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Assessment of the toxicity and antiproliferative activity of hemocyanins from *Helix lucorum*, *Helix aspersa* and *Rapana venosa*

I. Yankova[1], E. Ivanova[1], K. Todorova[1], A. Georgieva[1], V. Dilcheva[1], I. Vladov[1], S. Petkova[1], R. Toshkova[1], L. Velkova[2], P. Dolashka[2], I. Iliev[1]*

[1] *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria*

[2] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 9, 1113 Sofia, Bulgaria*

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Hemocyanins (Hcs) are respiratory, oxygen-carrying metalloproteins that are freely dissolved in the hemolymph of many molluscs and arthropods. The interest in hemocyanins has grown significantly since it was found that they can be successfully used in immunotherapy of neoplastic diseases as non-specific or active stimulators of the immune system. The present study aims to assess the cytotoxicity, *in vivo* toxicity and antiproliferative activity of hemocyanins isolated from marine snail *Rapana venosa* (RvH), garden snails *Helix lucorum* (HlH) and *Helix aspersa* (HaH). For *in vitro* safety testing, 3T3 Neutral Red Uptake (NRU) test was used. The experiments for antiproliferative activity of the hemocyanins were performed by MTT assay on a panel of cell lines - a model of breast cancer. The *in vivo* toxicological assessment was performed by regular clinical examinations of hemocyanin-treated laboratory mice and histopathological analysis of hematoxylin/eosin stained preparations of parenchymal organs. The evaluation of the *in vitro* cytotoxicity showed that the tested hemocyanins does not induce toxic effects in nontumorigenic epithelial cell lines. In contrast, significant reduction of the viability of human breast carcinoma cell lines was found after treatment with high concentrations of hemocyanins. The *in vivo* experiments showed no signs of organ and systemic toxicity in the hemocyanin-treated animals. The presented data indicate that Hcs show a potential for development of novel anticancer therapeutics due to their beneficial properties, biosafety and lack of toxicity or side effects.

Key words: hemocyanins (Hcs); cytotoxicity; antitumor activity; *in vivo* biosafety testing

INTRODUCTION

Breast cancer is the most common malignant diseases among women in Europe, accounting for more than 12% of the total number of new cases of cancer diagnosed in 2018 [1]. The frequency of newly diagnosed cases of breast cancer in Bulgaria is about 4 061 per year, 30% of which are fatal [2]. Despite the great advances of the modern medicine in the diagnosis and treatment of cancer, the number of cases has been growing every year, which is why breast cancer is the leading cause of death in women worldwide [3]. Due to the increasingly common multidrug resistance to cancer chemotherapy [4], it is necessary to search for new alternatives to the standard antineoplastic drugs. One such good alternative is the usage of hemocyanins. They are copper-containing respiratory glycoproteins with complex quaternary structure, localized in the hemolymph of some invertebrates belonging to the Mollusca and Arthropoda phyla [5, 6]. Hemocyanins have found an application as carrier proteins and adjuvants in antibody production and as non-specific immunostimulants in bladder cancer therapy [7, 8]. Among the hemocyanins, the Keyhole Lympet Hemocyanin (KLH), isolated from the marine snail *Megatura crenulata* has been most extensively studied and applied in immunotherapy [9, 10]. However, the restricted geographical distribution of *M. crenulata* does not allow the extraction of high quantities of Hcs.

As an alternative of KLH, we propose hemocyanins isolated from *H. aspersa*, *H. lucorum* and *R. venosa*, which are widespread throughout the world, including in Bulgaria. The structure and chemical composition of these hemocyanins have been studied in detail and described by Bulgarian research team [11-13]. The presence of a variety of carbohydrate components in hemocyanins can lead to a direct interaction with receptors on the surface of cancer cells. As a result, apoptosis or necrosis can be induced [14]. In addition, the carbohydrate components can induce antitumor immunity [15].

The aim of the present study was to determine the cytotoxicity and antiproliferative activity of HaH, HlH, and RvHI in permanent cell lines and to evaluate their biosafety *in vivo*.

