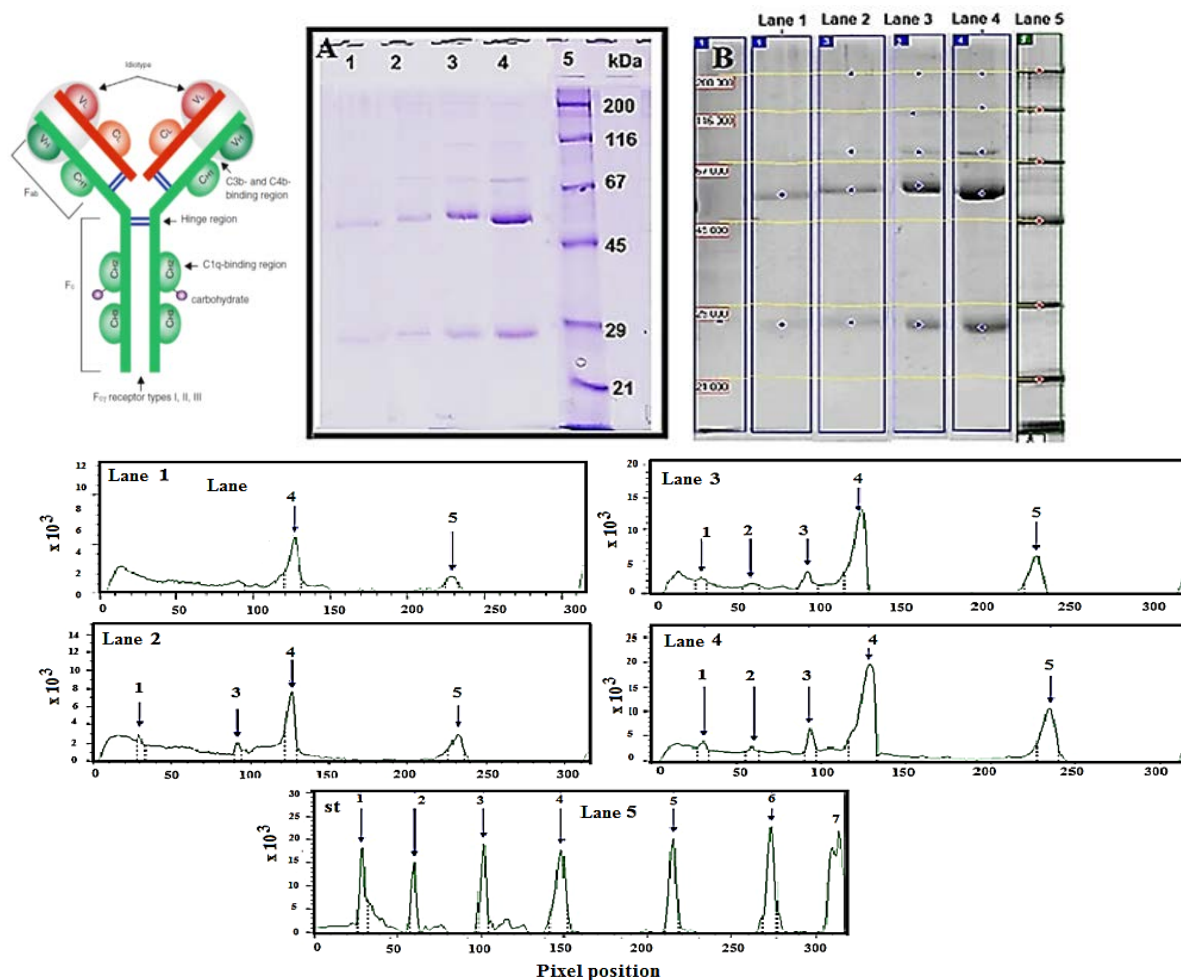
Collected plasma from mice before and 24 and 48 hours after induction of the local inflammatory response by a non-inflammatory mouse group and an inflammatory mouse group after a single intravenous administration of commercial IVIg and modified Fe+IVIg was purified through commercial human IgG purification columns. Control groups were injected with PBS only.

Using analysis intravenous immunoglobulin (IVIg) of SDS-PAGE and ImageQuant™ TL v8.2.0 software were calculated molecular masses of proteins from natural intravenous immunoglobulin (IvIg) without inflammation and after induced inflammation and of modified intravenous immunoglobulin (Fe-IvIg) without inflammation and after induced inflammation in mice. Significant changes were observed between the eluted samples of native intravenous immunoglobulin (IVIg) and of modified intravenous immunoglobulin in groups without induced inflammation. The analysis of the native IVIg shows the expression of 2 proteins with a molecular weight (MW) of 25.06 and 50.08 kDa, which probably corresponds to the heavy chain (H) and the light chain (L) of human IgG chain. There was a significant difference in the expression of 4 proteins with MW 196.96, 72.92, 52.85 and 26.20 kDa, eluted from the column IVIg after inflammation. The difference in the number of proteins expressed in sample Fe-IVIg without inflammation (MW of 193.74, 113.89, 72.21, 53.79 and 26.40 kDa) and sample Fe-IVIg with inflammation (MW of 196.87, 118.00, 71.88, 52.29 and 26.12 kDa) compared to group without inflammation treated native intravenous immunoglobulin (IVIg) were identified.

