Effect of extracts from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa* snails on Caco-2 colon cancer cells

Magdalena Matusiewicz [Corresp., 1], **Karolina Marczak** [1], **Barbara Kwiecińska** [1], **Julia Kupis** [1], **Klara Zglińska** [2], **Tomasz Niemiec** [2], **Iwona Kosieradzka** [2]

[1] Department of Nanobiotechnology, Institute of Biology, Warsaw University of Life Sciences, Warsaw, Poland

[2] Department of Animal Nutrition, Institute of Animal Sciences, Warsaw University of Life Sciences, Warsaw, Poland

Corresponding Author: Magdalena Matusiewicz
Email address: magdalena_matusiewicz@sggw.edu.pl

Background. Colorectal cancer is the third most commonly diagnosed cancer. Natural compounds, administered together with conventional chemotherapeutic agent(s) and/or radiotherapy, may be a novel element in the combination therapy of this cancer. Considering the anticancer properties of compounds derived from different tissues of various snail species confirmed earlier, the purpose of the present research was to evaluate the effect of extracts from eggs of *Helix aspera maxima* and *Helix aspersa aspersa* snails, and fractions of extracts containing particles of different molecular weights on Caco-2 human epithelial colorectal adenocarcinoma cells.

Methods. The extracts and fractions were analyzed for antioxidant activity, phenols and total carbohydrates using colorimetric methods. Lipid peroxidation products and glutathione in eggs were also examined using these methods. Crude protein and fat in eggs were determined. Molecular weights of egg proteins and glycoproteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Astaxanthin, selected vitamins and amino acids in eggs were measured using liquid chromatography methods, and minerals by emission spectroscopy, mass spectrometry or X-ray fluorescence. The action of extracts on the cell viability was determined by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) test, based on the mitochondrial oxidative activity, after 24 and 72 h of treatment. The influence of fractions on the cell viability was assayed after 24 h. The effect of extracts on the percentage of live and dead cells was evaluated by the trypan blue assay, in which live cells exclude trypan blue, while dead cells take up this dye, after 12, 24, 48 and 72 h of treatment. Their influence on the integrity of cell membranes was determined based on the activity of LDH (lactate dehydrogenase), released from damaged cells, after 24 and 72 h of treatment. Then, the effect of extracts on the content of lipid peroxidation products in cells was examined using colorimetric method, after 24 h of treatment. Their influence on types of cell death was determined by flow cytometry, after this time.

Results. The extracts and their fractions containing molecules <3 kDa decreased the cell viability, after 24 h of treatment. The extracts reduced the percentage of live cells (also after 48 h), increased the degree of cell membrane damage and the amount of lipid peroxidation products, induced apoptosis and reduced necrosis.

Conclusions. Antioxidants, phenols, lipid peroxidation products, anticancer peptides, restriction of methionine, appropriate ratio of essential amino acids to non-essential amino acids, vitamin D_3, Ca, Mg, S, Cu, Mn, Zn, Se and other bioactive compounds comprised in the extracts and their additive and synergistic effects may have influenced Caco-2 cells. Natural extracts or the chemical compounds contained in them might be used in the combination therapy of colorectal cancer, which requires further research.

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7 Magdalena Matusiewicz[1], Karolina Marczak[1], Barbara Kwiecińska[1], Julia Kupis[1], Klara
8 Zglińska[2], Tomasz Niemiec[2], Iwona Kosieradzka[2]

10 [1] Department of Nanobiotechnology, Institute of Biology, Warsaw University of Life Sciences,
11 Warsaw, Poland
12 [2] Department of Animal Nutrition, Institute of Animal Sciences, Warsaw University of Life
13 Sciences, Warsaw, Poland

15 Corresponding Author:
16 Magdalena Matusiewicz
17 Ciszewskiego 8, 02-786 Warsaw, Poland
18 Email address: magdalena_matusiewicz@sggw.edu.pl

Abstract

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31 were also examined using these methods. Crude protein and fat in eggs were determined.
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33 polyacrylamide gel electrophoresis. Astaxanthin, selected vitamins and amino acids in eggs were
34 measured using liquid chromatography methods, and minerals by emission spectroscopy, mass
35 spectrometry or X-ray fluorescence. The action of extracts on the cell viability was determined
36 by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) test, based on the mitochondrial
37 oxidative activity, after 24 and 72 h of treatment. The influence of fractions on the cell viability
38 was assayed after 24 h. The effect of extracts on the percentage of live and dead cells was

39 evaluated by the trypan blue assay, in which live cells exclude trypan blue, while dead cells take
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42 damaged cells, after 24 and 72 h of treatment. Then, the effect of extracts on the content of lipid
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46 viability, after 24 h of treatment. The extracts reduced the percentage of live cells (also after 48
47 h), increased the degree of cell membrane damage and the amount of lipid peroxidation products,
48 induced apoptosis and reduced necrosis.
49 **Conclusions.** Antioxidants, phenols, lipid peroxidation products, anticancer peptides, restriction
50 of methionine, appropriate ratio of essential amino acids to non-essential amino acids, vitamin
51 D_3, Ca, Mg, S, Cu, Mn, Zn, Se and other bioactive compounds comprised in the extracts and
52 their additive and synergistic effects may have influenced Caco-2 cells. Natural extracts or the
53 chemical compounds contained in them might be used in the combination therapy of colorectal
54 cancer, which requires further research.

Introduction

57 Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of
58 cancer death [1]. The incidence rates of colorectal cancer are over three-fold greater in countries
59 that have completed their socioeconomic transition, with high/very high Human Development
60 Index (HDI) comparing to transitioning countries, with low/medium HDI while the mortality
61 rates are over two-fold greater [1]. The greatest colon cancer incidence rates were found in
62 Europe, Australia/New Zealand, Northern America, Uruguay and Eastern Asia [1].
63 Colorectal cancer occurrence and progression are caused by many risk factors, the main of which
64 are age, gender, family and personal history, and region [2]. The risk of this cancer can be
65 reduced through proper dietary habits and lifestyle [3].
66 The main treatment for patients suffering from a potentially curable colorectal cancer is surgery,
67 and chemotherapy and/or radiation therapy may be given before/after it, depending on the stage
68 of the disease [4]. However, this treatment is not sufficient to control colorectal cancer as 30% of
69 stage I to III patients and up to 65% of stage IV patients relapse, underlining the urgent need to
70 find more effective therapies. Adverse effects of the above treatment impair the life quality, may
71 unfavorably influence on the course, outcomes and costs of treatment [3].
72 Treatment outcomes and life quality can be improved by application of natural products derived
73 from plants, animals and microorganisms, well tolerated and less toxic than conventional
74 chemotherapeutic agents [2]. There are nine anticancer drugs from marine organisms on the
75 market and other molecules from these organisms are being examined as anticancer drugs in
76 different phases of clinical trials [5].
77 Natural compounds, administered together with conventional chemotherapeutic agent(s) and/or
78 radiotherapy, may be a novel element in combination therapy of colorectal cancer [3,4]. Certain

79 compounds may sensitize to cytotoxic therapy, intensify the effective concentration of a drug,
80 enhance the combined effects of both therapeutics or exert a cytotoxic effect specifically on
81 cancer cells. Moreover, combination therapy targets many signaling pathways and uses a variety
82 of mechanisms to decrease the development of anticancer drug resistance.
83 Snails can provide many bioactive compounds for the pharmaceutical and cosmetics industries,
84 applicable in the development of novel preparations with lower toxicity and subsequent effects in
85 comparison with compounds commonly used for this purpose [6]. Information on the chemical
86 composition and nutritional value of mucus, foot tissues and shells of one of the most popular
87 edible land snails of the subspecies *Helix aspersa aspersa* is presented in the article by
88 Matusiewicz et al. [7]. The use of water extracts from lyophilized mucus and foot tissues of these
89 snails reduced the viability of Caco-2 human colorectal adenocarcinoma cells.
90 *In vitro* experiment of Ellijimi et al. demonstrated that the mucus extract derived from *Helix*
91 *aspersa maxima* snails decreased the content of melanin and tyrosinase activity in B16F10
92 murine melanoma cells and IGR-39 and SK-MEL-28 human melanoma cells [8]. It decreased the
93 viability of human melanoma cells and did not influence on the viability of HaCaT human
94 keratinocytes. It induced a caspase-dependent apoptosis of human melanoma cells, inhibited
95 their migration and invasion by decreasing the production of matrix metalloproteinase-2. It
96 strongly affected the adhesion of IGR-39 cells by blocking $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin functions and
97 by decreasing the production of α_v and β_1 integrins. The mucus and its two fractions derived
98 from *Achatina fulica* snail reduced the viability of MCF-7 breast cancer cells and Vero epithelial
99 cells [9].
100 Water extract from *H. a. aspersa* snails exhibited anticancer activity against Hs578T breast
101 cancer cells [10]. It induced necrosis of cancer cells, stimulated the mRNA expression of tumor
102 necrosis factor (TNF)-α and inhibited the expression of nuclear factor kappa-light-chain-
103 enhancer of activated B cells (NF-κB), phosphatase and tensin homolog (PTEN) and tumor
104 protein 53 (p53).
105 Many studies showed that mollusk and arthropod hemocyanins, oxygen-transporting hemolymph
106 glycoproteins, have significant anticancer activity which was demonstrated in both *in vitro* and *in*
107 *vivo* models. Hemocyanins have immunostimulatory activity, thanks to the structural properties,
108 they stimulate the immune system nonspecifically, they interact with macrophages, granulocytes,
109 CD4+ and CD8+ cells, induce potent cellular and humoral immune responses [11-22]. The
110 structural subunits of hemocyanins derived from the land snails *H. a. aspersa* and *Helix lucorum*,
111 the marine snail *Rapana venosa* and the mucus of *H. a. aspersa* snails reduced the viability of
112 HT-29 human colorectal adenocarcinoma cells [22]. The mucus and the α and βc subunits of *H.*
113 *a. aspersa* hemocyanin decreased the viability to the greatest extent. The half-maximal inhibitory
114 concentrations (IC$_{50}$) of above preparations for HT-29 cells were lower than for Balb/c3T3
115 fibroblasts. The mechanisms of their anticancer activity included apoptosis. The hemocyanins
116 isolated from the land snail *Helix pomatia* and the marine snail *Rapana thomasiana*
117 demonstrated strong anticancer and antiproliferative actions in a colon carcinoma murine model
118 [23]. The immunization with these hemocyanins resulted in prolonged survival of animals,

119 improved humoral anticancer response, inhibition of tumor growth, splenomegaly and
120 appearance of lung metastasis. Treatment with hemocyanins derived from *H. pomatia* and *R.*
121 *thomasiana* in other study resulted in the production of large amounts of antitumor IgGs, plasma
122 cells as well as tumor specific cytotoxic T cells, stimulation of the secretion of proinflammatory
123 cytokines, suppression of tumor size and growth, and prolongation of the life span of a colon
124 carcinoma murine model [24].
125 Considering the above anticancer properties of compounds derived from different tissues of
126 various snail species, the extracts from snail eggs, containing chemical compounds characterized
127 by potential anticancer activities, may affect the growth and development of cancer cells. The
128 purpose of the research was to evaluate the chemical composition of eggs from the popular
129 edible farm snails *Helix aspera maxima* and *Helix aspersa aspersa*, water extracts from these
130 eggs and fractions of extracts containing particles of different molecular weights. The action of
131 extracts and their fractions on the viability of Caco-2 human epithelial colorectal
132 adenocarcinoma cell line was determined. The effect of the extracts on the percentage of live and
133 dead cells, the integrity of cell membranes, the content of lipid peroxidation products in cells and
134 the types of cell death was then examined.
135

Materials & Methods

Animal material and preparation of lyophilizates

138 Two-day-old eggs (about 0.5 kg) of two snail subspecies, *Helix aspersa maxima* Taylor, 1883
139 ((*Cornu aspersum maxima* (Taylor, 1883)) and *Helix aspersa aspersa* Müller, 1774 ((*Cornu*
140 *aspersum aspersum* (Müller, 1774)), were obtained from the commercial breeding (Grudziądz,
141 Poland). Animal raw materials were collected in June 2018.
142 Fresh eggs, a few hours after harvest, were washed, homogenized and frozen at -80 °C, for two
143 days. Then, the raw materials were lyophilized (Lyovac GT 2 freeze-dryer, SRK Systemtechnik
144 GmbH, Riedstadt, Germany) for two days, in the dark. Lyophilized eggs were milled into fine
145 powder using a laboratory mill and stored in polypropylene tubes (-80 °C).

Preparation of extracts and their fractions for the determination of antioxidant indicators, phenols and total carbohydrates

148 Before each analysis, lyophilized eggs derived from *H. a. maxima* and *H. a. aspersa* snails were
149 homogenized in deionized water (concentration 100 mg/mL), by vortexing. The samples were
150 left for extraction (30 min, 4 °C), subjected to centrifugation (1600× *g*, 10 min) and the
151 supernatants (extracts) were collected. Then, the extracts were filtered using polyvinylidene
152 fluoride (PVDF) syringe filters (pore size 0.22 μm, EuroClone, Pero, Italy). The extracts were
153 fractionated, based on the molecular weight, using ultra centrifugal filter devices containing
154 regenerated cellulose membranes (Merck Millipore, Burlington, MA, USA), including 3, 10 and
155 50 kDa cutoffs, in compliance with the manufacturer's prescriptions regarding g-force and
156 centrifugation time. Four fractions were obtained: >50 kDa (>50 K), 10-50 kDa (10–50 K), 3–10
157 kDa (3–10 K) and <3 kDa (<3 K).

