

ANTI-TUMOUR ACTIVITY OF BIOACTIVE COMPOUNDS
ISOLATED FROM THE HEMOLYMPH AND MUCUS OF
THE GARDEN SNAIL *HELIX ASPERSA* AGAINST A
PANEL OF HUMAN CANCER CELL LINES

Maria Petrova, Zlatina Vlahova, Maria Schröder,
Alexander Tzintzarov, Lyudmila Velkova*, Dimitar Kaynarov*,
Aleksandar Dolashki*, Pavlina Dolashka*, Iva Ugrinova[#]

Received on June 2, 2023

Presented by I. Ivanov, Member of BAS, on June 27, 2023

Abstract

Cancer remains a significant global health concern, necessitating the search for new effective and safe anti-cancer agents. Natural products derived from plants and animals, including mollusks like snails, have gained attention as potential sources of novel anti-cancer compounds. Haemocyanins (Hcs), large copper-containing glycoproteins involved in oxygen transport in mollusks, have shown promise as anti-cancer agents. This study focuses on evaluating the in vitro anti-tumour efficacy of bioactive substances obtained from the hemolymph of the garden snail *Helix aspersa* on various human cancer cell lines representing different types of cancer. The results demonstrate that certain hemocyanin subunits from *H. aspersa* exhibit cytotoxic activity comparable to cisplatin, a widely used chemotherapy drug. Additional assays confirm the cytotoxic effects of the tested substances on cancer cells. The study underscores the potential of natural compounds from *H. aspersa* as alternative therapeutic agents for cancer treatment, while highlighting the need for further investigation. The

[#]Corresponding author.

This research was supported by the Bulgarian Ministry of Education and Science (Grant DO1-217/30.11.2018) under the National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)" approved by DO1-323/18.12.2019, DO1-358/17.12.2020 and DO1-278/03.12.2021.

DOI:10.7546/CRABS.2023.09.05

identification of specific proteins responsible for the observed anti-proliferative effects in the mucus of *H. aspersa* provides insights for the development of novel cancer therapies.

Key words: cancer, natural products, *Helix aspersa*, mucus, hemocyanin

Introduction. Cancer remains a major global health problem and despite advances in therapy, the search for new effective and safe anti-cancer agents remains a priority in medical research. In recent years, natural products derived from plants and animals have attracted much attention as potential sources of novel anti-cancer agents. In particular, mollusks, such as snails, have been shown to produce a variety of bioactive compounds with diverse properties, including anti-tumour and immunomodulatory activities. The snail mucus consists of complex multi-component mixtures containing a variety of biochemical and pharmacologically active substances with diverse masses and properties.

One class of compounds with promising anti-cancer activity that has been isolated from snails is hemocyanins (Hcs) [1–4]. Hemocyanins are large, copper-containing glycoproteins that are involved in oxygen transport in mollusks. They vary in molecular masses, quaternary structures, carbohydrate content, and composition. Some hemocyanins from mollusks have significant immunological and anti-tumour potential. Our previous studies have demonstrated that isoforms and functional units of hemocyanins from *Helix lucorum* (HlH) (previously called *Helix vulgaris* (HvH)) and hemolymph fraction with molecular weight 50–100 kDa from *Rapana venosa* have anti-tumour activity and immunological properties [5,6]. In particular, HlH and *Helix aspersa* (HaH) hemocyanins have been shown to have an anti-proliferative effect on CAL-29 and T-24 bladder cancer cell lines. The anti-tumour properties of HlH were found to be superior to keyhole limpet haemocyanin (KLH) investigated in clinical studies as a potential therapy for bladder cancer [7–9].

Some hemocyanins from molluscs have significant immunological and anti-tumour potential [10–12]. Research on snail mucus has established its antimicrobial properties and efficacy in wound healing, both in vitro and in clinical studies [13–15]. However, the potential anti-tumour effect of snail secretion was poorly investigated until recently. An in vitro study showed that snail slime from the *Helix aspersa* maxima species could not only treat melanogenesis but also provide anti-tumour activity against human melanoma cells [16]. Two fractions of mucus of another snail, the giant African snail *Achatina fulica*, decreased the viability of breast cancer cells (MCF-7) [17]. In turn, the mucus of *Actinia equina* exhibited a cytotoxic effect on human erythromyeloid leukemia-derived cells (K592) [18]. Extracts from tissues of *H. aspersa* were also shown to possess anti-cancer activity against breast cancer cells (Hs578T) [19]. Recently, the influence of extracts from mucus and their fractions of different molecular weights on the viability of Caco-2 cells have been evaluated [20].