* To whom all correspondence should be sent:
E-mail: *taparsky@abv.bg*

EXPERIMENTAL

Isolation of native hemocyanins from garden snails Helix aspersa and Helix lucorum and subunit RvH from marine snail Rapana venosa

The hemolymph from garden snails *Helix aspersa* and *Helix lucorum*, and marine snail *Rapana venosa* was collected after cutting the foot muscles. The rough particles and hemocytes were removed after filtration and centrifugation at 10,000 rpm (at 4 °C) for 20 min. It is known that hemolymph of these snails contained above 90% hemocyanin as a major protein with molecular mass around 9000 kDa. Therefore the crude hemolymph extract was subject to ultrafiltration on membrane 100 kDa (Millipore Ultrafiltration Membrane Filters) and the fraction above 100 kDa containing predominantly native hemocyanin was put to ultracentrifuge at 22,000 rpm (rotor Kontron-Hermle A8.24, centrifuge CENTRIKON) at 4 °C for 3 h, as a result the total hemocyanins were obtained as sediment. After removal of the supernatant, the precipitated total hemocyanins HaH and HlH were solubilized in 50 mM Tris buffer (pH 7.5) containing 20 mM $CaCl_2$ and further purified by gel filtration [16].

The native RvH was obtained also after ultracentrifugation at 22,000 rpm (at 4 °C) for 180 min and was purified by gel filtration. After dissociation of native RvH and purified on an ion-exchange chromatography, two subunit RvHI and RvHII (with molecular masses 420 kDa and 400kDa respectively) were obtained [16].

All the hemocyanins were filtered through a bacterial filter with a pore size of 0.2 μM (Corning®; Incorporated Life Sciences, St. Lowell, MA, USA) under sterile conditions.

Cell cultures

Two human mammary carcinoma cell lines MCF-7 and MDA-MB-231 were used as models for breast cancer. The MCF-7 cell line has some characteristics of differentiated mammary epithelium including expression of estrogen and progesterone receptors (ER^+, PR^+ and Her-2^-) [17, 18]. The MDA-MB-231 is a highly aggressive, invasive and poorly differentiated triple-negative (ER^-, PR^- and Her-2^-) breast cancer [19]. The cell lines BALB/c 3T3 (mouse embryonic fibroblasts) and MCF-10A (human breast epithelial cell line) were used as models for healthy tissue. Cells were cultured in Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Gibco, Austria), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Lonza, Belgium) in an incubator at 37 °C, 5% CO_2 and 95% humidity. Plastic flasks 25 cm^2 (Greiner, Germany), were used to grow the cells. The cells were kept in exponential phase of growth and after processing with trypsin-EDTA (FlowLab, Australia).

BALB/c 3T3 NRU cytotoxicity test

Neutral Red Uptake test (NRU-assay) is a colorimetric method for assessment of cell viability *in vitro* [20]. This method is based on the ability of living cells to include the Neutral Red dye in their lysosomes. BALB/c 3T3 cells were plated at a density of 1×10^4 cells in 100 μl culture medium in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with hemocyanins, dissolved in PBS and further diluted in culture medium. A wide concentration range was applied (from 7.5 to 1000 μg/ml) and the cells were incubated for additional 24 h. After treatment with Neutral Red medium for 3 h, washing and application of Ethanol/Acetic acid solution (NR Desorb), the absorption was measured on a TECAN microplate reader at wavelength 540 nm.

In vitro antiproliferative activity

The antiproliferative/antitumor activity testing was performed on MCF-10A, MCF-7 and MDA-MB-231 cell lines by MTT-dye reduction assay [21]. The assay is based on the metabolism of the tetrazolium salt MTT to formazan by mitochondrial reductases. The cells were plated at a density of 1×10^3 cells in 100 μl in each well of 96-well microplates and allowed to adhere for 24 h. The cells were than treated with hemocyanins applied at a concentration range from 7.5 to 1000 μg/ml for 72 h. The formazan absorption was registered using a microplate reader at λ=540 nm. For assessment of the antiproliferative activities, the IC_{50} values were calculated using non-linear regression analysis (GraphPad Prizm4 Software). The statistical analysis included application of One-way ANOVA followed by Bonferroni's post hoc test. $p<0.05$ was accepted as the lowest level of statistical significance. All results are presented as mean ± SD.

In vivo testing for toxic and pathological effects

In vivo studies were performed on 16 white laboratory mice with body weight about 25-30 g in order to test the biosafety of hemocyanins and to

exclude any toxicity or adverse pathological effects. The experimental animals were divided into four groups-one control and three experimental groups. The control animals were injected three times subcutaneously with saline solution. The animals from the experimental groups were injected three times subcutaneously as follows: the first group with the hemocyanin HaH, the second group with HlH and the third with RvHI at a dose of 1 mg/kg bw. All procedures were consistent with the recommendations for in vivo animal studies in accordance with the requirements of Regulation № 20/ 01.11.2012 regarding laboratory animals and animal welfare and European legislation.

