

SYNERGISTIC CYTOTOXICITY OF *RAPANA VENOSA*
HEMOLYMPH FRACTIONS WITH MITONAFIDE
IN LUNG CANCER CELLS

Zlatina Vlahova¹, Maria Petrova¹, Lazar Lazarov¹,
Evdokia Pasheva¹, Lyudmila Velkova², Aleksandar Dolashki²,
Pavlina Dolashka², Iva Ugrinova^{1✉}

Received on September 24, 2025

Accepted on October 28, 2025

Abstract

Lung cancer remains one of the most prevalent and lethal malignancies worldwide, and new therapeutic strategies are urgently needed. Marine-derived bioactive compounds have recently attracted attention as potential anticancer agents. In this study, we examined the anti-proliferative effects of two hemolymph fractions, with molecular weights below 30 kDa and above 50 kDa, isolated from the marine snail *Rapana venosa* after the removal of native hemocyanin. These fractions were evaluated in combination with two 1,8-naphthalimides (mitonafide and amonafide) using A549 (p53 wild-type) and H1299 (p53-null) lung cancer cell lines, as well as non-malignant MRC-5 fibroblasts. Both naphthalimides showed intrinsic cytotoxicity, with mitonafide being more potent and selective than amonafide. The hemolymph fractions alone exhibited weak cytotoxicity and low toxicity to normal cells. Remarkably, the fraction with MW > 50 kDa strongly potentiated mitonafide, reducing IC50 values and increasing selectivity indices (SI up to 26 in A549 and 13 in H1299), while amonafide combinations provided only modest improvements. Synergy analysis using the Highest Single Agent (HSA) model confirmed that the fraction with MW > 50 kDa enhanced mitonafide activity in lung cancer cells, with meaningful synergy observed in A549. Synergy was also detected in MRC-5

This research was supported by the Bulgarian National Science Fund, grant No. KP-06-H61/1 and grant No. KP-06-H81/6.

<https://doi.org/10.7546/CRABS.2025.12.08>

fibroblasts. The fraction with MW < 30 kDa similarly potentiated mitonafide and, to a lesser extent, amonafide.

Key words: *Rapana venosa* hemolymph, mitonafide, amonafide, lung cancer, synergy

Introduction. Cancer remains one of the leading causes of death worldwide, with lung cancer being among the most prevalent and lethal malignancies [1]. Despite advances in detection and therapy, prognosis remains poor due to the aggressive nature of the disease and the development of resistance to conventional treatments [2], underscoring the urgent need for novel therapeutic approaches. Natural products have long been a cornerstone of drug discovery. More than 60% of current anticancer drugs are derived from or inspired by natural sources [3].

Marine-derived bioactive compounds have recently gained attention as alternative or adjuvant antineoplastic agents. Our previous work identified a hemolymph fraction with MW > 50 kDa from the marine snail *Rapana venosa* with pronounced antitumour potential. This fraction reduced cancer cell viability, altered morphology, and induced autophagy, while showing lower toxicity toward non-malignant cells. Notably, it displayed synergistic and additive effects with cisplatin and tamoxifen, enhancing cisplatin cytotoxicity against TNBC (Triple-Negative Breast Cancer) cells nearly threefold [4].

In parallel, 1,8-naphthalimides have emerged as DNA-intercalating agents with broad biological activity, including antitumour effects [5]. Derivatives such as mitonafide, amonafide, and elinafide progressed to phase II clinical trials, although amonafide and elinafide were later discontinued due to toxicities [6, 7]. Mitonafide disrupts DNA replication and induces apoptosis [8], while also demonstrating potential to overcome drug resistance. However, clinical evaluations in NSCLC (Non-small cell lung cancer) and SCLC (Small-cell lung cancer) revealed limited efficacy, with mitonafide producing only a single partial response in a phase II study despite acceptable toxicity, and amonafide showing minimal activity with significant myelosuppression. These outcomes highlight the challenges of translating the promising preclinical activity of 1,8-naphthalimides into clinical benefit. Nevertheless, their unique mechanisms and ability to synergize with other chemotherapeutics justify continued exploration of optimized analogues and novel combination strategies.