The objective of this study is to assess the in vitro anti-tumour efficacy of bioactive substances obtained from the hemolymph of *H. aspersa*, a garden snail. The evaluation is performed on a diverse range of human cancer cell lines originating from different types of cancer. Specifically, MDA-MB-231 and MCF-7 cells represent human breast cancer, A549 and H1299 cells represent lung cancer, HeLa cells represent cervical cancer, A375 cells represent skin cancer, and SCC-9 cells represent head and neck cancer. These cell lines not only possess varied origins but also exhibit distinct genetic profiles.

Materials and methods. Cell cultures. All of the cell lines were obtained from ATCC, LGC Standards and cultivated in conditions of 5% CO₂ at 37 °C. To all basic growth media, 10% fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin (Thermo Fisher Scientific) were added. Basal mediums and some important characteristics of each human cancer cell lines used in this study are summarized in Table 1.

T a b l e 1

Cell line	Basal medium	Origin and basic characteristics
MDA-MB-231	Leibovitz's L-15 Medium	Triple-negative breast cancer cell line which is characterized by lack of expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptors (HER2). The cells expressed mutated TP53 gene and functional inactive p53 protein.
MCF-7	Eagle's Minimum Essential Medium, with 0.01 mg/ml human recombinant insulin	Breast cancer cell line with functional p53 gene and estrogen receptor (ER) expression.
A549	F-12K Medium	Epithelial-like lung cancer cell line, p53 expression. Non-small cell lung cancer.
H1299	RPMI-1640 Medium	Epithelial-like lung cancer cell line, homozygous partial deletion of the p53 protein, lack of expression of p53 protein. Non-small cell lung cancer.
HeLa	Eagle's Minimum Essential Medium	Adenocarcinoma. TP53 expression was reported to be low, and normal levels of pRB (retinoblastoma suppressor) were found.
A375	Dulbecco's Modified Eagle's Medium	Malignant melanoma, epithelial morphology.
SCC-9	Dulbecco's Modified Eagle's Medium: F12	Carcinoma, squamous cell, tongue.

Isolation of biologically active substances (BAS) from garden snail *H. aspersa*. The mucus was collected from the foot of *H. aspersa* snails, grown in Bulgarian eco-farms. For mucus production, each snail was stimulated by gently prodding the extended foot muscles with a glass rod. After a several step purification and homogenization process, subject of patent, including filtration

and centrifugation for removal of rough particles, the crude mucus extract was obtained [13]. The crude mucus extract was separated by ultrafiltration using Centrifugal Filter Units with different pore size (Amicon® Ultra-15 Centrifugal Filter Units 50K MWCO and Thermo Scientific™Pierce™Protein Concentrator PES, 20K MWCO) into two fractions containing compounds with Mw above 20 kDa (with concentration 1.5 mg/ml protein) and with Mw above 50 kDa (with concentration 1.7 mg/ml protein). Hemocyanin subunits α -HaH and β c-HaH were isolated from *H. aspersa* hemolymph as previously described [8].

SDS-PAGE electrophoresis. Protein fractions were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with the molecular weight marker ranging from 250 kDa to 10 kDa using a 5% stacking gel and 12% resolving gel, according to the Laemmli method. Equal volumes containing approximately 20 μ g/lane of the samples dissolved in Laemmli sample buffer and protein standard mixture (Precision Plus Protein™Standard All Blue, Bio-Rad Laboratories, Germany) were separated and visualized by staining with Coomassie Brilliant Blue G-250.