**Keywords:** intravenous immunoglobulin (IVIg), IgG, ImageQuant™ TL v8.2.0 software

**Acknowledgements:** This research was carried out with the support of a project under contract No. B 02-29/12.12.2014, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.

## Graphical abstract



Lane 1 - native intravenous immunoglobulin (IVIg) from a mouse group without induced inflammation; Lane 2 - native intravenous immunoglobulin lviIg from a mouse group with inflammation; Lane 3- modified intravenous immunoglobulin Fe-lviIg without inflammation; Lane 4-modified intravenous immunoglobulin Fe-IVIg with inflammation; Lane 5 - Serva Company Standard (10 µl quantity per the well) by ImageQuant™ TL v8.2.0. MW curve - corresponding software-generated cubic spLane standard curve with R2=0.9980.

**Calculated volumes and molecular weights of proteins from the native intravenous immunoglobulin (IVIg) without inflammation and after induced inflammation and of modified intravenous immunoglobulin (Fe-lviIg) without inflammation and after induced inflammation in mice, by ImageQuant™ TL v8.2.0.**

Band	Lane 1 lviIg		Lane 2 lviIg		Lane 3 lviIg		Lane 4 Fe-lviIg		Lane 5 Standart	
No	Cal.Vol (µg)	MW (kDa)	Cal.Vol (µg)	MW (kDa)	Cal.Vol (µg)	MW (kDa)	Cal.Vol (µg)	MW (kDa)	Cal.Vol (µg)	MW (kDa)
1	-	-	0.21	196.96	0.40	193.74	0.76	196.87	1.65	200.00
2	-	-	-	-	0.24	113.89	0.37	118.00	1.35	116.00
3	-	-	0.37	72.92	0.49	72.21	1.04	71.88	1.72	67.00
4	1.72	50.08	1.72	52.85	3.27	53.79	6.19	52.29	2.67	45.00
5	0.71	25.06	1.01	26.20	1.41	26.40	3.06	26.12	2.26	29.00
6	-	-	-	-	-	-	-	-	2.96	21.00

## PROTEOME ANALYSIS OF IMMUNOGLOBULINS ISOLATED FROM MICE WITH SEVERE INFLAMMATION, INJECTED WITH INTRAVENOUS IMMUNOGLOBULIN (IVIG)

**Lyudmila Velkova<sup>1</sup>**, Asya Daskalova<sup>1</sup>, Aleksandar Dolashki<sup>1</sup>, Dimitar Kaynarov<sup>1</sup>, Tchavdar Vassilev<sup>2</sup>, Pavlina Dolashka<sup>1</sup>

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Recent studies have shown, however, that polyspecific antibodies play an important role in the first-line innate defense against the dissemination of pathogens in the pre-immune host. Interestingly, their antigen-binding polyspecificity could be not only inherent, but also inducible.

The aim of the proteome analysis is to find out if the in vivo newly-generated polyspecific antibodies can bind to and block at least some products of the “genomic storm” in severe generalized inflammation experimental sepsis and with aseptic severe inflammation in mice.

The proteome analysis, of immunoglobulins isolated from mice without inflammation and after induced inflammation as well as immunoglobulins isolated from mice injected with modified intravenous immunoglobulin (Fe-IvIg) without inflammation and after induced inflammation, were conducted on one-dimensional gel electrophoresis (1D SDS-PAGE). The proteins were identified after tryptic digestion of cut off gel bands by mass spectrometry (MALDI-TOF-MS and MALDI-TOF-MS/MS). MALDI-MS specters were analyzed by Mascot Server - Peptide Mass Fingerprint. The analysis of MALDI-MS/MS specters confirmed the determined proteins.

The results from proteome analysis showed that the mice injected with IvIg and modified intravenous immunoglobulin (Fe-IvIg) after induced inflammation enhanced their antigen-binding polyspecificity that included binding to at pro-inflammatory cytokines, interleukins and antibodies. The latter finding suggested that the intravenous immunoglobulin and modified pooled IgG preparations could have a therapeutic effect when administered in experimental severe generalized inflammation (sepsis).