Building on these observations, we investigated whether combining amonafide and mitonafide with *R. venosa* hemolymph fractions could enhance antitumour activity in A549 and H1299 lung cancer cells. Both naphthalimides displayed intrinsic cytotoxicity, but their effects were significantly potentiated by the hemolymph fractions, resulting in synergistic and additive outcomes. These findings suggest that integrating DNA-intercalating agents with marine-derived biomolecules may represent a promising avenue for lung cancer therapy.

Materials and methods. Cell lines. Human NSCLC cell lines A549 (ATCC® CCL-185™) and NCI-H1299 (ATCC® CRL-5803™) and normal lung

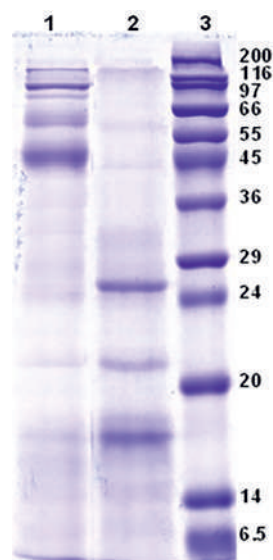
fibroblasts MRC-5 (ATCC® CCL-171™) were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). A549 and H1299 cells were cultured in complete media consisting of Dulbecco's MEM (DMEM) and RPMI-1640, respectively, modified with 10% Fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Thermo Fischer Scientific, Waltham, MA, USA). MRC-5 cells were grown in ATCC-formulated Eagle's Minimum Essential Medium modified with 10% Fetal bovine serum and 100 units/ml penicillin and 100 µg/ml streptomycin. All cells were grown in 5% CO₂ in an air-humidified incubator at 37 °C.

Isolation of bioactive fractions from the hemolymph of the marine snail *R. venosa*. Hemolymph was collected from *R. venosa* specimens inhabiting the Black Sea by incision of the foot muscle. The resulting fresh hemolymph was filtered and centrifuged at 6000 rpm (4400 × g, centrifuge VWR Micro Star 30R, with an angle rotor RA 6/50) for 20 min at 4 °C to remove coarse debris, hemocytes, and other cells. From the resulting supernatant, the native hemocyanin with MW 8000 kDa was obtained by ultracentrifugation at 18000 rpm for 3 h at 4 °C (centrifuge CENTRIKON with rotor Kontron-Hermle A8.24). The resulting supernatant was fractionated at 4 °C by ultrafiltration on Millipore membranes with pore sizes of 30 and 50 kDa. The two target fractions were obtained: fraction from the hemolymph from *R. venosa* with MW < 30 kDa (HRv < 30 kDa, 1.2 mg/mL protein) and fraction from hemolymph from *R. venosa* with MW > 50 kDa (HRv > 50 kDa, 2.4 mg/mL protein). Fractions were analyzed by SDS-PAGE and mass spectrometry as previously described [4].

Cytotoxicity assay and selectivity index. The cytotoxicity of the bioactive fractions alone or in combination with mitonafide (MedChemExpress LLC, Monmouth Junction, NJ, USA) or amonafide (Merck KGaA, Darmstadt, Germany) was assessed using the MTT assay [9]. Cells were seeded in 96-well plates and treated with serial dilutions of naphthalimides and hemolymph fractions, either individually or in combination (naphthalimide-to-fraction ratio 1:4). After 72 h, medium was replaced with phenol red-free medium containing 0.5 mg/mL MTT (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 4 h at 37 °C in 5% CO₂. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a VarioScan Lux microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). IC₅₀ values were determined using the “log concentration vs. normalized response (variable slope)” algorithm in GraphPad Prism v8.0.1 (GraphPad Software, Boston, MA, USA). Selectivity index (SI) was calculated as the ratio of IC₅₀ in non-tumourigenic MRC-5 cells to IC₅₀ in tumour cells. SI values > 2 were considered indicative of tumour selectivity.