Determination of antioxidant indicators

159 *Ferric-reducing antioxidant power*
160 Ferric-reducing antioxidant power of extracts and four fractions: >50 K, 10-50 K, 3-10 K and <3
161 K of extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was determined
162 using the modified Oyaizu method [25,26]. It involves reduction of Fe^{3+}, being in stoichiometric
163 excess over antioxidants, because of electron donation by them. The absorbance increase is noted
164 as the reduction capability is higher. Properly diluted extracts and fractions (2.5 mL) were mixed
165 with 0.2 M sodium phosphate buffer pH 6.6 (2.5 mL) and 1% potassium ferricyanide (2.5 mL).
166 The samples were incubated at 20 °C for 20 min and then 10% trichloroacetic acid (TCA, 2.5
167 ml) was added. The probes were centrifuged (3000× *g*, 5 min) and the supernatants (0.4 mL)
168 were combined with deionized water (0.4 mL) and 0.1% ferric chloride (160 μL). The
169 absorbance was measured at 700 nm, using microplate reader (Infinite M200, Tecan, Männedorf,
170 Switzerland). The standard curve was generated by applying various concentrations, ranging 0-
171 100 μM, of (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), a water-
172 soluble analog of vitamin E. The assay was conducted in three replicates (*n* = 3).
173 *2.2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS·⁺) scavenging*
174 *activity*
175 The relatively stable ABTS·⁺ discolors when is reduced. To determine ABTS·⁺ scavenging
176 activity of extracts and four fractions of extracts from *H. a. maxima* and *H. a. aspersa* eggs, the
177 procedure of Sun at al. with modifications was used [26,27]. This method is based on the
178 addition of antioxidants to ABTS·⁺ solution and spectrophotometric determination of the
179 remaining ABTS·⁺. To make ABTS reagent, 7 mM ABTS (5 mL) was combined with 140 mM
180 $K_2S_2O_8$ (88 μl). To generate free radicals, the mixture was put in the dark (16 h, room
181 temperature (RT)). The reagent was diluted using the absolute ethanol, to get the absorbance of
182 0.70 ± 0.02 at 734 nm (Infinite M200 microplate reader, Tecan, Männedorf, Switzerland).
183 ABTS·⁺ scavenging activity was assayed by combining of ABTS reagent (0.9 mL) with properly
184 diluted extracts or fractions of extracts from snail eggs (0.1 mL). After the incubation of samples
185 (6 min, RT), the absorbance was registered. The standard curve was obtained using TROLOX
186 (see Ferric-reducing antioxidant power section), *n* = 3.
187 *2.2-diphenyl-1-picrylhydrazyl radical (DPPH·) scavenging activity*
188 To evaluate DPPH· scavenging activity of extracts and fractions of extracts from *H. a. maxima*
189 and *H. a. aspersa* eggs, the method of Li et al. with some modifications was utilized [26,28]. The
190 procedure is based on donation of hydrogen atom or electron by antioxidants to the unpaired
191 electron of DPPH·, the absorbance falls proportionally to rise of DPPH non-radical form. 0.2
192 mM DPPH· in absolute methanol was mixed with properly diluted extracts and fractions of
193 extracts from eggs (2:1, v/v). After incubation of the samples (30 min, without access to light),
194 they were centrifuged (15000× *g*, 10 min). Thereafter, the supernatant absorbance was registered
195 at 517 nm (Infinite M200 microplate reader, Tecan, Männedorf, Switzerland). The standard
196 curve was obtained as in Ferric-reducing antioxidant power section, *n* = 3.
197 **Determination of phenols**

198   The method of determination of total phenols, the Folin-Ciocalteu method, is based on the
199   reading of the absorbance of the complex arising from the reduction of Folin-Ciocalateu reagent,
200   i.e. salts of hetero polyacids, phosphomolybdic and phosphotungstic [26,29]. Over the reaction
201   Mo (VI) ions are reduced to Mo (V) ions, resulting in a blue color of $[PMoW_{11}O_{40}]^{4-}$. To
202   evaluate the concentration of phenols, properly diluted extracts and fractions of extracts derived
203   from snail eggs (0.5 mL) were mixed with Folin-Ciocalteu reagent (diluted 1:10 in deionized
204   water, 2.5 ml). After incubation for 2 min, the samples were mixed with 7.5% Na_2CO_3 (2 mL)
205   and incubated in a water bath (50 °C, 10 min). Then, the absorbance was read at 760 nm
206   (Spectronic 20D cuvette spectrophotometer, Milton Roy, Rochester, NY, USA). The standard
207   curve was obatained using various quercetin levels (0-100 μg/mL), $n = 2$.
208   **Determination of total carbohydrates, crude protein and crude fat**
209   Concentration of total carbohydrates in extracts and fractions of extracts from *H. a. maxima* and
210   *H. a. aspersa* eggs was measured using the phenol-sulfuric acid method [26,30]. The absorbance
211   was registered at 490 nm and glucose was utilized as the standard, $n = 3$.
212   Content of crude protein in lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was
213   determined by the Kjeldahl method, according to AOAC International [7,31], $n = 2$.
214   Crude fat was extracted from lyophilized snail eggs by applying petroleum ether using the
215   Soxhlet method [7,31], $n = 2$.
216   **Determination of lipid peroxidation products**
217   Level of lipid peroxidation products – thiobarbituric acid reactive substances (TBARS) in
218   extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was determined using
219   the procedure of Uchiyama and Mihara [7,32]. Extracts were acquired after homogenization of
220   egg lyophilizates in radioimmunoprecipitation assay (RIPA) buffer and centrifugation ($1600\times g$,
221   10 min). The absorbance was recorded at 532 nm (Infinite M200 microplate reader, Tecan,
222   Männedorf, Switzerland). TBARS were expressed as the equivalents of malondialdehyde (MDA)
223   and its precursor - 1.2.3.3-tetraethoxypropane (TEP) was used as the standard, $n = 6$.
224   **Determination of glutathione**
225   Widespread in cells thiol tripeptide, glutathione (GSH), constitutes nearly 97% of non-protein
226   thiol compounds. GSH is evaluated quantitatively by assay of non-protein -SH groups in the
227   samples deproteinized by TCA. The method for the evaluation of non-protein -SH groups
228   consists in the Ellman's method, reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by
229   thiol compounds and formation of colorful 2-nitro-5-mercaptobenzoic acid, having an
230   absorbance maximum at 412 nm [26,33]. Lyophilized eggs of *H. a. maxima* and *H. a. aspersa*
231   snails were subjected to homogenization in 0.1 M phosphate buffer pH 7.4 and centrifugation
232   ($1600\times g$, 10 min). In order to deproteinize, to the supernatants (1.5 ml) was added 50% TCA
233   (78.96 μl) and samples were subjected to centrifugation (3000 rpm, 5 min). Then, deproteinized
234   supernatants (25 μl) were mixed with 0.2 M phosphate buffer pH 8.0 (200 μl) and with 6×10^{-3}
235   M DTNB (25 μl), directly on a 96-well plate. The absorbance was read by applying microplate
236   reader (Infinite M200, Tecan, Männedorf, Switzerland). The standard curve was generated using
237   different contents (0–75 nmol/mL) of GSH in 2.5% TCA, $n = 6$.

238 **Determination of astaxanthin and vitamins A, D$_3$, E and C**
239 Determination of the contents of astaxanthin and vitamins A, D$_3$ (cholecalciferol) and E in
240 lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was preceded by liquid extraction with
241 the application of ultrasound. The concentrations of above compounds were measured using
242 high-performance liquid chromatography coupled with UV-Vis detection (HPLC/UV-Vis),
243 based on the standard curves. The analysis of astaxanthin was performed using the Altus A-10
244 system (PerkinElmer, Waltham, MA, USA) and LiChroCART 250-4, C18 column (Merck &
245 Co., Inc., Kenilworth, NJ, USA). The analyses of vitamins A and E were carried out by
246 Shimadzu HPLC/UV-Vis system (Kyoto, Japan) and LiChroCART 125-4, C18 column (Merck
247 & Co., Inc., Kenilworth, NJ, USA). The analysis of vitamin D$_3$ was done using the Altus A-10
248 system (PerkinElmer, Waltham, MA, USA) and LiChroCART 125-4, C18 column (Merck &
249 Co., Inc., Kenilworth, NJ, USA). The analyses were carried out in a private analytical laboratory
250 (Olsztyn, Poland), $n = 3$.
251 The analysis of vitamin C, as the sum of ascorbic acid and dehydroascorbic acid, in lyophilized
252 snail eggs was carried out using HPLC/UV-Vis, in the laboratory which is accredited by the
253 Polish Centre for Accreditation [34]. In order to determine the total content of vitamin C in the
254 sample extracts, the reduction of dehydroascorbic acid to ascorbic acid was performed with
255 dithiothreitol. The analysis was carried using a chromatograph with Waters® 2487 Dual
256 Wavelength Absorbance Detector (Waters Corp., Miliford, MA, USA) with a Symmetry C18
257 column, 100 Å, 5 µm, 4.6 mm x 150 mm (Waters Corp., Miliford, MA, USA), column
258 temperature: 25 °C, injection volume: 25-30 µl. The analysis was carried out at 245 nm
259 wavelength and 0.8 mL/min mobile phase flow, $n = 2$.
260 **Analysis of molecular weights of proteins and glycoproteins**
261 For the preparation of protein extracts, lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails
262 were homogenized in phosphate-buffered saline (PBS) with inhibitors of proteases and
263 phosphatases (Sigma-Aldrich, St. Louis, MO, USA), in the ratio 10 mg lyophilizate/1 ml PBS –
264 for analysis of proteins or 119 mg lyophilizate/1 ml PBS – for analysis of glycoproteins, and then
265 centrifuged (1600× g, 10 min). For protein profile analysis, the total protein concentration was
266 equalized between extracts. Samples were submitted for sodium dodecyl sulfate-polyacrylamide
267 gel electrophoresis (SDS-PAGE), with the application of a 5% stacking gel and 12% resolving
268 gel – for separation of proteins or 10% resolving gel – for separation of glycoproteins, by the
269 Laemmli method [7,35] with modifications. The samples (30 µL) were subjected to denaturation
270 and reduction using the Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA)
271 with β-mercaptoethanol (30 µl) and then heating (95 °C, 5 min). Each sample (20 µL) and a
272 protein marker (5 µL, PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, Thermo
273 Fisher Scientific, Waltham, MA, USA - for analysis of proteins or ColorBurst™ Electrophoresis
274 Marker, Sigma-Aldrich, St. Louis, MO, USA – for analysis of glycoproteins) were loaded onto
275 two gels and resolved by electrophoresis (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell
276 System, Bio-Rad Laboratories, Hercules, CA, USA). In the case of glycoprotein analysis, a

277 postive control, 10 μl of horseradish peroxidase at a concentration of 2 mg/mL with the Laemmli
278 sample buffer, was also used.
279 To analyze the molecular weights of proteins, protein bands separated on the first gel were fixed,
280 stained (QC Colloidal Coomassie Stain, Bio-Rad Laboratories, Hercules, CA, USA) and
281 destained in accordance with the manufacturer's procedure (Bio-Rad Laboratories, Hercules,
282 CA, USA).
283 Sugar moieties of glycoproteins separated on the second gel were detected by applying the
284 commercial kit, according to the manufacturer's procedure (Pierce Glycoprotein Staining Kit,
285 Thermo Fisher Scientific, Waltham, MA, USA). Glycols present in glycoproteins are oxidized to
286 aldehydes when treated with periodic acid and are stained magenta.
287 The gels were visualized by applying Azure c400 imaging system (Azure Biosystems, CA,
288 USA).
289 **Analysis of amino acids**
290 The concentrations of the amino acids with the exception of tryptophan (Trp) in lyophilized eggs
291 of *H. a. maxima* and *H. a. aspersa* snails were assayed by ion-exchange chromatography with
292 spectrophotometric detection (IEC-VIS) [36]. The analysis was performed using an automatic
293 amino acid analyzer AAA 400 and an ion-exchange column (Ingos, Prague, Czech Republic).
294 The level of Trp was determined using high-performance liquid chromatography with
295 fluorescence detection (HPLC-FLD, Agilent 1100 Series, Agilent Technologies, Santa Clara,
296 CA, USA) [7,37]. Zorbax® ODS C18, 4.6 mm ID × 250 mm (5 μm) column (Agilent
297 Technologies, Santa Clara, CA, USA) was used.
298 Analysis of amino acids was carried out in the laboratory accredited by the Polish Centre for
299 Accreditation, $n = 2$.
300 The amino acid score (AAS), chemical score (CS) and essential amino acid index (EAAI) were
301 calculated using the equations below [7,38,39]:

1.
$$AAS = \frac{aa}{AA \text{ (standards)}}$$
    •

2.
$$CS = \frac{aa}{AA \text{ (egg)}}$$
    •

3.
$$EAAI = \sqrt[n]{\frac{100A}{AS} \times \frac{100B}{BS} \times \frac{100C}{CS} \times ... \times \frac{100H}{HS}}$$
    •

302 in which aa - the content of amino acid in the protein of snail eggs (%); AA (standards) - the
303 content of amino acid in the reference protein for 2-5 years old children (%) [38]; AA (egg) - the
304 content of amino acid in the whole egg reference protein (%) [39]; n - amino acid number; A, B,
305 C, H – the content of EAA (essential amino acids) in the protein of snail eggs (%); AS, BS, CS,
306 HS - the content of EAA in the reference protein (%).
307 **Analysis of minerals**
308 The contents of Ca, P, Na, K, Mg, Cu, Fe, Mn and Zn in lyophilized eggs of *H. a. maxima* and
309 *H. a. aspersa* snails were assayed by inductively coupled plasma—atomic emission spectroscopy
310 (ICP-AES, iCAP 6500, Thermo Fisher Scientific, Waltham, MA, USA) [40]. The concentrations

311   of Ni, Cr, Mo, B, Co, Se, V and Sn in snail eggs were determined by mass spectrometry with
312   ionization in inductively coupled plasma (ICP-MS, Varian 820-MS, Varian, Inc., Palo Alto, CA,
313   USA) [41]. The contents of S, Cl, Si, I and F in eggs were evaluated by wavelength-dispersive
314   X-ray fluorescence (WDXRF, Axios, PANalytical, Almelo, the Netherlands) [42]. The analyses
315   were carried out in the laboratories accredited by the Polish Centre for Accreditation, $n = 3$.