**Keywords:** intravenous immunoglobulin (IvIg), modified intravenous immunoglobulin (Fe-IvIg), severe inflammation in mice, IgG, proteome analysis.

**Acknowledgements:** *This research was carried out with the support of a project under contract No. B 02-29/12.12.2014, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.*

## TREATMENT OF HARD TO HEAL WOUND AND CHRONIC WOUNDS - A NEW APPROACH WITH NATURAL SUBSTANCES

**Momchil Kermedchiev<sup>1,2</sup>**, Irina Michael<sup>1</sup>, Evgenia Peneva<sup>1</sup>, Radka Lazarova<sup>1</sup>, Stela Jordanova<sup>1</sup>, Pavlina Dolashka<sup>2</sup>

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2 Institute of Organic Chemistry with Center for Phytochemistry, BAS, Sofia, **Bulgaria**

Some wounds do not heal normally and present a challenge. They can be considered difficult to heal and chronic wounds. There are many reasons for such wounds and when they occur, the physician must take into account all factors related to wound. We need to take appropriate and timely measures to correct the underlying cause for difficult to heal wound and it should be treated with a new methodology.

Knowledge of the therapeutic action of mucus from snails and various kinds of medicinal herbs in the treatment of wounds have been known since the time of the known written story reached us. Snail farms and their use in the treatment of wounds are described in Egyptian cryptograms and papyri, Pliny

the Elder (23 AD) writes that snails are "a sovereign means of treating pain associated with burns, abscesses and other wounds".

Patients with difficult to heal and chronic wounds of different age and genesis (Diabetic gangrene, Chronic venous insufficiency, Chronic arterial insufficiency and pressure ulcers), as well as with different comorbidities, were selected prospectively of different sexes and ages.

Applied is the same prospective new protocol, new wound antisepsis approach, new pace and type of dressings, treatment plan, training the patient and kinsfolk to self-help and dressings. Results of a number of parameters were reported during the treatment process: Local wound status, pH-metry, local inflammatory process, bacterial flora, pain level, healing rate. The new approach used in the treatment of difficult to heal wounds with snail mucus from *Helix Aspersa* and herbal essential oils shows promising results.

There is a significant reduction in the time for wound healing, rapid and effective reduction of the local inflammatory process and chronic pain. Improving the general and psycho-emotional state of the patient, significantly improving the quality of life. The obtained results suggest that the healing of such wounds is promoted not only by limiting bacterial infection, but also by stimulating the growth of tissues and providing them with an adequate local alkaline-acid, moist and nutritious environment. Early and regular application of our proposed new approach to treatment will lead to the prevention of amputation of the legs and would serve as a basic therapy for the treatment of chronic wounds.

**Keywords:** *Hard to heal wounds, chronic wounds, treatment, snail Helix Aspersa, Essential oils.*

## **ACKNOWLEDGEMENTS**

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# POSTERS

## PROTECTIVE EFFECT OF MICROPOROUS NATURAL CLINOPTILOLITE ON SUBCHRONIC HEAVY METAL INTOXICATION IN RATS

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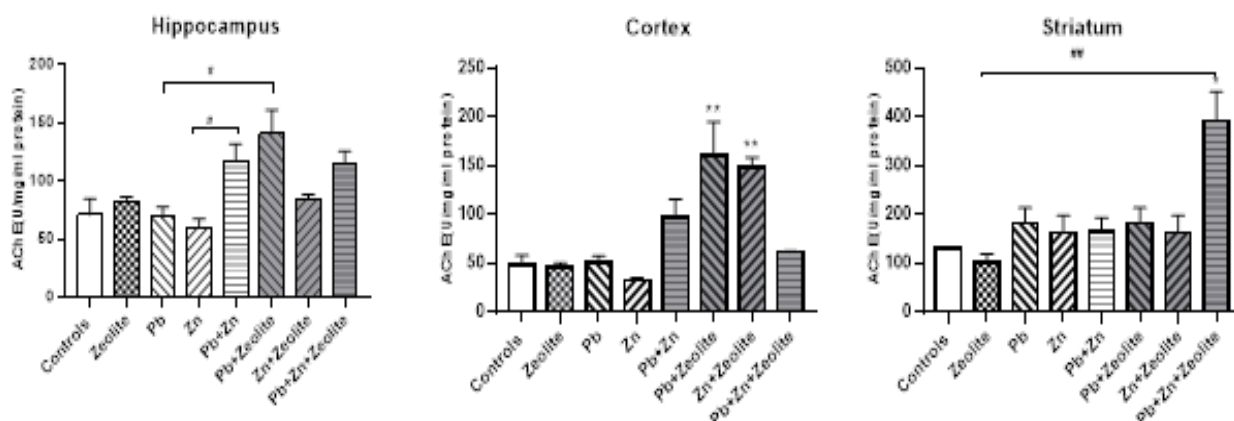
Zeolites are hydrated natural or synthetic microporous crystals with well-defined structures containing  $\text{AlO}_4$  and  $\text{SiO}_4$  tetrahedra. Zeolite use in medicine is a relatively recent subject of interest. Dietary zeolite supplementation reduces oxidative damage and plaque generation in the brain of an Alzheimer's disease mouse model.