Glycosylation screening. Isolated fractions from the mucus of garden snail *H. aspersa* were analyzed with the orcinol-sulphuric test to determine the carbohydrate content. About 2 μ l of the purified samples were applied to a thin layer plate and air dried. The plate was sprayed with orcinol/H₂SO₄ and heated for 20 min at 100 °. The orcinol/H₂SO₄ solution contained 0.02 g of orcinol, 20% H₂SO₄, and H₂O to a total volume of 10 ml.

Mass spectrometry analysis. The molecular masses of the isolated fractions were measured by an Autoflex™III, High-Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics) which uses a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. Analysis was carried out using α -cyano-4-hydroxycinnamic acid as a matrix. Two microlitres of the sample were mixed with 2.0 μ l of matrix solution (7 mg/ml of α -cyano-4-hydroxycinnamic acid (CHCA) in 50% CN containing 0.1% TFA) and only 1.0 μ l of the mixture was spotted on a stainless steel 192-well target plate. The samples were dried at room temperature and subjected to mass analysis. A total of 3500 shots were acquired in the MS mode and collision energy of 4200 was applied.

Cytotoxic assays. The cytotoxicity of biologically active substances (BAS) and with the chemotherapy drug cisplatin (Sigma-Aldrich) was determined by MTT test as previously described [6]. The absorbance was measured with a multimode microplate reader Varioskan Lux (Thermo Scientific). Results were analyzed with GraphPad Prism software v.7. Viability was also determined upon visual inspection via phase contrast microscopy and Trypan Blue staining. After staining for 5 min with 0.4% Trypan Blue solution (Thermo Fisher Scientific), the cells were counted in a Cell Counter (Corning, New York, NY, USA) chamber, and the percentage of live cells was determined.

Results and discussion. The present study aimed to investigate the cy-

tototoxic and cytostatic activities of newly isolated bioactive substances from the garden snail *Helix aspersa* on a large panel of human cancer cell lines. The results provide valuable insights into the potential of these natural substances as alternative therapeutic agents for cancer treatment.

The cytotoxicity assays, performed using the MTT test and Trypan Blue staining, revealed the effects of the tested compounds on cell viability. The MTT assay is widely used to assess cell metabolic activity and is based on the conversion of a yellow tetrazolium salt to a purple formazan product by viable cells. Trypan Blue staining, on the other hand, allows for the visualization and quantification of non-viable cells, indicating loss of membrane integrity.

In the MTT assay, the tested compounds were evaluated at various concentrations ranging from 32 µg/ml to 512 µg/ml. The obtained IC₅₀ values, representing the concentration required to inhibit 50% of cell viability, were presented in Fig. 1, panel A. Comparisons were made with the well-known chemotherapeutic agent cisplatin, which was included as a positive control. Interestingly, some