Preparation of extracts and their fractions for cell culture tests

317   Before cell culture tests, lyophilized eggs derived from *H. a. maxima* and *H. a. aspersa* snails
318   were homogenized in deionized water, at the concentration of 25 mg/mL ((tests described in
319   Effect of extracts on cell viability (MTT test), Effect of extracts on the percentage of live and
320   dead cells (trypan blue test), Effect of extracts on the integrity of cell membranes and Effect of
321   extracts on the types of cell death sections)) or 2.5 mg/mL (Effect of extracts on the content of
322   lipid peroxidation products section). The homogenates were left for extraction (30 min, 4 °C),
323   centrifuged ($1600 \times g$, 10 min) and the extracts were collected.

324   For the experiment described in Effect of fractions of extracts on cell viability (MTT test)
325   section, the extracts at the concentration of 100 mg/mL were prepared, filtered and fractionated
326   to obtain four fractions: >50 K, 10–50 K, 3–10 K and <3 K, as in Preparation of extracts and
327   their fractions for the determination of antioxidant indicators, phenols and total carbohydrates
328   section.

329   Extracts or fractions were sterilized (PVDF syringe filters, pore size 0.22 μm, EuroClone, Pero,
330   Italy) under the biological safety cabinet (TopSafe™ 1.2, class II, BIOAIR, Pavia, Italy). Then,
331   for some tests, appropriate dilutions were prepared with sterile deionized water.

Caco-2 cell culture

333   Human epithelial colorectal adenocarcinoma (Caco-2) cell line (ECCC, 55 passage, Sigma-
334   Aldrich, St. Louis, MO, USA) was cultivated in polystyrene plates designed for adherent cell
335   culture ((for the experiments described in Effect of extracts on cell viability (MTT test), Effect of
336   fractions of extracts on cell viability (MTT test) and Effect of extracts on the integrity of cell
337   membranes sections - in 96-well plates, at an initial density of 1×10^4 cells/100 μl, for the
338   experiment presented in Effect of extracts on the percentage of live and dead cells (trypan blue
339   test) section – in 24-well plates, at a density of 5.94×10^4 cells/594 μl and for the experiments
340   described in Effect of extracts on the content of lipid peroxidation products and Effect of extracts
341   on the types of cell death sections - in 6-well plates, at a density of 0.75×10^5 cells/1.5 mL)) in
342   Minimum Essential Medium (MEM) comprising 2 mM L-glutamine, 10% fetal bovine serum
343   (FBS), 1% non-essential amino acids (NEAA) and 1% antibiotic-antimycotic (all solutions were
344   purchased from Thermo Fisher Scientific, Waltham, MA, USA) [7,26]. The cells were kept at 37
345   °C in 5% CO_2 and 95% relative humidity in a CO_2 incubator (INCO 108 med, Memmert GmbH
346   + Co. KG, Schwabach, Germany) for 24 h. After incubation and reaching about 70% confluency,
347   they were starved in MEM with 1% FBS and 1% antibiotic-antimycotic (Thermo Fisher
348   Scientific, Waltham, MA, USA) overnight [7,26].

Effect of extracts on cell viability (MTT test)

350   90 µL of new medium (MEM with 1% FBS and 1% antibiotic-antimycotic; Thermo Fisher
351   Scientific, Waltham, MA, USA) and 10 µL of extracts from lyophilized eggs of *H. a. maxima*
352   and *H. a. aspersa* snails, at the concentrations of 25, 25×10^{-1}, 25×10^{-2}, 25×10^{-3}, 25×10^{-4} and
353   25×10^{-5} mg/mL were added to the cells. Equal volumes of deionized water (sterile) were
354   introduced into the control cells. Additional controls were also included. After 24 h and 72 h of
355   incubation in a CO_2 incubator (INCO 108 med, Memmert GmbH + Co. KG, Schwabach,
356   Germany; 37 °C, 5% CO_2 and 95% relative humidity), the MTT (methylthiazolyldiphenyl-
357   tetrazolium bromide) test was done using the method of Tada et al. with modifications [7,26,43].
358   Yellow MTT solution is converted to water-insoluble, dark blue MTT formazan, by
359   mitochondrial dehydrogenases of living cells. 15 µl of MTT reagent (Sigma-Aldrich, St. Louis,
360   MO, USA) in PBS (5 mg/mL) was added to the cells and they were incubated (37 °C, 4 h). Then,
361   100 µl of lysis buffer (10% SDS in 0.01 M HCl) was added and plates were incubated overnight
362   at 37 °C. The absorbance was measured at 570 nm (Infinite M200 microplate reader, Tecan,
363   Männedorf, Switzerland), $n = 6$.
364   **Effect of fractions of extracts on cell viability (MTT test)**
365   90 µL of new medium and 10 µL of four fractions: >50 K, 10–50 K, 3–10 K and <3 K of extracts
366   from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails, at the concentrations of 1.25 and
367   0.125 mg/mL were introduced into the cells. The same controls as in Effect of extracts on cell
368   viability (MTT test) section were applied. After 24 h incubation, the MTT test was done, as in
369   Effect of extracts on cell viability (MTT test) section. $n = 4$.
370   **Effect of extracts on the percentage of live and dead cells (trypan blue test)**
371   The trypan blue test is based on the fact that live cells have intact cell membranes which exclude
372   dyes such as trypan blue, while dead cells take up the dyes [44]. After mixing the cell suspension
373   with trypan blue, live cells have clear cytoplasms, while dead cells have blue ones. 535 µL of
374   new medium and 59 µL of extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa*
375   snails, at the concentration of 25 mg/mL were added to the cells. Equal volumes of sterile
376   deionized water were introduced into the control cells. After 12 h, 24 h, 48 h and 72 h of
377   incubation in a CO_2 incubator (INCO 108 med, Memmert GmbH + Co. KG, Schwabach,
378   Germany; 37 °C, 5% CO_2 and 95% relative humidity), the trypan blue test was done. Initially,
379   the cells were trypsinized, washed in cold PBS and centrifuged. The supernatants were removed
380   and the cell pellets were resuspended in PBS (100 µl). Then, 0.4% trypan blue (Sigma-Aldrich,
381   St. Louis, MO, USA; 50 µl) were mixed with the cell suspensions (50 µl). After incubation for 5
382   min, the samples (10 µl) were loaded into a Bürker chamber. Cells in the entire chamber were
383   photographed using a DMi8 inverted light microscope with a MC190 HD camera, employing the
384   LAS V4.10 software (Leica, Wetzlar, Germany). The results were expressed as the percentage of
385   live and dead cells in the groups treated with the extracts relative to the control group, $n = 5$.
386   **Effect of extracts on the integrity of cell membranes**
387   Cell membrane damage results in the release into the medium of the cytosolic enzyme, lactate
388   dehydrogenase (LDH). This enzyme can be quantified by applying a coupled enzymatic reaction.
389   It is a catalyst for the transformation of lactate to pyruvate by reduction of NAD^+ to NADH.

390   Thereafter, diaphorase utilizes NADH in order to reduce INT tetrazolium salt to red formazan
391   that content is measured (490 nm). The LDH test was carried out as recommended by the
392   commercial kit manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). 100 μL of new
393   medium and 10 μL of extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails, at
394   the concentrations of 25, 25×10^{-1}, 25×10^{-2}, 25×10^{-3}, 25×10^{-4} and 25×10^{-5} mg/mL were
395   added to the cells. Equal volumes of sterile deionized water were added to the control cells.
396   Additional controls were also included. After 24 h and 72 h of incubation in a CO_2 incubator
397   (INCO 108 med., Memmert GmbH + Co. KG, Schwabach, Germany; 37 °C, 5% CO_2 and 95%
398   relative humidity), LDH activity was evaluated and expressed as % of maximum LDH activity
399   (after cell lysis), $n = 4$.

400   **Effect of extracts on the content of lipid peroxidation products**
401   1.5 mL of new medium and 150 μL of extracts from eggs of two snail subspecies, at the
402   concentration of 2.5 mg/mL were introduced into the cells (each of the extracts was added to 18
403   wells). Equal volumes of sterile deionized water were introduced into the control cells. After 24
404   h incubation (CO_2 incubator INCO 108 med., Memmert GmbH + Co. KG, Schwabach,
405   Germany; 37 °C, 5% CO_2 and 95% relative humidity), the cells were trypsinized, washed in cold
406   PBS (two times) and centrifuged. The pellets were resuspended in RIPA buffer (130 μl) and
407   incubated (30 min, 4 °C) to lyse cells. Lysates were centrifuged ($14000 \times g$, 10 min., 4 °C),
408   supernatants were collected, frozen in liquid nitrogen and stored at -80 °C. Then, concentration
409   of TBARS in the supernatants was determined as in Determination of lipid peroxidation products
410   section. The total protein concentration in the supernatants was also determined, by the Bradford
411   method, using bovine serum albumin as the standard [45]. The results were expressed in μg/mg
412   total protein, $n = 3$.

413   **Effect of extracts on the types of cell death**
414   1.5 mL of new medium and 150 μL of extracts from lyophilized eggs of *H. a. maxima* and *H. a.*
415   *aspersa* snails, at the concentration of 25 mg/mL were added to the cells. Equal volumes of
416   sterile deionized water were added to the control cells. After 24 h of incubation in a CO_2
417   incubator (INCO 108 med, Memmert GmbH + Co. KG, Schwabach, Germany; 37 °C, 5% CO_2
418   and 95% relative humidity), the types of cell death were determined by flow cytometry, in
419   accordance with the procedure of the commercial kit with Alexa Fluor® 488 Annexin V and
420   propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA). The last one, a
421   fluorescent dye, binds to the nucleic acids staining dead cells and Annexin V conjugated to
422   Alexa Fluor® 488 fluorophore binds to phosphatidyl serine on the external surface of the
423   apoptotic cell membrane. Live cells do not stain, Annexin V-stained cells are considered early
424   apoptotic, PI/Annexin V-stained cells - late apoptotic and PI-stained cells - necrotic. The cells
425   were trypsinized and washed two times in cold PBS. Then, they were centrifuged, the
426   supernatants were removed and the pellets were resuspended in Annexin-binding buffer (100 μl).
427   Alexa Fluor® 488 Annexin V (5 μl) and PI working solution (1 μl) were introduced into the cell
428   suspensions. After incubation of the cells for 15 min (RT), Annexin-binding buffer (400 μl) was
429   introduced, the samples were mixed by pipetting and kept on ice. The cells were assayed using

430   BD FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), fluorescence
431   emission intensity was registered by FL1 channel for Alexa Fluor® 488, at 530 nm and FL2 for
432   PI, at 575 nm, with excitation at 488 nm. 10000 events were registered per sample. Flowing
433   Software 2.5.1 (Perttu Terho, Turku, Finland) was used to generate the plots, $n = 5$.
434   The cells were photographed using a DMi8 inverted light microscope with a MC190 HD camera,
435   employing the LAS V4.10 software (Leica, Wetzlar, Germany).

436   **Statistical analysis**
437   The results are shown as the mean ± the SEM (standard error of the mean). The results of
438   antioxidant indicators, content of phenols and total carbohydrates were subjected to a two-way
439   analysis of variance (ANOVA), the mean values were compared by applying the Tukey's post-
440   hoc test. Other results of the chemical composition were submitted to an unpaired Student's t-
441   test. Statgraphics Centurion software (StatPoint Technologies, Inc., Warrenton, VA, USA) was
442   employed. The cell test results were submitted to a one-way ANOVA, the means for groups
443   treated with the extracts were compared to groups treated with deionized water by applying the
444   Dunnett's post-hoc test. Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) was
445   used. The difference between the means at $p < 0.05$ was assumed statistically significant.

446

447   # Results
448   **Antioxidant indicators, content of phenols and total carbohydrates**
449   In the case of eggs of both snail subspecies, the fraction containing particles >50 kDa (K) was
450   marked by the greatest ferric-reducing antioxidant power, several times higher than the value for
451   the extract and fraction with particles 10-50 K (Table 1). The lowest ferric-reducing antioxidant
452   power was shown for fractions containing particles <10 K. The significant subspecies-fraction
453   interaction demonstrated that the fractions comprising particles >50 K were characterized by the
454   greatest ABTS·+ scavenging activity. The lower ability to reduce ABTS·+ was exhibited by
455   extract from eggs of *H. a. maxima*, followed by extract from eggs of *H. a. aspersa* and fractions
456   containing particles 10-50 K. The lowest ABTS·+ scavenging activity was noted in fractions with
457   particles <10 K.
458   The fractions with particles >50 K had the highest DPPH· scavenging activity. A lower DPPH·
459   scavenging activity was noted for extracts. Fractions containing particles <50 K did not show the
460   ability to reduce the DPPH·.
461   The statistically significant interaction demonstrated that the content of phenols was the highest
462   in the fractions with particles >50 K. This content was lower in the extracts and the extract from
463   *H. a. maxima* eggs comprised more phenols than the extract from *H. a. aspersa* eggs. Phenol
464   concentration in fractions comprising particles <50 K was slight or no such compounds were
465   detected.
466   The concentration of total carbohydrates in the lyophilized eggs from *H. a. maxima* was 17.97 ±
467   0.89 %, while in the lyophilized eggs from *H. a. aspersa* - 14.01 ± 0.33 %. Significantly the
468   highest concentration of total carbohydrates was observed in the fractions containing particles
469   >50 K. Fractions with particles <50 K contained few carbohydrates.