The substantial role of zeolite as a factor essentially reducing Pb, Zn and their combination bio-accumulation is established in some experiments. But how Zeolite affects the cholinergic function changed by heavy metals (HM) exposure remains unclear.

Aim of this study was to assess the effect of Zeolite on Acetylcholin esterase activity in rats with subchronic HM intoxication by Pb, Zn and their combination

Male Wistar rats were treated subchronically with HM salts ( $\text{ZnCl}_2$  and  $\text{Pb}(\text{CH}_3\text{COO})_2$  and their combination) 100 mg/kg in drinking water for 30 days. Half of the animals received also 2% Zeolite for 30 days with their regular food. Control group drink clean water. On the 24<sup>th</sup> hour after the last HM salts intake and Zeolite respectively, three brain structures cortex, hippocampus and striatum were isolated and the specific AChE activity was measured according Ellman's method (1961).

Results demonstrated that subchronic HM intoxication decreased AChE activity in the brain of heavy metals-exposed rats, indication for cholinergic synapses deficiency, most pronounced in hippocampus and cortex. Everyday Zeolite administration normalized AChE activity in the cortex, changed by Zn and combination. In striatum only the combination of Pb and Zn salts and Zeolite significantly increase the AChE activity.



Our results demonstrated restoring effect of Zeolite on AChE activity- reduced by heavy metals subchronic administration in some brain structures. The mechanisms of detoxifying effect of Zeolite against HM toxicity deserve further studies.

**Keywords:** Zeolite, heavy metals, toxicity, acetylcholine esterase

**Acknowledgments:** This work was in part supported by Farer EOD. We would like to thank to Mr. Slavcho Slavov for his technical assistance in our experiments.

## POTENTIAL APPLICATION IN MEDICINE OF BIOACTIVE COMPOUNDS FROM MARINE SNAIL *RAPANA VENOSA*

Lyudmila Velkova<sup>1</sup>, Spiro Konstantinov<sup>2</sup>, Aleksandar Dolashki<sup>1</sup>, Ventseslav Aatanasov<sup>1</sup>, Pavlina Dolashka<sup>1</sup>

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The priority for the next years will be centered on investigation of alternative biomolecules with a new mechanism of action for therapy and enhancing human longevity. The aim of this study was to investigate the anti-tumor effect of different fractions from hemolymph of *Rapana venosa* which is a complex mixture from various bioactive substances.

The hemolymph from *R. venosa*, was separated into several fractions, by ultrafiltration using membranes with different pore sizes. The native molecule of *R. venosa* hemocyanin (RvH) and its two structural subunits RvH1 and RvH2 were isolated and purified from fraction >100 kDa. The isolated components: RvH, RvH1 and RvH2, fraction below 10 kDa and fraction between 10-50 kDa were investigated *in vitro* for antitumor activity of human cell lines: bladder cancer (T-24) and cutaneous T-cell lymphomas (MJ, Mycosis fungoides and HuT-78, Sézary syndrome).

The results revealed a significant cytotoxic effect of structural subunit RvH2 against bladder cancer (tumor cells line T-24). The most effective inhibition against cutaneous T-cell lymphomas (MJ, Mycosis fungoides) was observed after treatment with fraction, containing bioactive components with Mw between 10-50 kDa from hemolymph of *R. venosa*.

The absence of toxic effects on *R. venosa* extracts makes them promising candidates for topical application in the bladder before and after transurethral resection. Our results may be considered as basic information for further investigations on bioactive compounds from hemolymph of *R. venosa* for creating new natural products with potential biomedical applications.