Synergy score determination. Dose–response data and synergy scores for drug combinations were calculated using the Bioconductor package SynergyFinderPlus [10] in RStudio, applying the Highest Single Agent (HSA) model.

Fig. 1. Electrophoretic analysis of target fractions from *R. venosa* hemolymph on 12% SDS-PAGE: 1) fraction with MW > 50 kDa (HRv > 50 kDa); 2) fraction with MW < 30 (HRv < 30 kDa); 3) protein marker in the range 6.5–200 kDa (SigmaMarker™, Sigma-Aldrich, Saint Louis, MO, USA)



Results and discussion. In this study, we investigated the anti-proliferative effects of biologically active fractions isolated from the hemolymph of the marine snail *Rapana venosa*. Two hemolymph fractions were examined: one with a MW < 30 kDa and another with MW > 50 kDa.

Characterization of the two fractions of the *R. venosa* hemolymph was carried out by 12% SDS-PAGE (Fig. 1) and revealed two completely different electrophoretic profiles.

In the fraction with MW > 50 kDa, the highest expression was observed for proteins with MWs ~ 50 kDa and ~ 93 kDa, followed by proteins with MWs ~ 130 kDa and ~ 63 kDa. These results are in good agreement with extracellular proteins previously identified in the 50–100 kDa region of the hemolymph fraction of *R. venosa* with antibacterial and antitumour activity [4, 11]. The proteins observed in this fraction were identified in our previous studies by proteomic analysis. In this way, it was established that the protein with the highest expression at 50 kDa (Fig. 1) corresponds to glycosylated functional units of *R. venosa* hemocyanin (RvH 2-e; RtH2-a), while the proteins determined at 93 kDa and 62 kDa correspond to peroxidase-like proteins and a protein with L-amino acid oxidase activity (LAAO), respectively [4, 11]. The protein presented at 130 kDa corresponds to fragments including two functional units of hemocyanin from *R. venosa* derived from endogenous proteolytic processes. We hypothesize that the proteins most substantially responsible for the antitumour activity of this fraction are the glycosylated functional units of *R. venosa* hemocyanin and the presence of proteins with L-AAO activity, such as aplycyanin A, which can specifically catalyze the oxidation of essential amino acids (L-arginine and L-lysine). It is the synergy between these proteins that is associated with the promising antitumour activity

of the fraction with MW > 50 kDa from *R. venosa* hemolymph against a panel of human breast cell lines [4].

In fraction with MW < 30 kDa, dominate proteins with MWs ~ 26 kDa, 17 kDa, and 22 kDa. In this fraction we have found proline-rich antimicrobial peptides with molecular weights between 3–10 kDa [12], and unknown extracellular proteins with MWs about 17, 21, and 27 kDa, which were determined based on a match search in the UniProt database of marine Mollusca. Based on the obtained results, we assume that proteins observed on bands at 21 and 27 kDa include lectins. Lectins exhibit diverse biological activities at the cellular and molecular levels, including antitumour, antifungal, antibacterial, and antiviral activities, as well as other possible medical applications, and thus could be used for the design and development of potent therapeutics [13]. The results of the search in the UniProt database showed matches with various C-type lectins with theoretical MW in the region of 25–27.5 kDa found in the hemolymph of marine gastropods, such as the C-type lectin domain with MW 27.170 kDa found in the hemolymph of *Lottia gigantea* (UniProt ID V4BQI3) and perlucin-like protein in the hemolymph of *Biomphalaria glabrata* (MW 25.616 kDa, UniProt ID A0A9W3BPT7), as well as galectin-like proteins with theoretical MWs of 26.006 kDa and 24.392 kDa found in the sea snail *Elysia chlorotica* (UniProt ID A0A3S0ZDA4 and UniProt ID A0A433SIR7). The protein expressed at 17 kDa is in good agreement with Galactose-binding lectins from *Mytilus californianus* and *Crenomytilus grayanus* with theoretical MWs 16.991 kDa (UniProt ID A0A0P0E482) and 17.023 kDa (UniProt ID H2FH31), respectively, as well as with lectins from the hemolymph of the sea snail *Elysia chlorotica* with theoretical MWs of 16.480 kDa, 17.452 kDa, and 18.529 kDa (with UniProt IDs A0A433TBR5, A0AAE0XNA, and A0A433SI60, respectively).