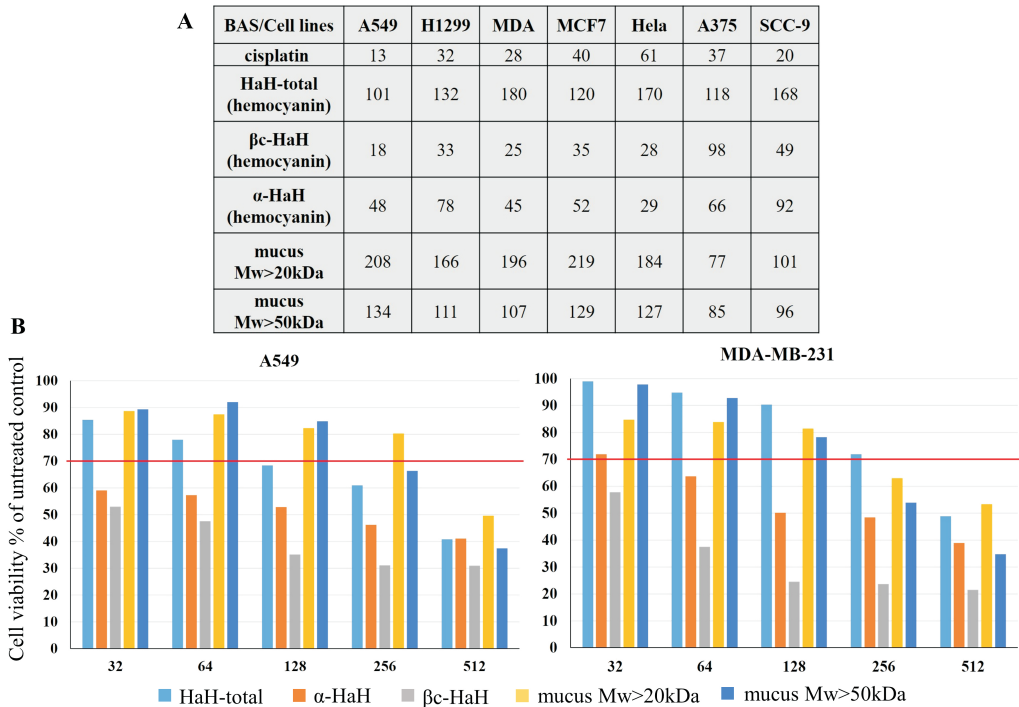


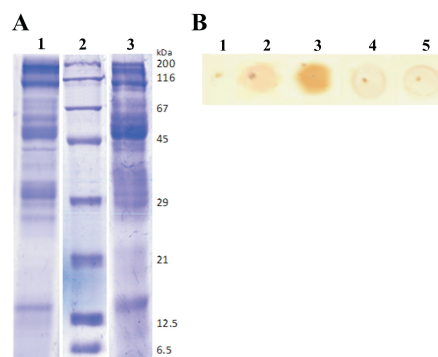
Fig. 1. Anti-proliferative effect of biologically active substances. **A.** The IC₅₀ values (in µg/ml) were determined using MTT assays to evaluate the potency of the biologically active substances (BAS) isolated from *H. aspersa* on seven different human cancer cell lines of various origins. As a positive control, the widely used chemotherapy drug cisplatin was included, and its IC₅₀ values were reported in µM. **B.** Cell viability in percentage of untreated control estimated by Trypan Blue staining

of the newly isolated substances, specifically the hemocyanin subunits α -HaH and β c-HaH, demonstrated comparable cytotoxic activity to cisplatin within the tested concentration range. This finding highlights the potential of these natural substances as effective anti-cancer agents.

To validate the MTT results, Trypan Blue staining was performed. This additional assay confirmed the trends observed in the MTT assay and provided complementary information on cell viability. Figure 1, panel B depicts the results of Trypan Blue staining at 72 h of incubation with the tested substances. The bar graph presents the percentage of cell viability relative to the untreated control. The red line in Fig. 1 indicates the threshold for cytotoxicity, set at 70% viability based on established ISO guidelines. According to this criterion, the HaH-total, 20kDa, and over 50kDa mucus fractions from *Helix aspersa* were considered non-toxic at most tested concentrations. However, at a concentration of 256 μ g/ml, these substances exhibited slight toxicity. Notably, at the highest concentration tested, all the substances demonstrated significant cytotoxicity, with viability below 50%.

Using SDS-PAGE electrophoresis followed by mass spectrometry analysis, we separated the proteins present in the biologically active mucus derived from *H. aspersa*. We focused on proteins with molecular weights greater than 20 kDa and 50 kDa, which exhibited either no or slight anti-proliferative effects, respectively. Our goal was to identify the specific proteins responsible for the observed biological activity. Figure 2, panel A illustrates the results of the electrophoresis, where multiple protein bands were detected. The analysis of the two mucus fractions (position 1 and 3) revealed prominent protein bands at approximately 220 kDa, 100 kDa, several bands around 50 kDa, as well as bands ranging from 26 to 35 kDa. Notably, in position 3 (Fig. 1A), we observed a protein band with a molecular weight of approximately 20 kDa, which could potentially correspond to a previously identified antibacterial protein found in the mucus of *Cornu aspersum*, as reported by PITT et al. [15]. Moreover, we detected additional proteins in the region spanning from 26 to 35 kDa, at approximately 43 kDa, between 45 and