**Keywords:** *marine snail Rapana venosa, hemolymph, R. venosa hemocyanin, anti-tumor activity*

**Acknowledgments:** *This work was supported by the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM № 658/14.09.2018 funded by the Bulgarian Ministry of Education and Science*

## BIOACTIVE COMPOUNDS FROM *RAPANA VENOSA* AND *HELIX ASPERSA* WITH ANTITUMOR PROPERTIES AGAINST TWO CANCER CELL LINES

Ventseslav Aatanasov<sup>1</sup>, Maria Petrova<sup>2</sup>, Jordana Todorova<sup>2</sup>, Maria Schröder<sup>2</sup>, Aleksandar Dolashki<sup>1</sup>, Lyudmila Velkova<sup>1</sup>, Iva Ugrinova<sup>2</sup>, Pavlina Dolashka<sup>1</sup>

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The object of our investigation are the hemolymph from marine snail *Rapana venosa* (living in Black sea) and the mucus from garden snail *Helix aspersa*, which are rich in various bioactive substances with potential application in medicine. The present study is the first report, which showed *in vitro* investigation and aimed to evaluate the antitumor activity of biologically active compounds from the hemolymph of marine snail *R. venosa* and from the mucus of garden snail *Helix aspersa* onto a panel of human breast cancer cell lines which differed by origin and by genetic profile. Breast cancer is the most common malignancy among women. Reduced cell viability was observed in both MCF-7 and MDA-MB-231 breast cancer cell lines after treatment with low concentrations of two mucus fractions from *H. aspersa* with Mw above 20 kDa and above 50 kDa, fraction with Mw between 50 and 100 kDa from the hemolymph from marine snail *R. venosa* and two isoforms (RvH1 and RvH2) of *R. venosa* hemocyanin. Interestingly lower toxicity was observed in MCF-10A cells which are commonly used as a model of



normal breast cells. The highest antiproliferative effect was established for the fraction with Mw between 50-100 kDa, isolated from hemolymph of *R. venosa*. Chemotherapy drugs cis-Platin and Tamoxifen were used alone and in combination with the active fractions from the hemolymph of *R. venosa* and the mucus of *H. aspersa* to find synergistic effect of action against the tumor cell lines. The results revealed that the synergistic anti-proliferative effect was highest when using the active fraction between 50-100 kDa (RvH1) from hemolymph of *R. venosa* in combination with cis Pt for the treatment of MDA-MB-231 (triple negative) cell line. Microscopic observations on MDA-MB-231 cells showed substantially disturbed morphology after treatment with the fraction from hemolymph RvH1 in combination with cis-Platin in comparison to non-treated cells or treated with RvH1 alone. These results may be considered as important information for further investigations of anticancer activity of novel bioactive compounds from hemolymph of marine snail *R. venosa*.

**Keywords:** matine snail *Rapana venosa*, garden snail *Helix aspersa*, MCF-7 and MDA-MB-231 breast cancer cell lines

**Acknowledgements:** This work was supported by the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM № 658/14.09.2018 funded by the Bulgarian Ministry of Education and Science.

## NEW ADVANCES IN ANALYSIS OF NATURAL EXTRACTS FROM MOLLUSCS

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Extracts and different biologically active compounds from several organisms were purified and analyzed either by one-dimensional or two-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with the target to find a mechanism of action for their antitumoral and antimicrobial effects. Hemolymph and mucus of *Cornu aspersum*, *Helix lucorum* and *Rapana venosa* are covered. Information about the molecular weight (MW), the amount and the isoelectric point (pI) of several protein spots were determined by using electrophoresis analyzation programs, which are further investigated.

**Keywords:** SDS-PAGE, natural extracts, protein analyzation software

**Acknowledgements:** This work was supported by the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)” approved by DCM № 658/14.09.2018 funded by the Bulgarian Ministry of Education and Science.

## IN THE SEARCH FOR ANTIFUNGAL ACTIVITY IN HEMOLYMPH, ISOLATED FROM MOLLUSCS AND ARTHROPODS

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Antibacterial and antifungal activity of bioactive compounds is a new direction of scientific searching. Mollusks have been known as a source of novel natural products with antibacterial activity.

But the compounds exhibiting antifungal effect are very rare found. At the same time different species, belonging to genera *Aspergillus*, *Mucor*, *Penicillium*, *Cladosporium*, *Fusarium*, and *Alternaria* have been reported as etiological agents of respiratory disorders, onychomycosis, asthma with fungal sensitization, etc.

The aim of the present study was to investigate the fungicidal and fungistatic activity of hemolymph fractions isolated from *Rapana venosa*, *Carcinus aestuarii*, and *Helix lucorum* against strains belonging to above mentioned genera.

The hemolymph from *R. venosa*, *C. aestuarii*, and *H. lucorum* was collected and purified using different membrane techniques. The crude extract was separated using Millipore filters by ultrafiltration into different fractions. Ten hemolymph fractions were tested for their antifungal activity against fungi belonging to 5 genera: *Mucor hiemalis*, *Fusarium oxysporum*, *Aspergillus niger*, *Penicillium tubingensis*, and *Alternaria solani*.

Clear inhibitory effect was established after treatment with dissociated hemocyanin from *C. aestuarii*, hemolymph fraction < 100 kDa from *R. venosa* and fraction < 100 kDa from *H. lucorum*. The microplate assay confirmed antifungal activity of mentioned fractions. Antifungal activity was species dependent. These three fractions demonstrated strong fungicidal effect for a long time, for 7 days in concentration 12 µL/well.