Some lectins are promising agents in antitumour therapy. For example, Galactose-binding lectin from *C. grayanus* exhibits a dose-dependent cytotoxic effect on the human globotriaosylceramide (Gb3)-expressing Burkitt's lymphoma (Raji) cell line by arresting the cell cycle in the G2/M phase and apoptosis [14]. In addition, this lectin also demonstrates antitumour activity on the Gb3-expressing human MCF-7 breast cancer cell line by recognizing Gb3 on the surface of breast cancer cells [15].

In this study, the IC₅₀ values and selectivity indices (SI) for mitonafide, amonafide, hemolymph fractions, and their combinations are summarized in Table 1. Mitonafide alone displayed strong cytotoxicity against A549 and H1299 cells (IC₅₀ = 0.202 and 0.154 µg/mL) with moderate selectivity (SI 4.7 and 6.2), whereas amonafide was less potent (IC₅₀ = 0.915 and 0.525 µg/mL) and less selective (SI 1.8 and 3.2). The hemolymph fractions alone had high IC₅₀ values and low or modest SI, confirming weak intrinsic cytotoxicity and low toxicity to non-malignant fibroblasts. Strikingly, mitonafide combined with the < 30 kDa fraction showed improved activity and selectivity (SI ~ 10 in both A549 and H1299).

T a b l e 1

Calculated IC₅₀ and SI values (μg/mL) for the tested compounds and their combinations.
Data represent mean ± SD from at least $n = 3$ independent experiments

Compounds	A549		H1299		MRC-5
	IC ₅₀ ± SD	SI	IC ₅₀ ± SD	SI	IC ₅₀ ± SD
Mitonafile	0.202 ± 0.004	4.7	0.154 ± 0.01	6.2	0.959 ± 0.005
Amonafile	0.915 ± 0.007	1.8	0.525 ± 0.006	3.2	1.67 ± 0.09
HRv MW < 30 kDa	214 ± 8.2	0.9	175 ± 5.8	1.1	193 ± 6.3
Mitonafile + HRv < 30 kDa	0.14 ± 0.02	10.7	0.15 ± 0.06	10	1.5 ± 0.3
Amonafile + HRv < 30 kDa	1.53 ± 0.7	4.1	2.3 ± 0.4	2.7	6.3 ± 0.8
HRv MW > 50 kDa	150 ± 2.6	1.9	100 ± 4.1	2.8	279 ± 3.2
Mitonafile + HRv > 50 kDa	0.08 ± 0.01	26.3	0.16 ± 0.03	13.1	2.1 ± 0.6
Amonafile + HRv > 50 kDa	1.44 ± 0.3	5.1	1.72 ± 0.8	4.2	7.3 ± 0.4

Even stronger effects were observed with the > 50 kDa fraction, where mitonafile achieved very low IC₅₀ values and SI values of 26 (A549) and 13 (H1299), indicating potent synergism and selective cytotoxicity. By contrast, amonafile combinations produced only modest SI improvements (between 2.7 and 5) without substantial gains in potency. These results demonstrate that *R. venosa* fractions, particularly the one with MW > 50 kDa, markedly potentiate mitonafile's anti-cancer activity and improve its selectivity profile, while their effect on amonafile remains limited.

Next, synergy was quantified using the Highest Single Agent (HSA) model, which is an effect-based method for evaluating drug combinations. Effect-based approaches compare the effects of individual drugs with those of their combinations to determine whether the interaction is synergistic. According to the HSA model, a combination is considered synergistic if its effect is greater than that of the most active single agent. Unlike models that consider additive contributions, HSA evaluates combinations only against the strongest individual effect. As a result, its main use is in situations where one of the drugs shows no activity across all tested concentrations [16]. Interpretation thresholds were set as: HSA score > 10 = meaningful synergy; between -10 and 10 = additivity; < -10 = antagonism.