470 **Content of crude protein, crude fat, lipid peroxidation products, glutathione, astaxanthin**
471 **and vitamins**
472 The data showed that lyophilized snail eggs comprised mainly crude protein and its content was
473 similar in the eggs of both subspecies (Table 2). Furthermore, the presence of lipid peroxidation
474 products – TBARS was noted in extracts from eggs. The content of GSH was statistically
475 significantly higher in the eggs of *H. a. maxima* than in *H. a. aspersa*. The presence of crude fat,
476 astaxanthin and vitamins A, C and E was not detected in the eggs of both subspecies. The
477 lyophilized eggs of *H. a. aspersa* contained significantly more vitamin D_3.
478 **Analysis of molecular weights of proteins and glycoproteins**
479 Profiles of proteins and peptides derived from extracts from lyophilized eggs of *H. a. maxima*
480 and *H. a. aspersa* separated by SDS-PAGE are shown in Fig. 1A. The results indicate that the
481 egg proteins and peptides had molecular weights in the range of protein standards: from 10 kDa
482 to 250 kDa. Proteins of molecular weight >55 kDa predominated. The comparison of the protein
483 and peptide profiles shows that *H. a. maxima* eggs (panel (b)) contained more components of
484 low molecular weights compared to *H. a. aspersa* eggs (panel (c)). Protein and peptide bands
485 between 10 kDa and 15 kDa can be observed in the case of both analyzed extracts.
486 The eggs of *H. a. maxima* (Fig. 1B, panel (b)) and *H. a. aspersa* (Fig. 1B, panel (c)) contained
487 glycoproteins of molecular weights ranging from 8 kDa to 220 kDa. Glycoproteins >50 kDa
488 predominated, especially in the range from 50 kDa to 100 kDa and the profile of glycoproteins
489 was similar in case of both subspecies.
490 **Analysis of amino acids**
491 The amino acid profiles of lyophilized eggs of *H. a. maxima* and *H. a. aspersa* are presented in
492 Table 3. Eighteen amino acids were identified in eggs, eight of which are essential amino acids
493 (EAA) for humans – leucine (Leu), lysine (Lys), phenylalanine (Phe), valine (Val), threonine
494 (Thr), isoleucine (Ile), methionine (Met) and tryptophan (Trp) [46]. The content of individual
495 amino acids, except for Thr and Trp, in the crude protein of the eggs of two snail subspecies
496 differed statistically significantly. AAS, CS and EAAI for snail eggs are shown in Table 4. AAS
497 for amino acids of snail eggs was >1.00, except AAS for Lys and Leu for *H. a. aspersa* eggs.
498 The first limiting amino acid, according to AAS, was His (histidine) for *H. a. maxima* eggs and
499 Lys for *H. a. aspersa* eggs. The contents of Phe + Tyr (tyrosine), Val and Ile in eggs were the
500 highest compared to FAO/WHO standard protein. CS for amino acids of snail eggs was <1.00,
501 except CS for Phe + Tyr and Lys for *H. a. maxima* eggs. The first limiting amino acid, according
502 to CS, was Met + Cys for eggs of both snail subspecies. The concentration of Met in eggs was
503 low compared to most amino acids (Table 3). The EAAI (FAO/WHO reference amino acid
504 pattern) for snail eggs was >100 and the EAAI (whole egg reference amino acid pattern) was
505 <100 (Table 4). This index had higher value in the case of *H. a. maxima* eggs.
506 Taking into account non-essential amino acids (NEAA), snail eggs had the highest content of
507 glutamic acid (Glu), aspartic acid (Asp) and serine (Ser) (Table 3).

508 Snail eggs were characterized by a high total concentration of delicious amino acids (DAA) -
509 Glu, Asp, Ala (alanine) and Gly (glycine). Gly content was the lowest in this group of amino
510 acids.
511 The concentrations of total amino acids (TAA), EAA, half-essential amino acids (HEAA),
512 NEAA and DAA were higher in the crude protein of *H. a. maxima* eggs than of *H. a. aspersa*
513 eggs. EAA/TAA and EAA/NEAA ratios were higher for *H. a. maxima* eggs and DAA/TAA ratio
514 was higher for *H. a. aspersa* eggs.
515 **Analysis of minerals**
516 The content of macroelements and microelements, except for Mg, Mn and Se, in the lyophilized
517 eggs of *H. a. maxima* and *H. a. aspersa* snails was statistically significantly different (Table 5).
518 The concentration of macroelements (descending order) in the eggs of both snail subspecies was
519 as follows: Ca, P, Na, K, Mg, S, Cl. The concentration of microelements (descending order) in
520 *H. a. maxima* eggs was as follows: Cu, Ni, Si, Fe, Mn, Cr, Mo, B, Zn, Co, V, Se, I, Sn and in *H.
521 a. aspersa* eggs: Si, Cu, Fe, Mn, Ni, B, Zn, Mo, Cr, Co, Se, I, V, Sn. No F was detected in the
522 eggs.
523 **Effect of extracts on cell viability (MTT test)**
524 The influence of various concentrations of water extracts from lyophilized eggs of *H. a. maxima*
525 and *H. a. aspersa* snails on the viability of Caco-2 cells was determined using the MTT test,
526 based on the mitochondrial oxidative activity in live cells. Treatment with an extract from *H. a.
527 maxima* eggs (at concentrations of 25, 25×10^{-1} and 25×10^{-4} mg/mL) and an extract from *H. a.
528 aspersa* eggs (25, 25×10^{-1} and 25×10^{-3} mg/mL), for 24 h, resulted in a statistically significant
529 reduction in the viability of Caco-2 colon cancer cells compared to control cells, treated with
530 deionized water (Fig. 2a). Treatment with extracts from eggs of both snail subspecies, at
531 different concentrations, for 72 h, did not statistically significantly affect the viability of Caco-2
532 cells compared to control cells (Fig. 2b).
533 **Effect of fractions of extracts on cell viability (MTT test)**
534 Treatment with fraction of an extract from *H. a. maxima* eggs containing particles of a molecular
535 weight <3 kDa (1.25 and 0.125 mg/mL), for 24 h, resulted in a statistically significant reduction
536 of the viability of Caco-2 cells compared to control cells (Fig. 3a). Treatment with fraction of an
537 extract from *H. a. aspersa* eggs containing particles <3 kDa (concentrations as above), for the
538 same time, resulted in a reduction of the viability of Caco-2 cells compared to control cells, but
539 this effect was not statistically significant (Fig. 3b).
540 **Effect of extracts on the percentage of live and dead cells (trypan blue test)**
541 The effect of extracts from eggs of *H. a. maxima* and *H. a. aspersa* on the percentage of live and
542 dead cells was evaluated by the trypan blue assay, in which live cells, having intact cell
543 membranes, exclude trypan blue, while dead cells take up this dye and have blue cytoplasms.
544 Treatment with extracts from eggs of both snail subspecies (25 mg/mL) for 12 h did not have a
545 statistically significant effect on the percentage of live Caco-2 cells in comparison with control
546 cells, treated with deionized water (Fig. 1a). Incubation with the extract from *H. a. maxima* eggs
547 significantly increased the percentage of dead cells. Treatment with both extracts for 24 h and 48

548   h resulted in a statistically significant reduction in the percentage of live Caco-2 cells as

549   compared to control cells and did not significantly affect the percentage of dead cells (Fig. 4b

550   and 4c). Treatment with these extracts for 72 h did not significantly affect the percentage of live

551   and dead Caco-2 cells as compared to control cells (Fig. 4d).

552   **Effect of extracts on the integrity of cell membranes**

553   Treatment with an extract from *H. a. maxima* eggs (25, 25×10^{-1} and 25×10^{-3} mg/mL) and an

554   extract from *H. a. aspersa* eggs (25×10^{-1} mg/mL), for 24 h, statistically significantly increased

555   the degree of damage to Caco-2 cell membranes, the activity of the cytosolic enzyme - LDH,

556   released from damaged cells, compared to control cells, treated with deionized water (Fig. 5a).

557   Treatment with extracts from the eggs of both snail subspecies ($25 - 25 \times 10^{-5}$ mg/mL), for 72 h,

558   did not significantly affect the activity of released LDH (Fig. 5b).

559   **Effect of extracts on the content of lipid peroxidation products**

560   Treatment of cells with extracts from the eggs of both snail subspecies (2.5 mg/mL), for 24 h,

561   increased the amount of lipid peroxidation products – TBARS compared to control cells, treated

562   with deionized water, but this effect turned out to be statistically insignificant (Fig. 6).

563   **Effect of extracts on the types of cell death**

564   Treatment of Caco-2 cells with extracts from eggs of both snail subspecies (25 mg/mL), for 24 h,

565   caused the induction of apoptosis and reduction of necrosis - an increase in the percentage of

566   early apoptotic cells (effect statistically significant for *H. a. aspersa* egg extract) and a decrease

567   in the percentage of necrotic cells compared to control cells, treated with deionized water (Fig.

568   7).

569

Discussion

571   High concentrations of reactive oxygen species (ROS) in cells may lead to oxidative stress,

572   damage to the cell membranes, proteins and DNA [47]. Antioxidants are necessary in prevention

573   of the formation and inhibition of the activity of ROS [48]. Some antioxidants and a greater total

574   antioxidant capacity are related to decreased risk of colorectal cancer [47,49,50]. These

575   compounds may be beneficial in the initiation and progression of cancer [48]. Their action may

576   include a positive effect on cell proliferation, apoptosis, metastasis and drug resistance. In the

577   current research, higher antioxidant activity, expressed as $ABTS^{\cdot +}$ scavenging activity, and

578   phenol content were noted in the extract from lyophilized eggs of *H. a. maxima* than of *H. a.*

579   *aspersa*. Antioxidants including phenols were concentrated in fractions >50 K. The differences

580   in the antioxidant potential of the fractions determined with the use of various methods result

581   from the complex kinetics of the contained antioxidants and the time they reach stable endpoints

582   [51]. Ferric-reducing antioxidant power and $ABTS^{\cdot +}$ scavenging activity are based on electron

583   donating capacity of bioactive compounds, and $DPPH\cdot$ scavenging activity is based on electron

584   and hydrogen atom transfer [52]. $ABTS^{\cdot +}$ is more reactive than $DPPH\cdot$. Ferric-reducing

585   antioxidant power differs from the two methods used to determine the concentration of

586   antioxidants, because the reduction of Fe^{3+} to Fe^{2+} is monitored and free radicals are not involved

587   [53]. The content of hydrophilic and high-pigmented antioxidants is better reflected by ABTS$\cdot^+$
588   scavenging activity than DPPH· scavenging activity.
589   Phenols are compounds that can initiate the process of autoxidation under certain conditions, and
590   therefore they can act as prooxidants [54,55]. Such conditions include, for example, high pH,
591   high contents of transition metal ions, Cu^{2+}, Fe^{3+} and the presence of oxygen molecules. The
592   high concentration of phenols also favors their prooxidative effect [55]. In addition, low
593   molecular weight phenols are easily oxidized and show prooxidative activity, unlike those with
594   high molecular weight, which have little or no prooxidative activity [54].
595   Cancer cells are characterized by a higher level of transition metal ions and the mobilization of
596   such endogenous metal ions as Fe and Cu may explain the selective toxicity of polyphenols
597   toward cancer cells [56]. Moreover, cancer cells of many solid tumors, because of the Warburg
598   effect, are characterized by a high glycolysis level, which leads to a decrease in pH, that exposes
599   Cu bound to chromatin to a potential attack of prooxidants [57].
600   Polyphenol-induced production of ROS may play a major role in apoptosis initiation, even
601   though ROS are also generated as a consequence of it [58-61]. The prooxidative activity of some
602   polyphenols, in non-cytotoxic concentrations, may sensitize cancer cells to other cancer therapies
603   [62]. Epigallocatechin gallate treatment of chemoresistant HT-29 colon cancer cells was related
604   to an increased ROS production, reduced proliferation and sensitization to 5-fluorouracil [63].
605   ROS induced by phenolic compounds, with/without the presence of transition metals, may
606   contribute to lipid peroxidation [64].
607   In the study by Kostadinova et al., the fraction of *H. a. aspersa* mucus containing the smallest
608   particles, <5 kDa, was characterized by a greater antioxidant potential compared to the other
609   fractions tested [65]. Low molecular weight peptides and free amino acids may contribute
610   significantly to the antioxidant potential [66]. About 70% of the antioxidant peptides had a
611   molecular weight in the range 400-650 Da [67]. In two fractions of *H. a. aspersa* mucus,
612   containing molecules <1 kDa and <3 kDa, similar metabolites with antioxidant activity were
613   detected [68]. The antioxidant activity of *H. a. maxima* eggs was demonstrated in the study of
614   Górka et al. [69].
615   The concentration of carbohydrates in the lyophilized eggs of the studied snail subspecies was
616   higher than in the lyophilized foot tissues of *H. a. aspersa* snails and similar to their content in
617   the lyophilized mucus of these snails [7]. Carbohydrates were also concentrated in the fraction
618   >50 K. Other authors' research shows that the shell of *H. aspersa* eggs consists of crystals of
619   calcium carbonate bound to the mucopolysaccharide matrix that are surrounded by the mucus
620   layer [70,71]. Hovingh and Linker showed that chondroitin sulfate is the major
621   mucopolysaccharide (glycosaminoglycan) present in the heart, mantle and kidney of *H. aspersa*,
622   and heparan sulfate was also present in smaller amounts in these organs [72]. Wu et al. proved
623   that chondroitin sulfate derived from sturgeon (*Acipenser*) reduced the proliferation of HCT 116
624   human colon carcinoma cells and induced extensive apoptosis [73]. Chondroitin sulfate also
625   inhibited the xenograft HCT 116 tumor development in mice by inhibition of proliferation and
626   induction of apoptosis. Many anticancer drugs based on glycosaminoglycans or their mimetics