Dissociated hemocyanin from *C. aestuarii* and peptide fraction < 100 kDa from *R. venosa* could be perspective compounds for the next investigations.

**Keywords:** mollusks, arthropods, hemolymph, antifungal activity, *Rapana venosa*, *Helix lucorum*, *Carcinus aestuarii*

**Acknowledgements:** This work was supported by the Bulgarian Ministry of Education and Science under the National Research Program “BioActivMed” approved by DCM № 658/14.09.2018.

## COMPARISON OF THE EFFECTS OF NATURAL VANILLIN AND NEUROTENSIN ANALOGUE ON SOME AFFECTIVE SYMPTOMS IN EXPERIMENTAL MODEL OF PARKINSON DISEASE

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Vanillin, the most used flavor agent, is phenolic aldehyde (Fig. 1A) which naturally occurs in many plants from genus *Vanilla* (Orchidaceae) and it has implications as an antioxidant in some experimental models of disease with high oxidative stress. Besides vanillin has been used as precursor of some catecholamine neuromediators as dopamine. Therefore it is expected to impact some of these neuromediators' networks, which partially is confirmed by some recent studies.

Neurotensin is natural neuromodulator. Some of its receptors are closely associated with dopamine receptors. However, the natural neurotensin has relatively short lifetime in the body and limited bioavailability which makes it unsuitable for clinical application. Therefore, its synthetic functional analogues are highly looked for.

Parkinson's disease is the second most common neurodegenerative disease whose classical symptoms are movement disturbances due to degradation of the dopaminergic system. However, in the recent decades data have been accumulated regarding significant affective impairments as depression, apathy etc. probably related to the mesocorticolimbic dopaminergic system disturbance.

The aim of the present study was to evaluate some effects of vanillin and neurotensin analogue on motor and affective behavior in experimental model of Parkinson's disease.

The neurotensin analogue was synthesized by using standard solid-phase method on a Wang resin with a Fmoc/Boc strategy as described in our previous studies (Poapatanasov, et al. 2017). The neurotensin analogue closely resembles the structure of the active part of the neurotensin – 8-13 fragment, except at the C-terminal Arg is replaced with the unnatural amino acid canavanine and lysine (Fig. 1B).

The rat model of Parkinson's disease was induced by intrastriatal injection of 6-OHDA. The animals were treated for 5 days with intraperitoneal injections of either vanillin (at dose 20 mg/kg) or neurotensin analogue (at dose 5 mg/kg) delivered once per day. Motor and affective behavior tests (Xu et al. 2015) were performed. There was improvement in some of the assessed motor (Fig. 2A) and non-motor symptoms (Fig. 2B) in the treated animals with either vanillin or neurotensin analogue in comparison to the non-treated rats.

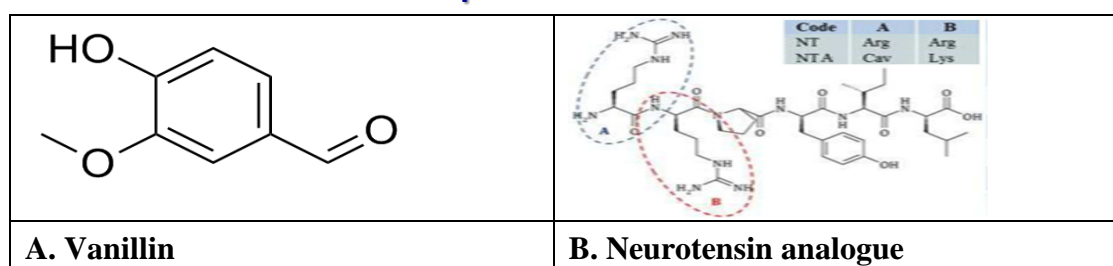
Probably this improvement in the neurotensin-treated group is related to the impact of the neurotensin-3 receptors which are involved in the regulation of some neuro-inflammatory processes. As for the vanillin its effects can be related directly to its anti-oxidant properties, its affinity to vanillin type or dopamine type receptors or its ability to elevate serotonin and dopamine in brain tissue as some recent studies revealed (Xu et al. 2015).

Based on the results obtained the substances deserve further evaluation for possible application in the management of some non-motor symptoms of Parkinson's disease.