Histological examination

Materials from livers, kidneys, spleen, lungs and heart were fixed in 10% buffered formalin (pH 7) (Alkaloid AD, Skopie), dehydrated, embedded in paraffin (Emmonya, Biotech Ltd, Bulgaria), sliced into 5-10 μm, dewaxed in xylene (Alkaloid AD, Skopie) and stained with Mayer's Hematoxylin (Emmonya, Biotech Ltd, Bulgaria) and Eosin (Bio optica, Milano, Italy) according to routine histological techniques. Investigations were performed on a microscope Leica DM 5000B, Germany and micrographs were taken.

RESULTS

In vitro effects of hemocyanins on the cell viability and proliferation

The Hcs were studied for cytotoxicity by standard method (3T3 NRU-test). The BALB/c 3T3 cells were incubated with the test compounds (total native hemocyanins HaH and HlH and structural subunit RvHI) at concentrations ranging from of 7.5 to 1000 μg/ml for 24 h, after which the cytotoxicity expressed as a percentage of the negative control was determined. The obtained results are shown in Fig. 1, A). The tested Hcs were non-toxic (the cytotoxicity values determined for all hemocyanin concentrations tested do not show a significant difference compared to the negative control), so CC_{50} values can not be determined at the applied concentration range.

Cell viability testing was performed by standard MTT dye reduction assay. Normal human mammary epithelial cells (MCF-10A) were incubated with the test hemocyanins at concentrations ranging from 7.5 to 1000 μg/ml for 72 h. The cell viability expressed as a percentage of the negative control was determined. The dose-response curves are shown in Fig. 1, B). HaH induced a slight reduction of the viability of MCF-10A cells. At the highest concentration studied, the cell viability was decreased by only 6%. The other two hemocyanins (HlH and RvHI) showed a significant increase in the cell viability of about 12 and 8%, respectively, compared to the negative control.

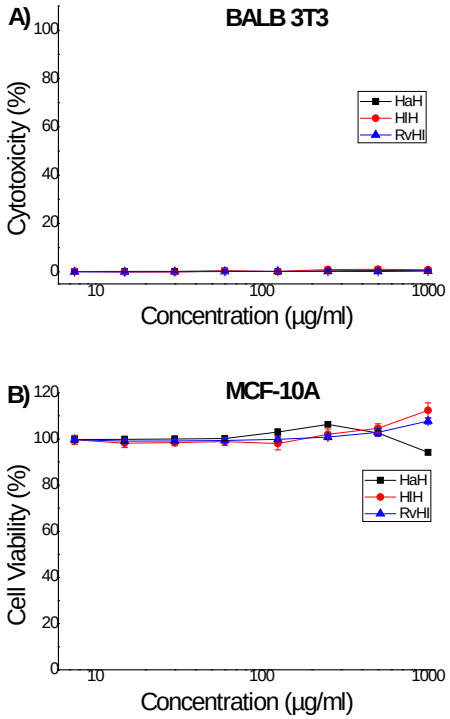

Fig. 1. *In vitro* cytotoxic (A) and proliferative (B) activity of hemocyanins (HaH, HlH and RvHI).

In vivo toxicological investigations

All animals from the experimental groups showed good general condition and appetite throughout the test period. Livers, spleen, lungs, kidneys and heart of the hemocyanin-treated experimental mice did not show any histopathological signs of toxicity at the doses used (Fig. 2). LD_{05} was not reached and the concentrations were assessed in the region of LD_0. In the spleen of the treated mice, no pathological changes were observed. The histoarchitecture was normal with preserved white and red pulp ratio. Myeloid and erythroid hyperplasia with megakaryocytes was also found, evidential of extramedullary hematopoiesis, which is commonly observed in rodents, especially mice, as a normal component of the pulp of the spleen (Fig. 2, B).