627 have been developed with promising results on animal models and in clinical trials [74]. Nicolai
628 et al. demonstrated that galactogen is the main compound contained in the eggs of *H. a. aspersa*
629 snails, and its concentration turned out to be about six times greater than that of glycogen [75]. In
630 the study of Górka et al., *H. a. maxima* eggs did not contain glucose [69].
631 Our research showed that the lyophilized eggs of both snail subspecies consisted mainly of crude
632 protein and were practically fat-free. Protein requirement for maintenance is 0.66 g/kg/d [76].
633 The concentration of crude protein in the lyophilized eggs of the studied snails turned out to be
634 nearly two times lower than in the lyophilized mucus of *H. a. aspersa* and over two and a half
635 times lower than in the lyophilized foot tissues of these snails [7].
636 Murine squamous cell carcinoma VII and human colorectal carcinoma grew slower in mice that
637 were administered low-carbohydrate and high-protein diet comparing to a Western diet –
638 relatively high-carbohydrate and low-protein [77]. This diet reduced glycolysis, on which cancer
639 cells depend to a large extent. In turn, feeding a low-protein diet decreased tumor growth in
640 mouse cancer models [78].
641 The lyophilized mucus of *H. a. aspersa* snails comprised almost no crude fat, and the foot tissues
642 contained less than 4% [7]. The fat comprised in the lyophilized foot tissues, however, had a
643 high nutritional value. The crude protein content in lyophilized eggs of the studied snails was
644 lower than the total protein content in the dry matter of *H. a. maxima* eggs in the study of Górka
645 et al. [69]. In the studies by Maćkowiak-Dryka et al., the fat content in *H. a. maxima* eggs was
646 0.04%, while in *H. a. aspersa* eggs was 0.03%, however, this fat turned out to have a low
647 nutritional value [79]. Other authors detected the slight presence of triacylglycerols in *H. a.*
648 *aspersa* eggs [75] and cholesterol in *H. a. aspersa* and *H. a. maxima* eggs [69,75].
649 The concentration of carbohydrates in *H. a. maxima* and *H. a. aspersa* eggs is greater compared
650 to caviar and the raw materials from which other caviar substitutes are obtained [80,81]. On the
651 other hand, the fat content is much lower and the fat is not a good source of polyunsaturated fatty
652 acids (PUFA).
653 In our study, in egg extracts were detected lipid peroxidation products.
654 The product of lipid peroxidation, 4-hydroxynonenal (HNE) can have a stronger cytotoxic,
655 pronecrotic and proapoptotic effect on cancer cells than on normal cells because cancer cells
656 have a lower PUFA content in the cell membrane, to protect against lipid peroxidation [82]. It
657 influences oxidative homeostasis and growth regulation. MDA, formed by lipid peroxidation,
658 reacts with bases of nucleic acids and forms adducts that can cause apoptosis [83]. 4-HNE could
659 suppress growth of Caco-2 and HT-29 cells [83]. The anticancer action might be related to
660 oxidative stress alterations and consequent apoptosis induction. Glutathione conjugates of HNE
661 are used as chemotherapeutic agents in cancer models.
662 GSH, a major intracellular antioxidant, regulates cellular redox state, protects cells from lesions
663 induced by lipid peroxides, ROS, reactive nitrogen species, as well as xenobiotics [84]. GSH is
664 an important controller of cell apoptosis, ferroptosis, proliferation, differentiation and immune
665 function. Alterations in GSH antioxidant system at the molecular level and disturbances in
666 homeostasis of this compound are related to cancer initiation, progression and response to

667   treatment. Increased GSH concentrations in cancer cells are connected with cancer progression
668   and elevated resistance to chemotherapeutics. In our investigation, *H. a. maxima* eggs contained
669   more GSH than *H. a. aspersa* eggs.
670   The proteins and peptides of snail eggs had molecular weights from 10 kDa to 250 kDa, and
671   proteins >55 kDa predominated. *H. a. maxima* eggs contained more components of low
672   molecular weights compared to *H. a. aspersa* eggs. Proteins and peptides of a molecular weight
673   between 10 kDa and 15 kDa were present in the eggs of both subspecies.
674   Sixteen putative cationic and amphipathic anticancer peptides were predicted in two fractions of
675   *A. fulica* mucus, which decreased the viability of MCF7 and Vero cells [7,9]. Nine of the
676   peptides had a molecular weight <3 kDa, three - in the range of 3–10 kDa, two - in the range of
677   10–50 kDa and two - >50 kDa. The presence of anticancer peptides was also predicted in the
678   mucus of several mollusks, including land snails [85].
679   The eggs of both snail subspecies presented the similar profile of glycoproteins, of molecular
680   weights from 8 kDa to 220 kDa and glycoproteins >50 kDa predominated, especially in the range
681   from 50 kDa to 100 kDa.
682   The glycoprotein *Helix aspersa* agglutinin, member of the family of H-type lectins identified in
683   invertebrates, an element of the perivitelline fluid of snail eggs, may be useful in drug delivery
684   systems targeting colorectal cancer, due to binding to the surface of cancer cells [86].
685   EAA enable endogenous synthesis of NEAA and their availability is the major limiting factor in
686   protein synthesis [87]. AAS for Lys in *H. a. aspersa* lyophilized eggs was <1.00, so this amino
687   acid concentration was lower than in FAO/WHO standard [38]. According to this score, the first
688   limiting amino acid of lyophilized *H. a. maxima* eggs was His and of lyophilized *H. a. aspersa*
689   eggs - Lys. For lyophilized foot tissues and mucus of *H. a. aspersa* more than one amino acid
690   had AAS <1.00, with Trp being the first limiting amino acid for foot tissues and Lys for mucus
691   [7]. The concentrations of Phe + Tyr, Val and Ile in lyophilized eggs were dominating. In the
692   case of lyophilized foot tissues and mucus of *H. a. aspersa*, these were Ile, Thr and Val [7]. CS
693   for amino acids of lyophilized snail eggs was <1.00, except CS for Phe + Tyr and Lys for *H. a.*
694   *maxima* eggs, so their contents were smaller than in whole egg protein pattern. According to this
695   score, the first limiting amino acid of lyophilized eggs was Met + Cys. In the case of lyophilized
696   foot tissues and mucus of *H. a. aspersa*, CS for all amino acids was <1.00, while the first
697   limiting amino acids were for the foot tissues - Met + Cys, and for the mucus - Trp and Met +
698   Cys [7]. Met content in lyophilized eggs was low comparing to most amino acids. The EAAI
699   (FAO/WHO standard) of lyophilized snail eggs was >100 and the EAAI (whole egg standard)
700   was <100. This index was greater for *H. a. maxima* eggs and significantly greater than for
701   lyophilized foot tissues and mucus of *H. a. aspersa* [7].
702   Dietary restriction of Met may be a main strategy to control cancer growth [88]. Furthermore,
703   Met restriction resulted in killing Met-dependent cancer cells co-cultured with normal cells.
704   Animal research in which diets restricted by Met were studied showed inhibited cancer growth
705   and extend healthy life-span. Met depletion in Met-dependent cancer cells can contribute to cell

706   cycle arrest in late S/G2 phase, the susceptibility of cells to death and their hypersensitivity to
707   chemotherapy.
708   Glu, Asp and Ser are NEAA found in the highest concentrations in lyophilized snail eggs. Gly
709   content was about two times lower than in the lyophilized foot tissues and mucus of *H. a.*
710   *aspersa* [7]. The deprivation of Gly and Ser in the diet inhibits the growth of some cancers,
711   including intestinal [89,90]. Depletion of Gly in the diet limits one route for Ser synthesis.
712   Limiting Gly and Ser may reduce the cancer's capability to cope with ROS. In addition, a
713   combination of Gly and Ser deprivation with radiation, a treatment inducing ROS may prove
714   effective. Gly and Ser deprivation limits single-carbon units for nucleotide biosynthesis and this
715   might enhance the effectiveness of drugs that target nucleotide synthesis. Moreover, Gly limited
716   growth of cancer in model animals [91].
717   Branched-chain amino acids (Ile, Leu, Val), administered to obese, diabetic rats, showed the
718   ability to reduce preneoplastic changes.
719   The ratios of EAA/TAA and EAA/NEAA for lyophilized *H. a. maxima* eggs were higher than
720   for *H. a. aspersa* eggs and these ratios were higher for the examined eggs than for the
721   lyophilized foot tissues and mucus of *H. a. aspersa* [7]. The effect of EAA and the mixture of
722   amino acids comprised 85% EAA and 15% NEAA on various cancer cells, including Caco-2 and
723   HCT 116, and MCF 10A human breast epithelial cells was examined [87]. Both EAA and the
724   mixture containing EAA and NEAA showed antioproliferative and cytotoxic activities, including
725   activation of autophagy and apoptosis. Changing the ratio of EAA to NEAA may be an
726   anticancer strategy leading to the selective cancer cell death. Moreover, EAA administration,
727   with/without the chemotherapeutic drug doxorubicin, increased the mortality of various cancer
728   cells, including HCT 116 [92]. EAA increased the concentration of apoptotic markers. Higher
729   EAA/NEAA ratio may limit the survival of cancer cells and their proliferation.
730   The lyophilized *H. a. aspersa* eggs contained more vitamin D_3 than the lyophilized *H. a. maxima*
731   eggs and both could be a valuable source of this vitamin.
732   Vitamin D_3 is produced mainly in the skin that is exposed to ultraviolet-B radiation of the sun,
733   but can also be acquired from diet and supplements [93]. In combination with vitamin D binding
734   protein in circulation, vitamin D_3 (provitamin cholecalciferol) moves to the liver and is
735   metabolized to 25-hydroxyvitamin D [25-hydroxycholecalciferol, calcidiol, calcifediol,
736   25(OH)D]. 25(OH)D is transported to the kidneys where it is metabolized to calcitriol
737   [1,25(OH)$_2$D] which is converted to metabolites with less activity.
738   It seems rational to suggest a vitamin D supplementation at a dose of 800–2000 IU/day (20-50
739   µg/day) [94].
740   Vitamin D and calcium affect additively incidence of colorectal adenoma, its malignant
741   transformation and progression [95]. Calcitriol affects different colon cancer cell lines containing
742   an adequate level of vitamin D receptors (VDR) [96]. Its transcription-independent (non-
743   genomic) activities, mediated by extranuclear VDR and alternative receptors, have also been
744   reported. It inhibits proliferation of above cells through different mechanisms and sensitizes
745   them to apoptosis. It induces *CST5*/cystatin D expression which limits proliferation [97].

746 Calcitriol modulates the expression of histone H3 lysine-27 demethylase Jumonji C domain-
747 containing protein 3 (*JMJD3*) which mediates the activities of calcitriol in colorectal cancer cells
748 including decrease of proliferation [98]. Calcitriol sensitizes colon cancer cells to apoptosis
749 induction through the increase of expression of proapoptotic genes, the decrease of expression of
750 survival genes and *via* interference with the secretion of IL-1ß by macrophages [99]. VDR
751 agonists enhance the action of chemotherapeutics in animal and cell models of colorectal cancer
752 [100]. Calcitriol inhibits the production of *DKK-4*, promoting chemoresistance [101,102]. It was
753 demonstrated that *miR*(*microRNA*)-22, induced in a VDR-dependent manner, decreases
754 proliferation of colorectal cancer cells and tumor growth [103,104].
755 The SUNSHINE trial investigated vitamin D_3 supplementation in patients undergoing
756 chemotherapy with unresectable advanced and metastatic colorectal cancer [105,106].
757 Participants receiving high-dose vitamin D_3 (firstly 8000 IU/day during 2 weeks and then 4000
758 IU/day), in comparison to the standard dose (400 IU/day), experienced a 2-month increase
759 (statistically nonsignificant) in median progression-free survival. In adjusted analyses,
760 participants receiving high-dose experienced progression and death less frequently during the
761 22.9 month-median follow-up. The AMATERASU trial included participants with luminal
762 gastrointestinal cancer, stage I to III, after tumor resection [105,107]. Patients were
763 supplemented with vitamin D_3 (2000 IU/day) or placebo and a post hoc analysis showed a
764 significant benefit from supplementation.
765 The content of Ca in the lyophilized eggs of *H. a. maxima* and *H. a. aspersa* was about 17 times
766 higher than in the lyophilized foot tissues of *H. a. aspersa* and more than 1.5 times higher than in
767 the lyophilized mucus of this subspecies [7]. The tested egg lyophilizates comprised about 2
768 times less P than the lyophilizate from *H. a. aspersa* foot tissues, while they were over 2.5 times
769 richer in this element compared to the lyophilizate from the mucus of these snails [7]. It was
770 shown that Ca possesses properties against colorectal cancer, it limits proliferation and induces
771 apoptosis [108]. Furthermore, Ca and P demonstrated a protective effect at different adenoma-
772 carcinoma sequence steps [109]. In turn, Arnst and Beck Jr showed that an excess of inorganic
773 phosphate can influence and promote the cancer phenotype [110]. Higher Ca, K, Mg, Mn, Zn, Se
774 and I intakes and lower P, Na, Cu and Fe intakes may be connected with lower colorectal cancer
775 risk [111,112]. The action of the first group of elements may be related to their antioxidative and
776 other anticarcinogenic effects and the action of the second group of elements - to prooxidative
777 and other procarcinogenic effects. The concentration of Na in lyophilized snail eggs was about 4
778 times lower than in the lyophilized foot tissues of *H. a. aspersa* and several dozen times lower
779 than in their lyophilized mucus [7]. The WHO recommends a consumption of Na <2 g/day for
780 adults [113]. As for other macroelements, the content of K and Mg in lyophilized eggs turned out
781 to be several - a dozen times lower than in the lyophilized foot tissues and mucus of *H. a.*
782 *aspersa*, and the content of S and Cl – several – a dozen times lower than in lyophilized foot
783 tissues of these snails [7]. The WHO recommends the intake of K for adults in an amount of at
784 least 3.51 g/day [114]. Recommended nutrient intake (RNI) for Mg for males (19–65 years) is
785 260 mg/day and for females (19–65 years) - 220 mg/day [115]. Mg and 25(OH)D may act