Fig. 2. **A.** SDS-PAGE (12.0%) analysis of proteins fractions isolated in mucus: **position 1** – *Helix aspersa* mucus with Mw >20 kDa; **position 2** – Protein marker with molecular weights 6.5–200 kDa; **position 3** – *Helix aspersa* with Mw >50 kDa; **B.** Carbohydrate test with orcinol/H₂SO₄: **position 1** – negative control (H₂O); **position 2** – positive control (mannose 0.5 mg/ml); **position 3** – positive control (mannose 1 mg/ml); **position 4** – mucus *H. aspersa* >20 kDa; **position 5** – mucus *H. aspersa* >50 kDa



60 kDa, and within the range of 100 to 250 kDa. Several of these proteins have previously been identified in studies investigating mucus fractions of *C. aspersum*. For instance, a protein with a molecular weight of about 50 kDa, exhibiting anti-pseudomonal properties, has been reported in prior research [15].

The presence of proteins with molecular weights ranging from 50 to 60 kDa aligns with the findings of MATUSIEWICZ et al. [20], who conducted SDS-PAGE analysis on mucus from *Helix aspersa* Müller. It is likely that some of the proteins observed around 30–40 kDa and 50–60 kDa in Fig. 2A are lectins, which are common components of gastropod mucus. Lectins belong to various classes and play a role in the immune response of invertebrates, as highlighted by Pitt et al. [15]. In mollusk mucus, lectins contribute to functions such as substrate binding/attachment and viscosity. They have also been found to exhibit properties related to binding mammalian cells, as reported by Pitt et al. and ELLIJIMI et al. [15,16].

Additionally, the mucus of the African Giant Land snail, *Achatina fulica*, has been shown to contain a 160 kDa glycoprotein called “achacin”. In the region above 100 kDa, the proteins may correspond to glycoproteins known as mucins, which have been detected in the mucus of *Helix aspersa* and *Helix pomatia* [19]. Previous data also support the anti-tumour effects of mucus extracts from *Helix aspersa*, as demonstrated [16,19,20]. Next we tested the carbohydrate content of the two mucus fractions by standard orcinol-sulphuric test. After conducting the orcinol/H₂SO₄ test, we discovered that the two mucus fractions from *H. aspersa*, specifically those above 20 kDa and above 50 kDa, contain high levels of glycoproteins (Fig. 2B). Finally, we performed a MALDI-TOF-MS analysis (Fig. 3), which enabled the determination of the precise molecular masses of proteins in the mucus fraction

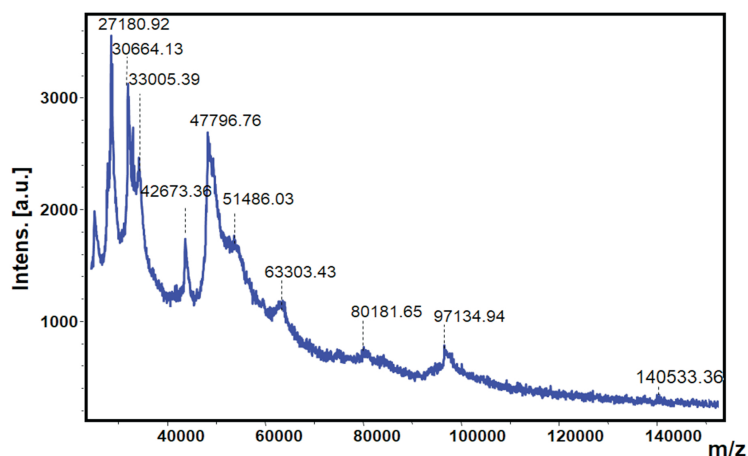


Fig. 3. MALDI-TOF-MS spectrum of fraction with Mw>20 kDa. Proteins with masses in wide m/z region between 20–150 kDa were detected

with a molecular weight greater than 20 kDa. Our analysis of the proteins presents in the mucus fraction with $M_w > 20$ kDa using MALDI-TOF-MS confirmed the molecular masses of the proteins detected through SDS-PAGE.

The identification of specific proteins with anti-proliferative activity may contribute to the development of novel therapeutic agents for various types of cancer. In conclusion, the proteins present in the mucus of *H. aspersa* exhibit anti-tumour activity against multiple human cancer cell lines, likely through a combination of different proteins with varying functions. Further research is warranted to identify the specific mechanisms through which these proteins exert their biological effects.