Megakaryocytes are more common in young than in adult animals, but in certain circumstances,

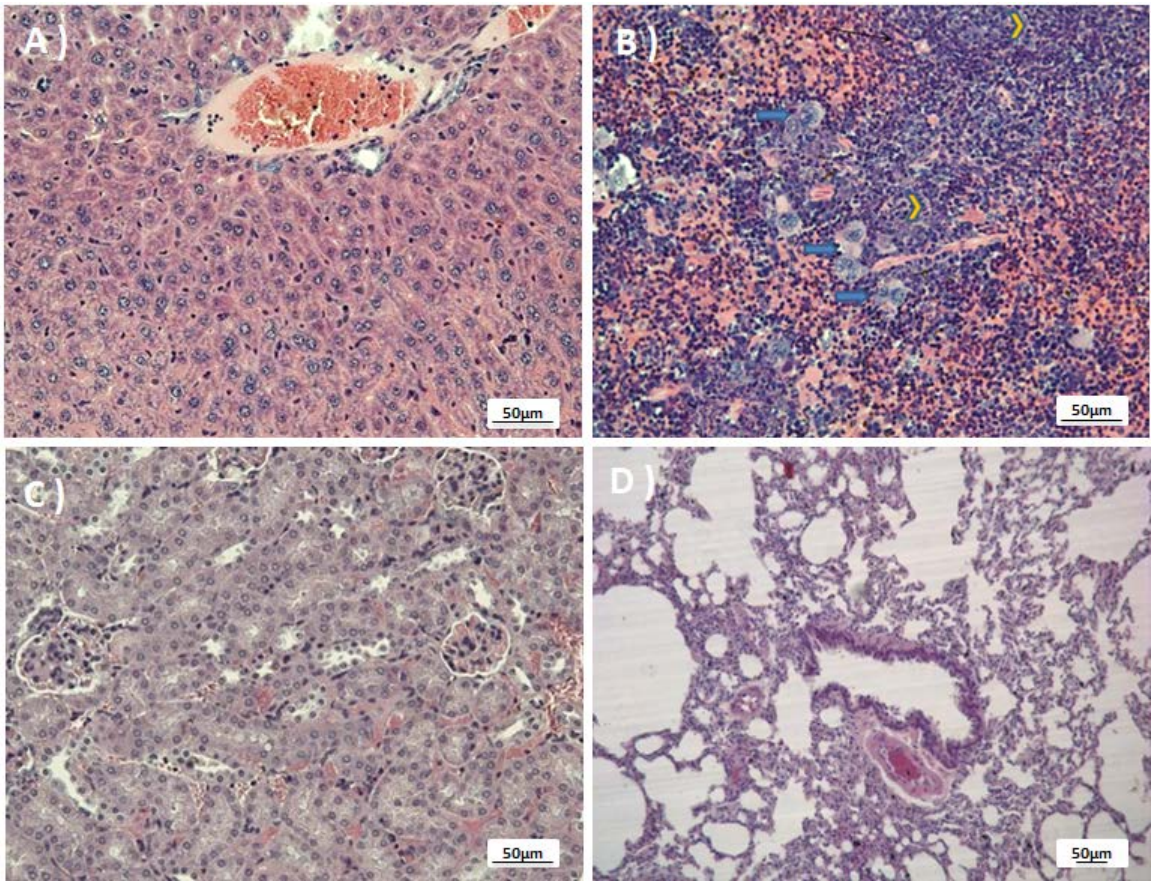

Fig. 2. *In vivo* toxicity screening and organ pathology. Animals were injected three times subcutaneously with 1mg/kg bw HaH. A) livers, B) spleen, C) kidneys and D) lungs. In the spleen, two small germinative centers (yellow arrows) with a thin marginal zone (black arrow) adjacent to the red pulp were observed. White to red pulp ratio were maintained. Myeloid and erythroid hyperplasia with megakaryocytes (blue arrows). Livers, kidneys and lung showed normal morphology.