786 synergistically in reducing the all-cause mortality risk in colorectal cancer patients [116]. Mg
787 plays the crucial role in biochemical reactions involved in vitamin D synthesis and metabolism.
788 In addition, every 100 mg/day increase in Mg intake was connected with 13% lower colorectal
789 adenoma risk and 12% lower colorectal tumor risk. Moreover, Mg deficiency and high Ca:Mg
790 intake have been linked to a higher colon cancer incidence and mortality. Ca:Mg >2.6–2.8 may
791 have a negative influence on colorectal adenoma outcomes and Ca:Mg <1.7 may have negative
792 consequences, and were connected with an increased total mortality risk [117]. Mg deficiency
793 may increase intracellular Ca concentration promoting ROS generation and it may blunt
794 antioxidant capacity to promote oxidative stress [116]. Cell cultures and animal studies suggest
795 Mg role in carcinogenesis through affecting cell proliferation, apoptosis and other processes
796 [118]. In turn, Fujii et al. showed that in colorectal cancer hypomagnesemia is a predictor of the
797 effectiveness of therapy based on anti-epidermal growth factor receptor antibodies [119]. One
798 study showed the capability of S to induce apoptosis of non-small cell lung carcinoma cells that
799 are resistant to drugs [120]. Moreover, S is an element of non-enzymatic antioxidants, cancer
800 therapeutics [121]. Excess Cl, by reaction with water and mineral compounds, creates
801 trihalomethane, which causes cancer [122].
802 The concentration of Cu in the lyophilized *H. a. maxima* eggs was slightly higher compared to
803 the lyophilized *H. a. aspersa* foot tissues, and in lyophilized *H. a. aspersa* eggs – slightly lower
804 [7]. Other authors showed that Cu was cytotoxic to HT-29 cells, which was connected with
805 activation of apoptosis, increased oxidative stress, alterations in β-oxidation in mitochondria and
806 changes in lipid and energy metabolism [123]. It was also demonstrated that Cu exerted
807 toxicological effect on Caco-2 cells [124]. Research on rats indicated that low intake of Cu is a
808 risk factor for the development of colon tumor, induced by 3,2'-dimethyl-4-aminobiphenyl and
809 reduced the activities of Cu,Zn-superoxide dismutase (SOD) and ceruloplasmin [125]. According
810 to other authors, progression to colon cancer in rats was related to low Zn concentration and
811 lower Cu,Zn-SOD activity in blood plasma [126]. Zn modulates folding and misfolding of p53
812 which is associated with cancer [127]. According to others, higher Cu and Zn in blood serum
813 were associated with increased risk of colorectal cancer development. Se, selenoprotein P and Se
814 to Cu ratio were connected with decreased risk [128]. In turn, Cu metabolism may be a
815 promising target for colorectal cancers harboring KRAS mutations [129].
816 The level of Ni in lyophilized *H. a. aspersa* eggs turned out to be slightly lower compared to
817 lyophilized *H. a. aspersa* foot tissues, and in lyophilized *H. a. maxima* eggs – higher [7]. Contact
818 with Ni can cause different cancers and oxidative stress and mitochondrial dysfunctions can have
819 a crucial role in its toxicity [130]. Ni compounds can induce apoptosis in different cancer and
820 normal cells.
821 The Fe content in lyophilized eggs of both snail subspecies was lower compared to that in
822 lyophilized foot tissues and mucus of *H. a. aspersa* [7]. RNI for Fe is 19.6-58.8 mg/day for
823 males (18+) and 9.1-27.4 mg/day for females (18+), and is dependent on its bioavailability [115].
824 An excess of intestinal Fe increases the risk of developing colorectal cancer by increasing the
825 proliferation of cancer cells, contributing to colon damage induced by oxidative stress and

826 enhancing oncogenic signaling [131]. Research in rodents showed that increased dietary Fe
827 enhances colonic crypt cell proliferation and colorectal tumor development [131-133]. Fe may be
828 able to produce ROS that increase oxidative stress, which causes, among others, lipid
829 peroxidation [131]. Excess oxidative stress can result in colonic inflammation which can lead to
830 colorectal cancer [131,134]. In turn, Fe deficiency is widespread in colorectal cancer patients and
831 may result in a diminished immunosurveillance response and changed tumor immune
832 microenvironment, which may potentially lead to cancer progression [131,135]. However, Bird
833 et al. showed a U-shaped connection between Fe intake and adenomatous polyps, demonstrating
834 that people who ingest low (<11.6 mg/day) or high (>27.3 mg/day) Fe amounts had increased
835 colorectal cancer risk, in comparison with people consuming an adequate Fe amount [131,136].
836 Intake of Fe from white meat and plants, containing less heme Fe than red meat, were inversely
837 connected with colorectal cancer risk [137].
838 The concentration of Mn in the examined lyophilized snail eggs was higher than in the
839 lyophilized *H. a. aspersa* mucus [7]. The mitochondrial Mn-SOD inhibited growth of different
840 cancer cells [138]. According to other authors, overexpression of Mn-SOD decreased HCT 116
841 cell growth by inducing cell senescence [139]. The rats that were administered low Mn in a diet
842 had 23% larger preneoplastic lesions and rats ingesting high Fe in a diet - 18% higher [140].
843 The concentration of Cr in lyophilized *H. a. maxima* eggs was higher than in lyophilized *H. a.*
844 *aspersa* foot tissues and mucus, and in lyophilized *H. a. aspersa* eggs - lower [7]. Cr (III)
845 ameliorated the healing of colitis in mice by promoting antioxidant potential, suppressing ROS
846 and inflammation [141].
847 The Mo content in the lyophilized *H. a. maxima* eggs was higher than in the lyophilized foot
848 tissues and mucus of *H. a. aspersa*, and in the lyophilized *H. a. aspersa* eggs – lower [7]. The
849 incidence and development of induced esophageal tumors were lower in rats which were
850 administered high-Mo diet compared to animals which were fed low-Mo diet [142]. Xanthine
851 oxidase could have played an important function in inhibiting carcinogenesis.
852 The level of B in lyophilized eggs of both snail subspecies was lower than in lyophilized *H. a.*
853 *aspersa* foot tissues and mucus [7]. Low-B diet could increase cancer risk [143].
854 The content of Zn in lyophilized eggs was lower compared to lyophilized *H. a. aspersa* foot
855 tissues and mucus [7]. RNI for Zn for males (19-65 years) is 4.2-14.0 mg/day and for females
856 (19-65 years) is 3.0-9.8 mg/day, and is dependent on its bioavailability [115]. Zn stabilizes the
857 structure and regulates NF-κB which induces the expression of various genes related to cell
858 proliferation, apoptosis inhibition, resistance to chemotherapeutics and other processes, promotes
859 tumor formation [144]. Activation of NF-κB can be blocked by affecting redox status of cells,
860 the application of competitive inhibitors binding to its DNA site, such as Zn or Cr. Zn could
861 protect healthy cells from the cytotoxic and genotoxic effects of H_2O_2, but enhances its toxicity
862 in tumor tissue [144,145]. Zn, as an element of CuZn-SOD, has a strong antioxidant potential
863 [144]. CuZn-SOD and Mn-SOD affects tumor formation and development [144,146,147].
864 Another mechanism of the antioxidant activity of Zn comprises its antagonism against minerals
865 participating in lipid peroxidation (Fe and Cu) [144]. It also protects the protein -SH groups from

866 oxidation by chelate formation. Zn ions play an important role in the induction of
867 metallothioneins, contributing to cancer development or inhibition. It stabilizes the zinc finger
868 structures, which play important functions in, among others, proliferation and apoptosis. Zn
869 deficiency may result in a higher cancer initiation and progression risk [144,145]. It plays a
870 major role in the growth and division of cells and programmed death [144]. Zn has
871 multidirectional role in the initiation and inhibition of apoptosis. Endogenous Zn is likely
872 indispensable in autophagy induction, under oxidative stress. A meta-analysis of six studies
873 showed that higher Zn intake was associated with reduced colorectal cancer risk [148]. Rat
874 studies suggest that Zn deficiency contributes to colorectal cancer development and progression
875 [149]. Application of Zn to rats treated with dimethylhydrazine (DMH) reduced tumor incidence,
876 size and multipilicity [150]. Furthermore, increased Zn concentrations inhibited growth of cells
877 that represent various colon cancer stages and induced their death [151]. The main mechanism
878 turned out to be oxidative stress related to mitochondria disorders and plasma membrane
879 damage. Cell death was cell line dependent; the cells showed signs of apoptosis, necrosis,
880 autophagy and mixed types of cell death.
881 The concentration of Co in lyophilized eggs of *H. a. maxima* and *H. a. aspersa* was higher in
882 comparison with lyophilized *H. a. aspersa* foot tissues and mucus [7]. High Co consumption
883 may be associated with an increased colon cancer risk and high Se consumption with decreased
884 risk [152].
885 V was also present in the lyophilized eggs of both snail subspecies. Vanadium N-(2-hydroxy
886 acetophenone) glycinate demonstrated cytotoxic action on human colorectal carcinoma cells and
887 other cancer cells, without toxic influence on normal fibroblasts [153]. It induced apoptosis of
888 human colorectal carcinoma cells by mechanisms including mitochondrial damage and higher
889 ROS level.
890 The content of Se in lyophilized snail eggs was lower compared to lyophilized *H. a. aspersa* foot
891 tissues and mucus [7]. RNI for Se for males (19–65 years) is 34 µg/day and for females (19-65
892 years) is 26 µg/day [115]. Se is an element of selenocysteine which is part of selenoproteins
893 (SELENO), as glutathione peroxidase (GPX) and thioredoxin reductase (TXNRD) [154]. Their
894 important functions include maintaining cellular redox homeostasis. Other selenoproteins, as
895 SELENOF and SELENOS, in turn, participate in the folding and degradation of proteins. There
896 was an inverse correlation between Se intake and colorectal cancer mortality [154,155]. Elevated
897 SELENOP level was inversely correlated with colorectal cancer risk [154,156]. It seems that
898 suboptimal Se concentration is a cancer risk factor [154,157]. The mechanism of Se's anticancer
899 activity may be increased expression of selenoproteins, which prevent oxidative damage to DNA
900 [154]. SELENOH downregulated colorectal cancer cells showed increased growth properties
901 [154,158], and loss of GPX2 [154,159] and SELENOF [154,160] impaired growth of colorectal
902 cancer cells. The probability of relapse-free survival in colorectal cancer patients was lower in
903 the case of high expression of GPX2 in the tumor [154,159]. The same relationship was noted for
904 high content of intratumoral SELENOF [154,161]. Moreover, the studies demonstrated the
905 anticancer potential of dietary Se by inhibiting the tumor growth of colon cancer cells [162-165].

906   Its chemopreventive effect could be triggered by various molecular mechanisms including
907   antioxidant protection, cell cycle suppression and apoptosis induction [162,166]. A high intake
908   of dietary Se (2.29 μg/g) reduced the growth of tumor of HT-29 colon cancer cells transplanted
909   into immunosuppressed mice and induced cancer cell apoptosis [162]. Se was hypothesized to
910   decrease lipid oxidation via GPX. In turn, the effect of Se on colorectal cancer in mice, induced
911   by 1,2-dimethylhydrazine, was mediated by its influence on, among others, increasing oxidative
912   stress, MDA concentration and apoptosis [167]. Depending on the concentration, Se can act as
913   an antioxidant and a pro-oxidant in different experimental conditions [167-169].
914   The content of Ca, Mg and Cu in the lyophilized eggs of *H. a. maxima* and *H. a. aspersa* was
915   higher than the content of these elements in the dry matter of *H. a. aspersa* eggs in the Beeby
916   and Richmond study [170]. The authors also detected the presence of Zn in these eggs. The
917   concentration of Zn and Mn in the lyophilized eggs of *H. a. maxima* and *H. a. aspersa* also
918   turned out to be higher compared to the concentration of these minerals in the dry matter of *H. a.*
919   *maxima* eggs in the study of Górka et al. [69], and the content of Ca, Mg, Cu, Fe and Cr in the
920   tested lyophilized snail eggs was similar to the content in the dry matter of *H. a. maxima* eggs.
921   The extract from *H. a. maxima* eggs (25, 25×10^{-1} and 25×10^{-4} mg/mL) and the extract from *H.*
922   *a. aspersa* eggs (25, 25×10^{-1} and 25×10^{-3} mg/mL) decreased the viability of Caco-2 cells after
923   24 h of treatment. Cell viability was reduced by fraction of an extract from *H. a. maxima* eggs
924   containing particles of a molecular weight <3 kDa (1.25 and 0.125 mg/mL) and fraction of an
925   extract from *H. a. aspersa* eggs comprising particles of the same molecular weight, at the same
926   concentrations, but the effect was not statistically significant in case of the second extract. The
927   extract from *H. a. maxima* eggs (25 mg/mL), after 12 h of incubation, increased the percentage
928   of dead Caco-2 cells and the extracts from eggs of both snail subspecies, after 24 and 48 h of
929   treatment, reduced the percentage of live cells. In addition, the extract from *H. a. maxima* eggs
930   (25, 25×10^{-1} and 25×10^{-3} mg/mL) and the extract from *H. a. aspersa* eggs (25×10^{-1} mg/mL),
931   after 24 h of incubation, increased the degree of damage to Caco-2 cell membranes, LDH
932   release. The effective concentrations of extracts depended on the content of individual chemical
933   compounds at the respective concentrations and on their interactions. Lack of statistically
934   significant effect of the same concentrations of extracts on cell viability, percentage of live cells
935   and LDH activity after 72 hours compared to the situation after 24 hours may have been
936   associated with a different cell density, cell growth rate and secretion of metabolites by the cells
937   [171]. The composition of the extracts may also have changed during incubation. The extracts
938   from the eggs of both snail subspecies (2.5 mg/mL), after 24 h of incubation, increased the
939   amount of lipid peroxidation products in cells, but this effect turned out to be statistically
940   insignificant. The extracts from eggs (25 mg/mL) caused the induction of apoptosis and
941   reduction of necrosis - an increase in the percentage of early apoptotic cells (effect statistically
942   insignificant for *H. a. maxima* extract) and a decrease in the percentage of necrotic cells (effect
943   statistically insignificant). The effect of extracts from snail eggs on the cell viability and the
944   induction of apoptosis may be assigned to the presence of antioxidants. The phenols contained in
945   the extracts may have shown a prooxidative activity, influenced on the greater degree of lipid