In conclusion, the investigation of natural compounds derived from the garden snail *Helix aspersa* has revealed their potential as alternative therapeutic agents for cancer treatment. Specifically, the hemocyanin subunits α -HaH and β c-HaH have demonstrated significant cytotoxic activity against various human cancer cell lines, comparable to the widely used chemotherapy drug cisplatin. These findings support the growing body of evidence suggesting that natural products from mollusks, such as snails, can serve as a valuable source of novel anti-cancer agents.

The study also highlights the need for further research to fully understand the mechanisms of action, safety, and potential side effects of these compounds. Additional investigations, including in vivo studies using animal models or clinical trials, are necessary to evaluate the efficacy and safety of these substances in a more physiologically relevant context.

Moreover, the identification of specific proteins in the mucus of *H. aspersa* with anti-proliferative effects provides valuable insights for the development of novel therapeutic strategies for various types of cancer. These proteins, which include hemocyanin subunits, lectins, mucins, and potentially other unidentified components, contribute to the observed anti-tumour activity. Future studies should aim to elucidate the precise mechanisms by which these proteins exert their biological effects and explore their potential applications in cancer therapy.

Overall, this research underscores the importance of exploring natural compounds from diverse sources, such as molluscs, in the search for effective and safe anti-cancer agents. The findings offer a promising foundation for further investigation and provide motivation for the development of innovative cancer treatments based on natural substances.

REFERENCES

- [¹] DECKER H., N. HELLMANN, E. JAENICKE, B. LIEB, U. MEISSNER et al. (2007) Minireview: Recent progress in hemocyanin research, Integr. Comp. Biol., **47**, 631–644.

- [2] MARKL J. (2013) Evolution of molluscan hemocyanin structures, *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, **1834**(9), 1840–1852.
- [3] GATSOGIANNIS C., O. HOFNAGEL, J. MARKL, S. RAUNSER (2015) Structure of mega-hemocyanin reveals protein origami in snails, *Structure*, **23**, 93–103.
- [4] DOLASHKA P., F. ZAL, A. DOLASHKI, L. MOLIN, P. TRALDI et al. (2012) ESI MS and MALLS analysis of quaternary structure of molluscan hemocyanins, *J. Mass Spectrom.*, **47**, 940–947.
- [5] DOLASHKA-ANGELOVA P., T. STEFANOVA, E. LIVANIOU, L. VELKOVA, P. KLIMENTZOU et al. (2008) Immunological potential of *Helix vulgaris* and *Rapana venosa* hemocyanins, *Immunol. Invest.*, **37**, 822–840.
- [6] PETROVA M., Z. VLAHOVA, M. SCHRÖDER, J. TODOROVA, A. TZINTZAROV et al. (2023) Antitumor Activity of Bioactive Compounds from *Rapana venosa* against Human Breast Cell Lines, *Pharmaceuticals*, **16**(2), 181.
- [7] BOYANOVA O., P. DOLASHKA, D. TONCHEVA, H. G. RAMMENSEE, S. STEVANOVIĆ (2013) In vitro effect of molluscan hemocyanins on CAL-29 and T-24 bladder cancer cell lines, *Biomed. Rep.*, **1**, 235–238.
- [8] ANTONOVA O., P. DOLASHKA, D. TONCHEVA, H. G. RAMMENSEE, M. FLOETENMEYER et al. (2014) In vitro antiproliferative effect of *Helix aspersa* hemocyanin on multiple malignant cell lines, *Z. Naturforsch. C J. Biosci.*, **69**, 325–334, doi:10.5560/znc.2013-0148.
- [9] DOLASHKI A., P. DOLASHKA, A. STENZL, S. STEVANOVIĆ, W. K. AICHER et al. (2019) Antitumour activity of *Helix* hemocyanin against bladder carcinoma permanent cell lines, *Biotechnol. Biotechnol. Equip.*, **33**(1), 20–32.
- [10] LAMMERS R. J., W. P. WITJES, M. H. JANZING-PASTORS, C. T. CARIS, J. A. WITJES (2012) Intracutaneous and intravesical immunotherapy with keyhole limpet hemocyanin compared with intravesical mitomycin in patients with non-muscle-invasive bladder cancer: results from a prospective randomized phase III trial, *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, **30**, 2273–2279, doi:10.1200/jco.2011.39.2936.
- [11] KRUG L. M., G. RAGUPATHI, C. HOOD, M. G. KRIS, V. A. MILLER et al. (2004) Vaccination of patients with small-cell lung cancer with synthetic fucosyl GM-1 conjugated to keyhole limpet hemocyanin, *Clin. Cancer Res.*, **10**, 6094–6100.
- [12] LAMM D. L., J. I. DEHAVEN, D. R. RIGGS (2000) Keyhole limpet hemocyanin immunotherapy of bladder cancer: laboratory and clinical studies, *Eur. Urol.*, **37**, 41–44.
- [13] DOLASHKA P., A. DOLASHKI, L. VELKOVA, S. STEVANOVIĆ, L. MOLIN et al. (2015) Bioactive compounds isolated from garden snails, *J. BioSci. Biotechnol.*, 147–155.
- [14] PITT S., M. GRAHAM, C. DEDI, P. TAYLOR-HARRIS, A. GUNN (2015) Antimicrobial properties of mucus from the brown garden snail *Helix aspersa*, *Br. J. Biomed. Sci.*, **72**, 174–181.
- [15] PITT S., J. HAWTHORNE, M. GARCIA-MAYA, A. ALEXANDROVICH, R. SYMONDS et al. (2019) Identification and characterisation of anti-*Pseudomonas aeruginosa* proteins in mucus of the brown garden snail *Cornu aspersum*, *Br. J. Biomed. Sci.*, **76**, 129–136.
- [16] ELLIJIMI C., M. B. HAMMOUDA, H. OTHMAN, W. MOSLAH, J. JEBALI et al. (2018) *Helix aspersa* maxima mucus exhibits antimelanogenic and antitumoral effects