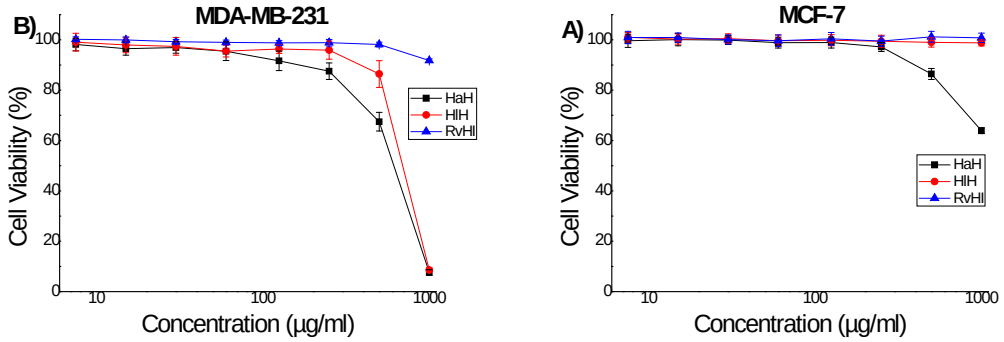

Fig. 3. Cell viability of breast carcinoma cells treated with hemocyanins (HaH, HlH and RvHI) for 72 h. A) MCF-7 and B) MDA-MB-231.

different external agents may provoke their increase. The obtained results showed that hemocyanins – HaH-total, HlH-total and subunit RvHI applied three times at a dose of 1 mg/kg bw did not cause any pathohistological changes indicative of toxic damage to the parenchymal organs. The degree of hyperplasia in the spleen was within the normal range and was similar to those observed in the controls. Lungs, kidneys, heart and livers also showed normal morphology.

Antitumor activity

The *in vitro* antitumor activity of the hemocyanins was determined by MTT dye reduction assay on two cancer cell lines MCF-7 and MDA-MB-231 (Fig. 3). In the MCF-7 cell line, the

hemocyanins showed similar activity to those found in MCF-10A cells (Fig. 3, A). Low antitumor activity of HaH-total (36% decrease of the cell viability) was observed at the highest concentration studied (1000 µg/ml). For the other tested hemocyanins (HlH-total and subunit RvHI), no difference was observed compared to the negative control.

In the MDA-MB-231 cell line, the hemocyanins (HaH and HlH) showed a weak antitumor effect (Fig. 3, B), with IC_{50} values 644 ±24 and 732 ± 19 µg/ml, respectively. No significant decrease of cell viability in the carcinoma cells treated with subunit RvHI was observed.

DISCUSSION

Molluscan Hcs are oligomeric glycoproteins with complex didecameric quaternary structures and heterogeneous glycosylation patterns, primarily consisting of *N*-glycans, which contribute to their structural stability and immunomodulatory properties in mammals [22]. Hemocyanins from different molluscan species have been considered for use in diverse biomedical and clinical applications.

Gastropod Hcs including tested hemocyanins are glycoproteins with a high carbohydrate content of about 9% (w/w) that may contain unusual monosaccharides, such as a methylated hexoses (for example, *O*-methyl-D-mannose and *O*-methyl-D-galactose), *β*(1,2)-linked xylose, *α*(1,3)-linked fucose, *α*(1,6)-linked fucose or hexuronic acid [22]. The glycan moieties play diverse roles in biological systems that make them relevant for use as biotherapeutics.

The tested hemocyanins differ significantly in their structural organization, molecular weight, carbohydrate structure and monosaccharide composition. The native *R. venosa* hemocyanin (RvH), like KLH, is composed of two polypeptide structural subunits RvHI and RvHII with molecular weight 420 and 400 kDa, respectively [23]. Total hemocyanins from garden snails *H. aspersa* and *H. lucorum* (HaH-total; HlH-total) are with molecular weight about 9MDa and consist of three structural subunits – two *α*- and one *β*c-isoforms [24]. Each of them ranging from 350 to 450 kDa, includes eight globular folded domains known as functional units (FUs) with molecular masses of about 47-65 kDa. Native HlH and HaH as well as subunit RvHI exhibit a predominant didecameric structure as revealed by electron microscopy [23-25].