Figure 2 shows the synergy analysis of mitonafile (A) or amonafile (B) combined with the MW < 30 kDa fraction. Mitonafile demonstrated synergy in A549 (score: 11.82) and even stronger effects in MRC-5 (HSA score: 19.26) and additivity in H1299 (HSA score: 3.5). Amonafile combinations showed additive effects across all tested cell lines (A549 - 2.99, H1299 - 6.82, MRC-5 - 4.14). These results suggest that the < 30 kDa fraction enhances mitonafile activity in lung cancer cells, especially A549, although the parallel synergy in MRC-5 indicates that further refinement is necessary to improve cancer selectivity.

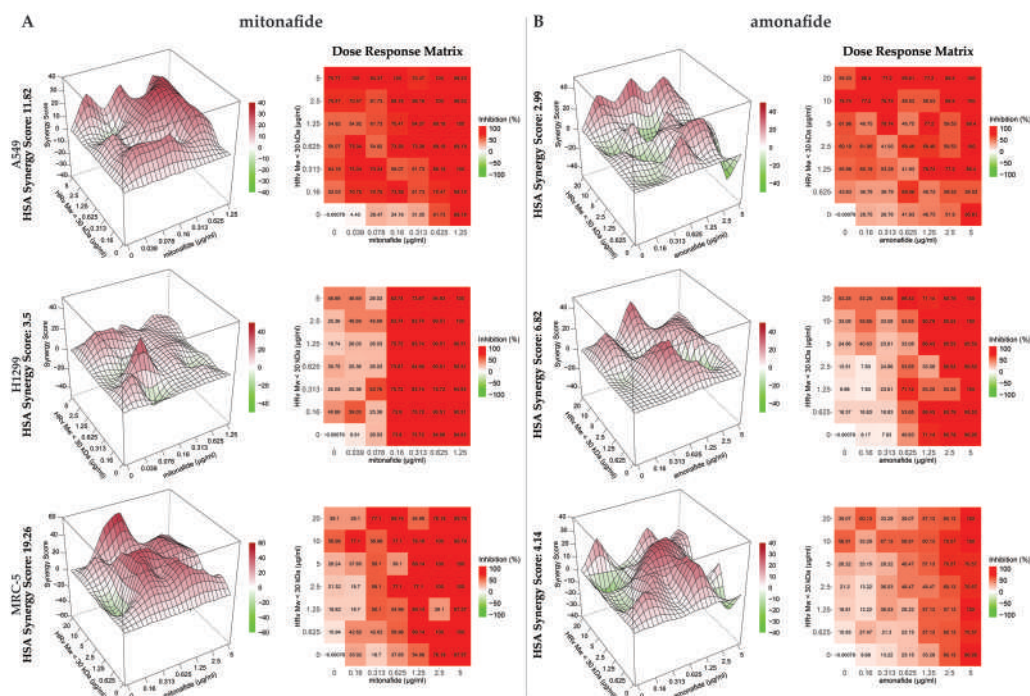


Fig. 2. Combination landscapes and dose-response matrices of A549, H1299, and MRC-5 cell lines treated with hemolymph fraction of *R. venosa* with MW < 30 kDa in combination with mitonafide (A) or amonafide (B)

Figure 3 shows an analysis of the synergy between mitonafide (A) or amonafide (B) when combined with the hemolymph fraction with MW > 50 kDa. Mitonafide combinations exhibited clear synergistic effects in A549 (HSA score: 12.57) and MRC-5 (HSA score: 16.79), but only an additive effect in H1299 (HSA score: 5.08). Amonafide combinations were less effective, with relatively high synergy scores but still showed additivity across all tested lines (A549 – 9.18, H1299 – 9.05, MRC-5 – 9.2). Therefore, although the fraction with MW > 50 kDa can enhance both naphthalimides, the observed synergy of mitonafide in non-malignant fibroblasts highlights the importance of optimizing dose-effect strategies to achieve better cancer selectivity.