- against melanoma cells, *Biomed. Pharmacother.*, **101**, 871–880.
- [¹⁷] TEERASAK E., P. THONGARARM, S. ROYTRAKUL, L. MEESUK, P. CHUMNANPUEN (2016) Prediction of anticancer peptides against MCF-7 breast cancer cells from the peptidomes of *Achatina fulica* mucus fractions, *Comput. Struct. Biotechnol. J.*, **14**, 49–57.
- [¹⁸] STABILI L., R. SCHIROSI, M. G. PARISI, S. PIRAINO, M. CAMMARATA (2015) The mucus of *Actinia equina* (Anthozoa, Cnidaria): An unexplored resource for potential applicative purposes, *Marine drugs*, **13**, 5276–5296.
- [¹⁹] EL OUAR I., C. BRAICU, D. NAIMI, A. IRIMIE, I. BERINDAN-NEAGOE (2017) Effect of *Helix aspersa* extract on TNF α , NF- κ B and some tumor suppressor genes in breast cancer cell line Hs578T, *Pharmacognosy Magazine*, **13**, 281.
- [²⁰] MATUSIEWICZ M., I. KOSIERADZKA, T. NIEMIEC, M. GRODZIK, H. ANTUSHEVICH et al. (2018) In Vitro Influence of Extracts from Snail *Helix aspersa* Müller on the Colon Cancer Cell Line Caco-2, *Int. J. Mol. Sci.*, **19**, 1064.

Institute of Molecular Biology
 “Acad. Roumen Tsanev”
 Bulgarian Academy of Sciences
 Akad. G. Bonchev St, Bl. 21
 1113 Sofia, Bulgaria
 e-mail: mhristova84@abv.bg
 vlahova94@gmail.com
 marias82@abv.bg
 alexander_imb@abv.bg
 ugryiva@gmail.com

**Institute of Organic Chemistry*
 with Centre of Phytochemistry
 Bulgarian Academy of Sciences
 Akad. G. Bonchev St, Bl. 9
 1113 Sofia, Bulgaria
 e-mail: lyudmila_velkova@abv.bg
 dimiter.kaynarov@orgchm.bas.bg
 adolashki@yahoo.com
 pda54@abv.bg