946   peroxidation in the cells treated with these extracts. The potential cytotoxic activity of phenols,
947   related to the production of ROS may have played an important role in activating apoptosis.
948   Lipid peroxidation products may have also been responsible for the cytotoxic, prooxidative and
949   consequent proapoptotic effects of extracts on Caco-2 cells. Decreased cell viability may be
950   attributed to anticancer peptides, especially those of a molecular weight <3 kDa, which requires
951   further research. The glycoproteins agglutinins, belonging to the H-type lectins family, present in
952   the extracts may have bound to the surface of cancer cells and facilitated the delivery of other
953   compounds to them, which also requires further studies. The restriction of Met was most likely
954   associated with the reduction of cell viability. Moreover, the appropriate ratio of EAA to NEAA
955   may have also been responsible for limiting the cell viability by the extracts, their cytotoxic and
956   proapoptotic effects. Furthermore, vitamin D may have contributed to the reduction of cell
957   viability and apoptosis. Additionally, Mg may have had a positive effect on the synthesis and
958   metabolism of vitamin D. Ca may have been responsible for limiting viability and inducing
959   apoptosis. S may have contributed to the induction of apoptosis by the extracts. The cytotoxic
960   effect on cells, related to the activation of apoptosis and the increase of oxidative stress, may
961   have been demonstrated by Cu. Caco-2 cell growth may have been inhibited by Mn, the
962   component of mitochondrial Mn-SOD. Zn may have been responsible for limiting cell viability.
963   It may have contributed to the induction of oxidative stress, causing mitochondria disorders and
964   damage to the cell membranes, to the activation of apoptosis. In turn, Se present in extracts may
965   have increased oxidative stress, the degree of lipid peroxidation and apoptosis. Other bioactive
966   compounds contained in the extracts and their additive and synergistic effects most likely also
967   influenced Caco-2 cells.
968

Conclusions

970   Extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails, due to the content of
971   antioxidants which are necessary in prevention of the formation and inhibition of the activity of
972   ROS, are able to reduce oxidative stress in the body and may be beneficial in the initiation and
973   progression of colorectal cancer. Reduction of the viability of Caco-2 colon cancer cells after
974   application of snail egg extracts, increase of the degree of damage to cell membranes, the amount
975   of lipid peroxidation products generated by cells, induction of apoptosis and reduction of
976   necrosis may be attributed to the presence of antioxidants, phenols, lipid peroxidation products,
977   anticancer peptides, restriction of Met, appropriate ratio of EAA to NEAA, vitamin D, Ca, Mg,
978   S, Cu, Mn, Zn and Se in them. Other bioactive compounds and their additive and synergistic
979   effects most likely also influenced Caco-2 cells. Differences in the effects on cells of the extracts
980   from eggs of two snail subspecies may result from differences in the content of bioactive
981   compounds in them.
982   It is planned to examine the composition of extracts and fractions containing molecules <3 kDa
983   in terms of the content of peptides with anticancer properties, using mass spectrometry and
984   bioinformatics tools, as well as other elements of the chemical composition of the extracts. It is
985   planned to investigate the effect of extracts and <3 kDa fractions on cell metabolome and to

986 examine other mechanisms of their anticancer activity, and to deepen research on their impact on
987 cell death (apoptosis, necrosis, autophagy).
988 Natural extracts from snail eggs or the chemical compounds contained in them might be used in
989 the combination therapy of colorectal cancer, targeting many signaling pathways and using a
990 variety of mechanisms to decrease the development of anticancer drug resistance. However, it
991 should be determined whether the extracts/chemical compounds contained in them may sensitize
992 to cytotoxic therapy, intensify the effective concentration of a drug, enhance the combined
993 effects of both therapeutics or exert a cytotoxic effect specifically on cancer cells.
994

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997

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Antioxidant indicators and concentration of phenols and total carbohydrates in extracts and fractions of extracts from lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*.

Data are expressed as mean ± standard error of the mean. Statistically significant effect: values of one indicator are statistically significantly different when $p < 0.05$. n (number of replicates) = 3 (all indicators except phenols), n = 2 (phenols). ABTS$^{·+}$ - 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation, DPPH· - 2.2-diphenyl-1-picrylhydrazyl radical, K – kDa, ND – not detected.

1 **Table 1 Antioxidant indicators and concentration of phenols and total carbohydrates in**
2 **extracts and fractions of extracts from lyophilized eggs of *Helix aspersa maxima* and *Helix***
3 ***aspersa aspersa*.**

Factor	Ferric-reducing antioxidant power (mg TROLOX/g)	ABTS·+ scavenging activity (mg TROLOX/g)	DPPH· scavenging activity (mg TROLOX/g)	Phenols (mg quercetin/g)	Total carbohydrates (%)
Subspecies					
H. a. maxima	8.94 ± 3.00	0.60 ± 0.17	2.03 ± 0.93	4.42 ± 2.06	12.81 ± 4.85
H. a. aspersa	7.49 ± 2.56	0.53 ± 0.16	1.63 ± 0.69	4.07 ± 2.09	11.28 ± 4.42
Fraction					
Extract	7.67 ± 0.55^B	0.92 ± 0.11^C	1.67 ± 0.07^A	4.85 ± 0.46^B	15.99 ± 0.98^B
>50 K	27.76 ± 2.88^C	1.61 ± 0.04^D	7.50 ± 1.00^B	16.14 ± 0.14^C	43.77 ± 3.46^C
10-50 K	2.71 ± 0.24^{AB}	0.25 ± 0.02^B	ND	0.05 ± 0.03^A	0.22 ± 0.03^A
3-10 K	1.42 ± 0.20^A	0.02 ± 0.01^A	ND	0.19 ± 0.12^A	0.20 ± 0.11^A
<3 K	1.52 ± 0.10^A	0.03 ± 0.01^A	ND	0.01 ± 0.01^A	0.03 ± 0.01^A
Subspecies × fraction					
H. a. maxima × extract	8.47 ± 0.64	1.10 ± 0.14	1.77 ± 0.07	5.64 ± 0.20	17.97 ± 0.89
H. a. maxima × >50 K	29.94 ± 4.84	1.59 ± 0.05	8.39 ± 2.00	16.04 ± 0.29	45.40 ± 6.35
H. a. maxima × 10-50 K	3.14 ± 0.14	0.25 ± 0.04	ND	0.05 ± 0.05	0.24 ± 0.03
H. a. maxima × 3-10 K	1.61 ± 0.28	0.03 ± 0.01	ND	0.38 ± 0.12	0.39 ± 0.16
H. a. maxima × <3 K	1.54 ± 0.12	0.02 ± 0.02	ND	0.01 ± 0.01	0.04 ± 0.02
H. a. aspersa × extract	6.87 ± 0.68	0.75 ± 0.10	1.57 ± 0.11	4.06 ± 0.04	14.01 ± 0.33
H. a.	25.58 ± 3.64	1.62 ± 0.06	6.61 ± 0.45	16.24 ± 0.10	42.15 ± 4.09

aspersa × >50 K					
H. a. aspersa × 10-50 K	2.28 ± 0.29	0.25 ± 0.02	ND	0.05 ± 0.05	0.20 ± 0.05
H. a. aspersa × 3-10 K	1.23 ± 0.28	0.01 ± 0.01	ND	ND	0.01 ± 0.01
H. a. aspersa × <3 K	1.49 ± 0.17	0.04 ± 0.00	ND	ND	0.02 ± 0.02
Main effects, *p*					
subspecies	0.254	0.123	0.344	0.001	0.328
fraction	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
subspecies × fraction	0.811	0.032	0.585	< 0.001	0.863

4 **Notes.**
5 Data are expressed as mean ± standard error of the mean. Statistically significant effect: values
6 of one indicator are statistically significantly different when $p < 0.05$. n (number of replicates) =
7 3 (all indicators except phenols), $n = 2$ (phenols). ABTS$^{\cdot +}$ - 2.2'-azino-bis(3-
8 ethylbenzthiazoline-6-sulfonic acid) radical cation, DPPH$^{\cdot}$ - 2.2-diphenyl-1-picrylhydrazyl
9 radical, K – kDa, ND – not detected.

Content of crude protein, crude fat, thiobarbituric acid reactive substances (TBARS), glutathione (GSH), astaxanthin and vitamins in lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*.

Data are expressed as mean ± standard error of the mean. Statistically significant effect: values of one indicator are statistically significantly different when $p < 0.05$. $n = 2$ (crude protein, crude fat, vitamin C), $n = 3$ (astaxanthin, vitamins A, D$_3$, E), $n = 6$ (TBARS, GSH).

1   **Table 2 Content of crude protein, crude fat, thiobarbituric acid reactive substances**
2   **(TBARS), glutathione (GSH), astaxanthin and vitamins in lyophilized eggs of *Helix aspersa***
3   ***maxima* and *Helix aspersa aspersa*.**

Parameter	*H. a. maxima*	*H. a. aspersa*	*p*
Crude protein (%)	29.2 ± 0.0	29.0 ± 0.0	-
Crude fat (%)	< 0.2	< 0.2	-
TBARS (µg/g)	0.543 ± 0.026	0.481 ± 0.033	0.174
GSH (µg/g)	69.64 ± 1.55	44.58 ± 3.15	< 0.001
Astaxanthin (µg/g)	< 0.2151	< 0.2151	-
Vitamin A (IU/g)	< 0.1566	< 0.1566	-
Vitamin C (µg/g)	< 150	< 150	-
Vitamin D_3 (µg/g)	0.4908 ± 0.0060	0.8048 ± 0.0028	< 0.001
Vitamin E (µg/g)	< 4.5252	< 4.5252	-

4   **Notes.**
5   Data are expressed as mean ± standard error of the mean. Statistically significant effect: values
6   of one indicator are statistically significantly different when $p < 0.05$. $n = 2$ (crude protein, crude
7   fat, vitamin C), $n = 3$ (astaxanthin, vitamins A, D_3, E), $n = 6$ (TBARS, GSH).

Amino acid composition of lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa aspersa* (mg/g crude protein).

Data are expressed as mean ± standard error of the mean. * for humans, # delicious amino acids. Statistically significant effect: values of one parameter are statistically significantly different when $p < 0.05$. $n = 2$.

1 **Table 3 Amino acid composition of lyophilized eggs of *Helix aspersa maxima* and *Helix***
2 ***aspersa aspersa* (mg/g crude protein).**

Amino acids	*H. a. maxima*	*H. a. aspersa*	*p*
Essential amino acids (EAA) *			
Leucine	72.84 ± 0.17	65.90 ± 0.04	< 0.001
Lysine	71.73 ± 0.39	53.81 ± 0.26	< 0.001
Phenylalanine	52.33 ± 0.24	45.57 ± 0.02	0.001
Valine	51.25 ± 0.36	49.48 ± 0.07	0.040
Threonine	45.57 ± 0.06	45.90 ± 0.07	0.064
Isoleucine	40.84 ± 0.33	38.64 ± 0.09	0.023
Methionine	18.79 ± 0.40	15.78 ± 0.10	0.018
Tryptophan	14.49 ± 0.25	13.43 ± 0.13	0.064
Half-essential amino acids (HEAA) *			
Arginine	53.80 ± 0.28	44.52 ± 0.45	0.003
Histidine	20.75 ± 0.10	20.12 ± 0.23	0.123
Non-essential amino acids (NEAA) *			
Glutamic acid [#]	104.54 ± 0.63	105.71 ± 1.12	0.459
Aspartic acid [#]	104.45 ± 0.01	90.12 ± 1.19	0.007
Serine	57.00 ± 0.02	59.16 ± 0.30	0.018
Tyrosine	49.45 ± 0.48	42.98 ± 1.23	0.039
Alanine [#]	40.62 ± 0.31	36.07 ± 0.01	0.005
Proline	38.68 ± 0.05	28.21 ± 0.39	0.001
Glycine [#]	33.26 ± 0.06	27.95 ± 0.03	< 0.001
Cysteine	14.79 ± 0.07	14.17 ± 0.42	0.281
Amino acid groups and ratios			
Total amino acids (TAA)	885.18 ± 0.96	797.50 ± 2.95	0.001
Essential amino acids (EAA)	367.84 ± 0.03	328.51 ± 0.02	< 0.001
Half-essential amino acids (HEAA)	74.55 ± 0.37	64.63 ± 0.23	0.002
Non-essential amino acids (NEAA)	442.80 ± 0.63	404.36 ± 3.19	0.007
Delicious amino acids (DAA)	282.87 ± 1.00	259.84 ± 2.29	0.012
EAA/TAA	0.42	0.41	-
EAA/NEAA	0.83	0.81	-
DAA/TAA	0.32	0.33	-

3 **Notes.**
4 Data are expressed as mean ± standard error of the mean. * for humans, [#] delicious amino acids.
5 Statistically significant effect: values of one parameter are statistically significantly different
6 when $p < 0.05$. $n = 2$.