Our study demonstrates that hemolymph fractions from *R. venosa* can markedly potentiate the antitumour activity of mitonafide, especially in A549 and H1299 lung cancer cells. The fraction with MW > 50 kDa showed the most favourable profile, strongly enhancing mitonafide's potency and selectivity, while the <30 kDa fraction also had synergistic effects but with a higher impact on non-malignant fibroblasts. In contrast, amonafide displayed only modest improvements when combined with hemolymph fractions.

Conclusion. In summary, *R. venosa* hemolymph fractions, particularly the one with MW > 50 kDa, selectively potentiate mitonafide's cytotoxicity against

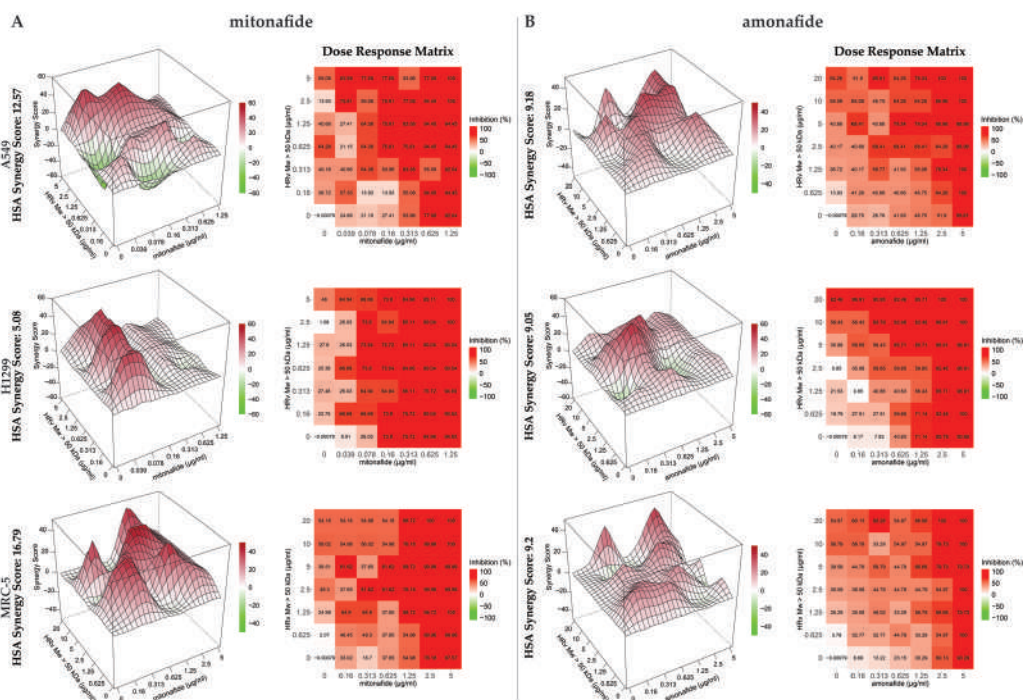


Fig. 3. Combination landscapes and dose-response matrices of A549, H1299, and MRC-5 cell lines treated with hemolymph fraction of *R. venosa* with MW > 50 kDa in combination with mitonafide (A) or amonafide (B)

lung cancer cells and improves its therapeutic profile. While additional work is required to identify the active components, our results highlight the potential of combining marine-derived biomolecules with classical naphthalimides as a novel strategy for lung cancer treatment.

REFERENCES

- [1] FERLAY J., M. COLOMBET, I. SOERJOMATARAM et al. (2021) Cancer statistics for the year 2020: An overview, *Int. J. Cancer*, **149**(4), 778–789.
- [2] USAMA M., B. RAZA, M. WU, S. JI (2025) Targeting the toll-like receptor signaling pathway in lung cancer: therapeutic opportunities and challenges, *Curr. Drug Targets*, **26**(15), 1112–1120, <https://doi.org/10.2174/0113894501414090250903070446>.
- [3] SALEH M. A., H. M. M. MASOUD, M. R. HABIB et al. (2025) Marine-derived peptides from *Rapana venosa* inhibit breast cancer cell growth through synergistic mechanisms with doxorubicin, *Sci. Rep.*, **15**(1), 32451.
- [4] PETROVA M., Z. VLAHOVA, M. SCHRÖDER et al. (2023) Antitumor activity of bioactive compounds from *Rapana venosa* against human breast cell lines, *Pharmaceuticals*, **16**(2), 181.