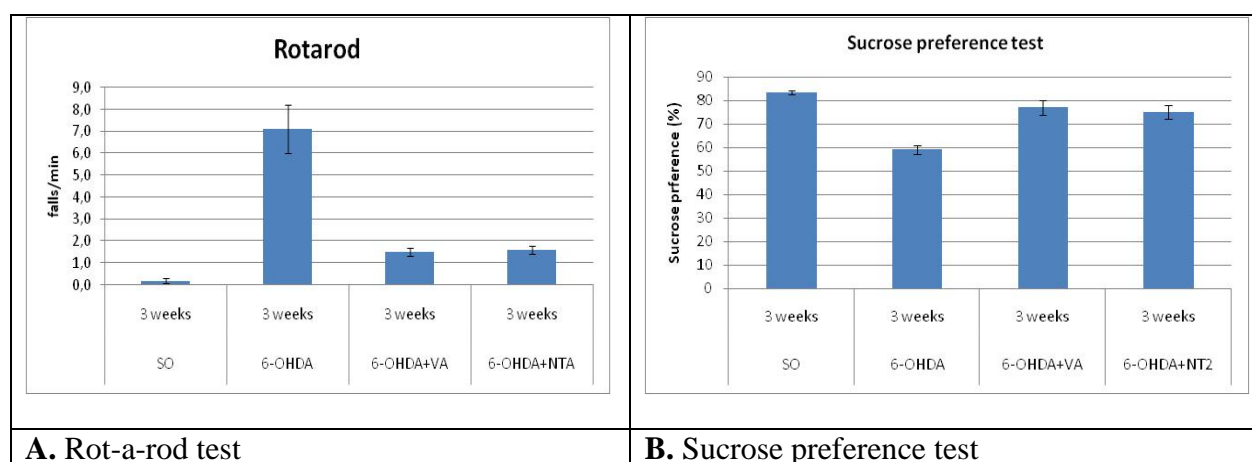
**Key words:** Vanillin, neurotensin, Parkinson's disease

**Acknowledgments:** This study was supported by the International Scientific Cooperation between Tel Aviv University in Israel and Institute of Neurobiology at the Bulgarian Academy of Sciences under joint research project "Directed synthesis of new neurotensin analogue and studies on its preventive effects in experimental rat models of Alzheimer's disease".

### Graphical abstract



**Fig. 1.** Two-dimensional chemical structure of the used substances



**Fig. 2.** Behavioral tests three weeks after the surgery

# ANTI-HERPES ACTIVITY OF DIFFERENT HEMOLYMPHATIC COMPOUNDS FROM CLASSES GASTROPODA AND MALACOSTRACA

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By our study – more than 12 extracts from *Carcinus aestuarii*, *Helix aspera*, *Helix lucorum*, *Rapana venosa* and *Eriphia verrucosa* have been thoroughly investigated and their antiviral activity against human viral models have been studied. A complete set for screening of natural products have been observed for detailed study ranging from the replication of the virus models into the host cell to the virucidal activity in the extracellular environment and first stages of viral life cycle -adsorption and penetration.

The aim of this comprehensive research was to determine not only the immediate effect over the virus but its influence over the stages of viral replication and mechanism of its action. More than 20 efficiencies have been observed with strongest candidate for in-depth study can be picked – the hemolymph from *Rapana venosa* 30-100 kDa.

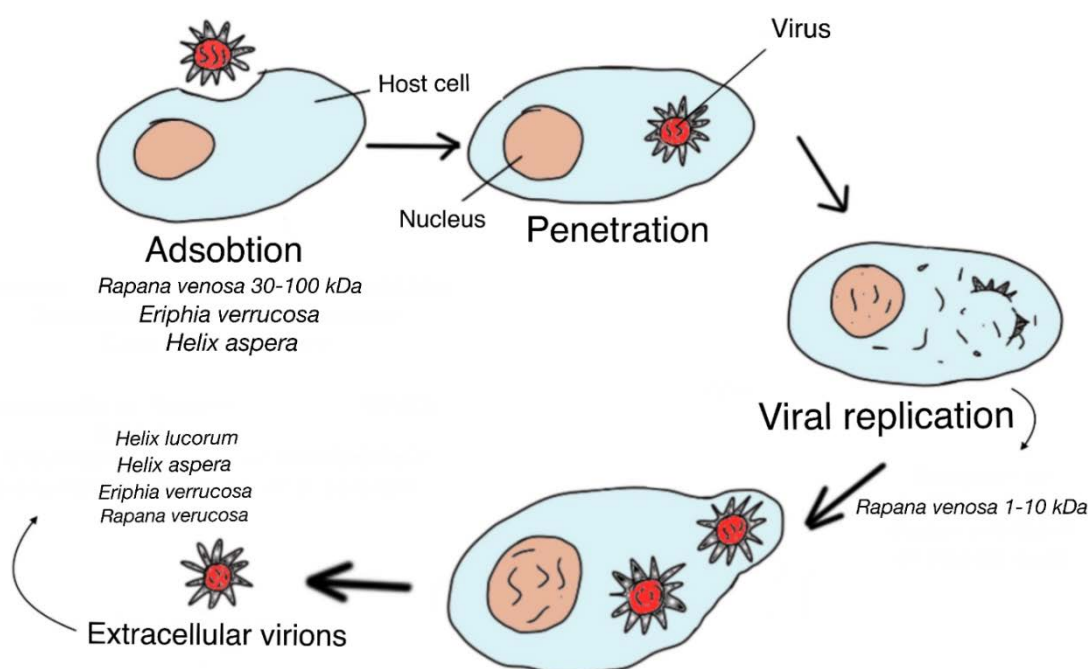
Pharmacological potential of this extract is determined by its overall stronger activity (more than 99% inactivation of HSV-1) and 99,53% activity against viral adsorption. This activity is observed in very low concentrations – 2  $\mu$ g/ml for rare side effects.