One of the main characteristics of the hemocyanins from snails *R. venosa*, *H. aspersa* and *H. lucorum* are their carbohydrate structures. Recent structural studies of the two isoforms of RvH demonstrate the presence mainly of high mannose, complex and hybrid-type glycan structures as well as unusual N-glycan structures with an internal fucose residue (*β*1-2) binding GalNAc and hexuronic acid [22]. The presence of mono- and bi-antenna *N*-linked glycan structures from complex and high mannose type were established in hemocyanins from *H. lucorum* and *H. pomatia* [26, 27]. Most of them contain variously methylated glycan structures of 3-*O*-methyl-D-mannose and 3-*O*-methyl-D-galactose as well as core modification - mainly with *β*1-2-linked xylose to *β*-mannose and/or *α*1-6-fucosylation of GlcNAc residue (the Asn-bound GlcNAc). The methylated structures are heterogeneous in terms of involved monosaccharides and position of methylation. Most of the analyzed glycans of *β*-HlH contain mainly a terminal and/or inner MeHex residue, in some cases even up to four such residues are present [26]. The chemical composition of hemocyanins (copper ions, oligosaccharide and amino acid sequences) determines their low toxicity [28]. In practice, hemocyanins toxicity has been studied on different cell lines under *in vitro* conditions, mainly in experiments to determine antiviral activity [29, 30]. Therefore, only cell lines suitable for the replication of the respective viruses were used, but not suitable for the determination of cytotoxicity, which would give us correct information about possible toxicity and pathological effects under *in vivo* conditions. In this study, we examined the cytotoxicity of total hemocyanins HaH and HlH, and subunit RvHI on the BALB/c 3T3 cell line by the NRU-test. This method has been approved for the determination of cytotoxicity by the ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) that coordinates U.S. federal government evaluation of new, revised, and alternative test methods [31]. The results obtained by this *in vitro* method show that the tested hemocyanins are non-toxic even at the highest concentrations used (1000 µg/ml). In addition, through this *in vitro* method, we predicted the absence of toxicity at *in vivo* conditions, which we proved by toxicological experiments and histological analyses. The results of *in vivo* experiments showed that the livers, spleen, lungs, kidneys and heart of the hemocyanin-treated mice remained intact and no histopathological signs of toxicity were observed at the doses used. Moreover, in experiments to determine cell viability of the

normal epithelial cell line (MCF-10A), we found that HlH and RvHI even increase cell proliferation. MCF-10A is a reliable model for normal human mammary epithelial cells, with functional cellular mechanisms of apoptosis and cell proliferation. As a result, they are very sensitive to external influences. The observed increase of the proliferative activity of treated cells is evidence of the regenerative properties of hemocyanins known from the traditional medicine [32]. This is the reason why hemocyanins are widely used in cosmetics and medicine. Most biological studies related to hemocyanins focus on their immunological properties. Due to the strong activation of humoral immunity, they are used as adjuvants and molecular carriers of weakly immunogenic antigens, such as tumor antigens [33]. Therefore, there is great interest in the research related to the use of hemocyanins in the immunotherapy of neoplastic diseases. In addition, there is evidence of successful treatment of bladder cancer by direct application of hemocyanins to the affected tissue. Moreover, *in vitro* experiments were performed to determine the antitumor activity of hemocyanins on tumor cell lines (647-V, T-24 and CAL-29) -models of bladder cancer [34]. All these literature data showd the presence of structural and chemical features of the hemocyanin molecule that may directly affect the proliferation and viability of cancer cells. Therefore, we performed *in vitro* experiments to determine the antitumor activity of the studied hemocyanins on the cell lines MCF-7 and MDA-MB-231, which are models of hormone-dependent and hormone-independent breast cancer, respectively. The results showed that the MCF-7 cell line was not sensitive to the studied hemocyanins, while the triple-negative cell line MDA-MB-231 showed a significant growth inhibition after treatment with high concentrations of HaH and HlH. Since it is known that MDA-MB-231 cell line has a very high proliferative potential and metabolic activity, it is likely that the observed antitumor effect is due to an interaction between the hemocyanins and the cytoplasmic membrane that decrease its permeability and block the transmembrane transport of metabolites, thus leading to reduced vitality and cell death.

We hypothesize that the difference in the antitumor activity of the investigated hemocyanins is most likely due to the difference in their specific carbohydrate moieties, which are located on the surface of the didecmeric structures [22]. The observed higher antitumor activity of HlH and HaH compared to RvHI is probably due to the high content of methylated *N*-glycans as well as β1-2-linked xylose to β-mannose in inner core in these Hcs. It is known that the glycans carrying a xylose residue linked to the inner core are often found in plants and demonstrate a high immunogenicity for mammalian species [35].

CONCLUSION

The results obtained and the subsequent analysis showed that the tested hemocyanins were not cytotoxic and did not cause pathological changes in the treated experimental animals. The increased viability of the normal cells and the observed growth inhibitory effect in mammary carcinoma cells with high proliferative potential indicate the possibility of application of hemocyanins in the field of regenerative medicine and cosmetics, as well as in adjunctive therapy of neoplasms with high proliferative index.

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