- [5] RYKOWSKI S., D. GURDA-WOŻNA, M. ORLICKA-PŁOCKA et al. (2021) Design, synthesis, and evaluation of novel 3-carboranyl-1,8-naphthalimide derivatives as potential anticancer agents, *Int. J. Mol. Sci.*, **22**(5), 2772.
- [6] TOMCZYK M. D., K. Z. WALCZAK (2018) 1,8-Naphthalimide based DNA intercalators and anticancer agents. A systematic review from 2007 to 2017, *Eur. J. Med. Chem.*, **159**, 393–422.
- [7] KAMAL A., N. R. BOLLA, P. S. SRIKANTH et al. (2013) Naphthalimide derivatives with therapeutic characteristics: A patent review, *Expert Opin. Ther. Pat.*, **23**(3), 299–317.
- [8] SINHA B. K., J. STRONG, N. W. GIBSON et al. (1985) Mechanism of DNA strand breaks by mitonafide, an imide derivative of 3-nitro-1,8-naphthalic acid, *Biochem. Pharmacol.*, **34**(21), 3845–3852.
- [9] MOSMANN T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, **65**(1–2), 55–63.
- [10] ZHENG S., W. WANG, J. ALDAHDOOH et al. (2022) SynergyFinder plus: toward better interpretation and annotation of drug combination screening datasets, *Genom. Proteom. Bioinform.*, **20**(3), 587–596.
- [11] KIRILOVA M., Y. TOPALOVA, L. VELKOVA et al. (2024) Antibacterial action of protein fraction isolated from *Rapana venosa* hemolymph against *Escherichia coli* NBIMCC 8785, *Pharmaceuticals*, **17**(1), 68.
- [12] DOLASHKA P., V. MOSHTANSKA, V. BORISOVA et al. (2011) Antimicrobial proline-rich peptides from the hemolymph of marine snail *Rapana venosa*, *Peptides*, **32**(7), 1477–1483.
- [13] AHMED M. K., S. BHOWMIK, S. G. GITERU et al. (2022) An update of lectins from marine organisms: characterization, extraction methodology, and potential biofunctional applications, *Mar. Drugs*, **20**(7), 430.
- [14] CHERNIKOV O., A. KUZMICH, I. CHIKALOVETS et al. (2017) Lectin CGL from the sea mussel *Crenomytilus grayanus* induces Burkitt's lymphoma cells death via interaction with surface glycan, *Int. J. Biol. Macromol.*, **104**(Pt A), 508–514.
- [15] LIAO J. H., C. T. CHIEN, H. Y. WU et al. (2016) A multivalent marine lectin from *Crenomytilus grayanus* possesses anti-cancer activity through recognizing globotriose Gb3, *J. Am. Chem. Soc.*, **138**(14), 4787–4795.
- [16] DUARTE D., N. VALE (2022) Evaluation of synergism in drug combinations and reference models for future orientations in oncology, *Curr. Res. Pharmacol. Drug Discov.*, **3**, 100110.

¹*Institute of Molecular Biology “Acad. Roumen Tsanev”, Bulgarian Academy of Sciences, Akad. G. Bonchev St, Bl. 21, 1113 Sofia, Bulgaria
e-mails: vlahova94@gmail.com, mhristova84@abv.bg, lazarutko@gmail.com, eva@bio21.bas.bg, ugrayiva@gmail.com*

²*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Akad. G. Bonchev St, Bl. 9, 1113 Sofia, Bulgaria
e-mails: lyudmila_velkova@abv.bg, adolashki@yahoo.com, pda54@abv.bg*