The research of mollusk extracts is a result of long-term cooperation between BAS and Sofia University. This comprehensive study has great scientific value and can be used as a foundation for future research in the field.

**Keywords:** hemocyanin, HHV-1, HHV-2, antiviral activities

**Acknowledgments:** This work was supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme “Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)”.

## Graphical abstract



## PROAPOPTOTIC ACTIVITY OF MOLLUSCAN HEMOCYANINS ON EHRlich ASCITES CARCINOMA CELL LINE

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The search for novel safe and effective antitumor therapeutics with natural origin is one of the main trends of contemporary oncology research. In the present study, a permanent cell line established from Ehrlich Ascites Carcinoma in mice was used as *in vitro* model system to assess the antitumor activity of total hemocyanins isolated from *Helix aspersa*, *Helix lucorum* (HaH-total and HIH-total) and their subunits ( $\alpha$ -HaH;  $\beta$ c-HaH;  $\alpha$ -HIH;  $\beta$ c-HIH), subunits of *Rapana venosa* hemocyanin (RvH I and RvH II) and *Helix aspersa* mucus. The viability and proliferation of hemocyanin-treated tumor cells were evaluated by a standard colorimetric MTT test. The selectivity of the antiproliferative and cytotoxic effects of the tested samples was assessed in parallel experiments on the nontumorigenic cell line of mice embryonal fibroblasts Balb/c3T3. The proapoptotic activity of the tested bioactive substances was analyzed by double fluorochroming with acridine orange and ethidium bromide (AO/EB) and the alterations in the nuclear morphology of the tumor cells was studied by DAPI staining. The results obtained indicated that the subunits  $\alpha$ -HaH,  $\beta$ c-HIH and *Helix aspersa* mucus show highest *in vitro* antitumor activity of all tested bioactive substances. The fluorescence microscopy analysis of the hemocyanin-treated Ehrlich ascites carcinoma cells revealed typical morphological features of apoptosis, such as cell membrane blebbing, chromatin condensation and margination, nuclear fragmentation and formation of apoptotic bodies. These findings lay grounds for further studies of molluscan hemocyanins as potential anticancer therapeutic agents.

**Keywords:** hemocyanins, Ehrlich ascites carcinoma, antiproliferative activity, apoptosis

**Acknowledgement:** This study was supported by contract ДО1-358/17.12.2020 г., Program "Innovative Low-Toxic and Biologically Active Means for Precision Medicine" – BioActiveMed. Ministry of Education and Science of the Republic of Bulgaria.

## EFFECT OF TRANSIENT TEMPERATURE DOWNSHIFT ON OXIDATIVE CELL RESPONSE OF *PENICILLIUM GRISEOFULVUM* ISOLATED FROM ANTARCTICA

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Antarctic fungi are capable of developing at extreme low temperatures using specific adaptive strategies. The increasing antioxidant defense is one such strategy. There are experimental evidences to conclude that the protection to low temperature includes changes in oxidative stress biomarkers and antioxidant enzyme activity. However, there is a lack of studies on the relationship between the transient temperature downshift and oxidative cell response in low eukaryotes such as filamentous fungi.

Here we compared the effect of short-term temperature downshift from optimal temperature to 15 or 6°C on antioxidant cell response in Antarctic fungus *Penicillium griseofulvum*. The results indicated typical oxidative stress reactions: significant reduction of biomass production; increase in the levels of oxidative damaged proteins, lipid peroxidation and accumulation of storage carbohydrates (glycogen and



trehalose) in comparison to growth at optimal temperature. In addition, significantly enhanced superoxide dismutase and catalase activity was evaluated. This response is cold-shock degree-dependent. During the phase of temperature recovery, cells resumed growth and protein synthesis, but maintained high level of reserve carbohydrates, damaged proteins, and antioxidant enzyme activity compared to the control.

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