Amino acid score (AAS), chemical score (CS) and essential amino acid index (EAAI) of lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*.

Grey fields – the first limiting amino acids.

1Table 4 Amino acid score (AAS), chemical score (CS) and essential amino acid index
2(EAAI) of lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*.

Amino acids	AAS		CS	
	H. a. maxima	*H. a. aspersa*	*H. a. maxima*	*H. a. aspersa*
Leucine	1.10	1.00	0.85	0.77
Lysine	1.24	0.93	1.02	0.77
Phenylalanine + tyrosine	1.62	1.41	1.09	0.95
Valine	1.46	1.41	0.78	0.75
Threonine	1.34	1.35	0.97	0.98
Isoleucine	1.46	1.38	0.76	0.72
Methionine + cysteine	1.34	1.20	0.59	0.53
Tryptophan	1.32	1.22	0.85	0.79
Histidine	1.09	1.06	0.94	0.91
EAAI	132.03	120.38	85.95	78.37

3**Notes.**

4Grey fields – the first limiting amino acids.

Elements detected in lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*.

Data are expressed as mean ± standard error of the mean. Statistically significant effect: values of one element are statistically significantly different when $p < 0.05$. $n = 3$.

1 **Table 5 Elements detected in lyophilized eggs of *Helix aspersa maxima* and *Helix aspersa***
2 ***aspersa*.**

Elements	*H. a. maxima*	*H. a. aspersa*	*p*
Macroelements (g/kg)			
Ca	110 ± 1	116 ± 2	0.030
P	5.005 ± 0.070	4.776 ± 0.038	0.045
Na	1.835 ± 0.026	1.337 ± 0.011	< 0.001
K	1.714 ± 0.010	0.745 ± 0.031	< 0.001
Mg	0.693 ± 0.004	0.685 ± 0.009	0.455
S	0.348 ± 0.002	0.374 ± 0.005	0.008
Cl	0.2357 ± 0.0009	0.1006 ± 0.0028	< 0.001
Microelements (mg/kg)			
Cu	35.5 ± 1.0	27.6 ± 0.6	0.002
Ni	25.87 ± 0.62	6.15 ± 0.07	< 0.001
Si	25.0 ± 0.2	31.6 ± 0.8	0.001
Fe	23.8 ± 0.7	18.5 ± 1.6	0.035
Mn	9.55 ± 0.32	10.05 ± 0.05	0.201
Cr	7.75 ± 0.17	1.48 ± 0.02	< 0.001
Mo	7.00 ± 0.05	1.65 ± 0.02	< 0.001
B	5.91 ± 0.05	4.95 ± 0.10	< 0.001
Zn	4.32 ± 0.10	2.85 ± 0.04	< 0.001
Co	0.407 ± 0.013	0.255 ± 0.002	< 0.001
V	0.118 ± 0.002	0.040 ± 0.002	< 0.001
Se	0.109 ± 0.011	0.119 ± 0.004	0.436
I	0.026 ± 0.002	0.108 ± 0.003	< 0.001
Sn	0.020 ± 0.000	0.023 ± 0.000	0.008
F	< 10	< 10	-

3 **Notes.**
4 Data are expressed as mean ± standard error of the mean. Statistically significant effect: values
5 of one element are statistically significantly different when $p < 0.05$. $n = 3$.

Figure 1

SDS-PAGE profile of (A) proteins and (B) glycoproteins isolated from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*.

(A) Panel (a) – molecular weights of standard proteins (Thermo Fisher Scientific, Waltham, MA, USA); panel (b) – extract from eggs of *H. a. maxima* and panel (c) – extract from eggs of *H. a. aspersa*. (B) panel (a) – molecular weights of standard proteins (Sigma-Aldrich, St. Louis, MO, USA); panel (b) – positive control (horseradish peroxidase); panel (c) – extract from eggs of *H. a. maxima* and panel (d) – extract from eggs of *H. a. aspersa*.

(A)

(B)
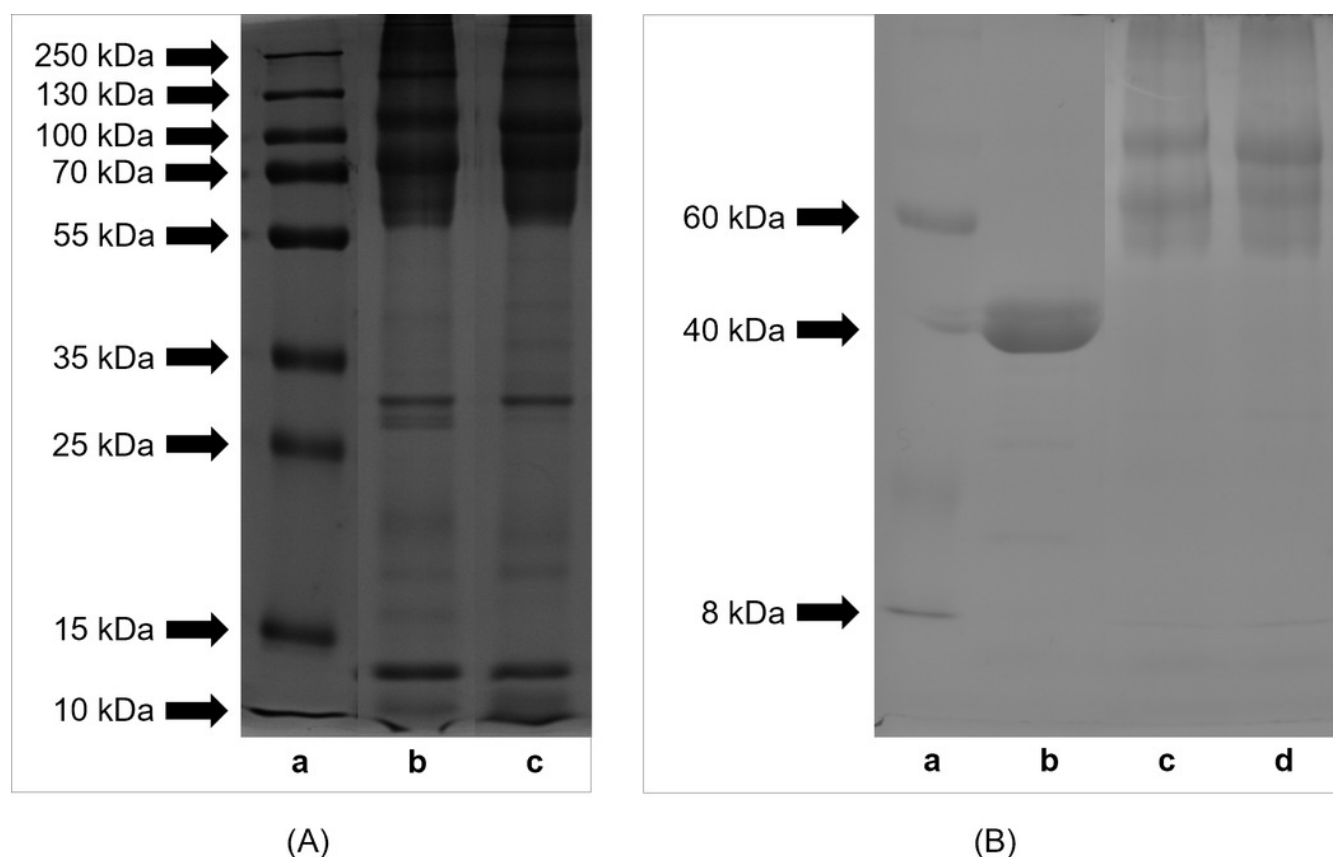
Figure 2

Viability of Caco-2 cells after treatment for (a) 24 h and (b) 72 h with extracts from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*, at different concentrations.

C – control cells (treated with deionized water). Error bars indicate standard error of the mean. Statistically significant effect: ** represents values that differ from control at $p < 0.01$, *** represents values that differ from control at $p < 0.001$. $n = 6$.
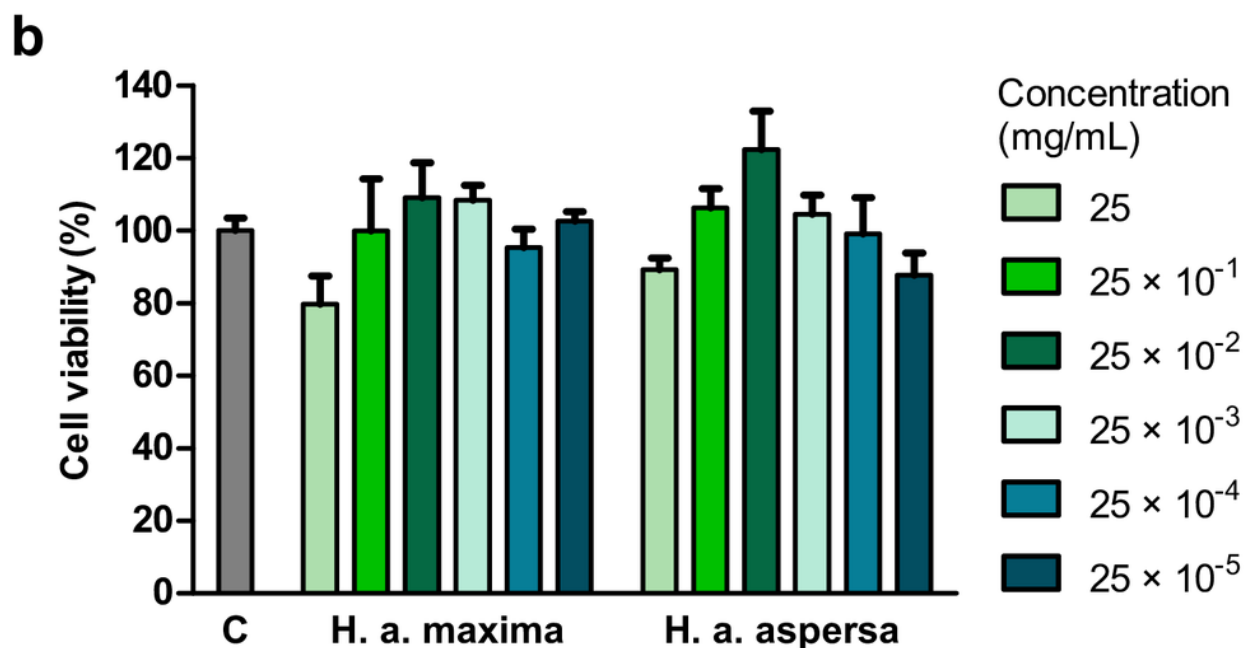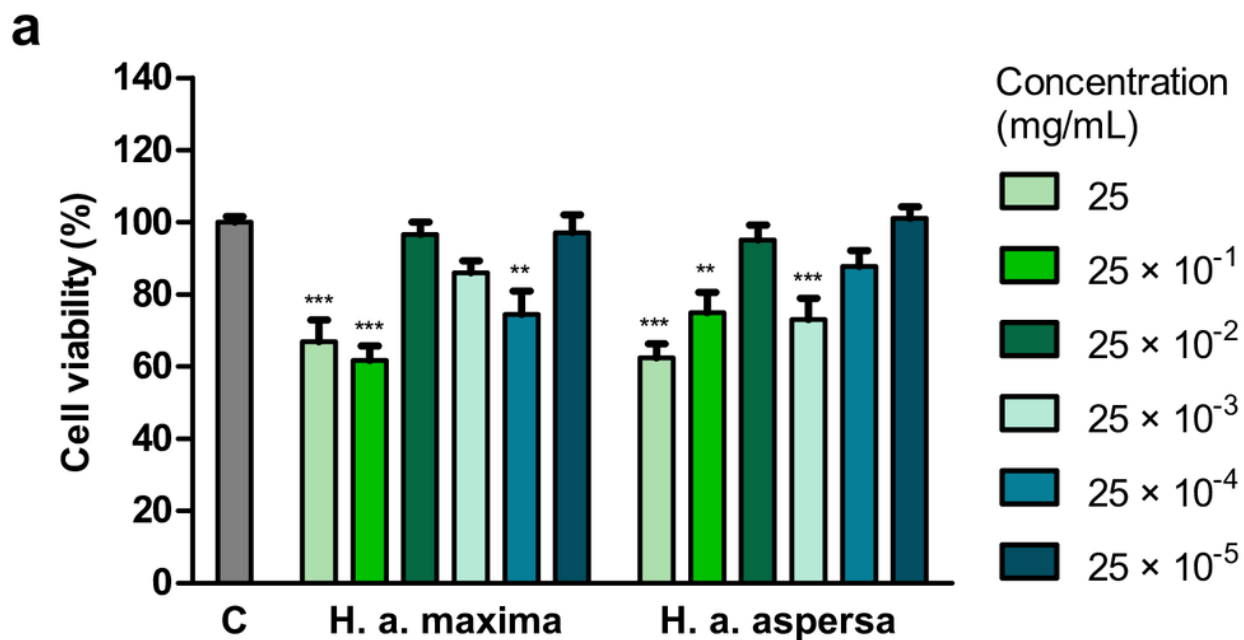
Figure 3

Viability of Caco-2 cells after 24 h of treatment with fractions >50 kDa (>50 K), 10-50 kDa (10-50 K), 3-10 kDa (3-10 K) and <3 kDa (<3 K) of extracts from eggs of (a) *Helix aspersa maxima* and (b) *Helix aspersa aspersa*,

at two concentrations. C – control cells (treated with deionized water). Error bars indicate standard error of the mean. Statistically significant effect: * represents values that differ from control at $p < 0.05$, ** represents values that differ from control at $p < 0.01$. $n = 4$.
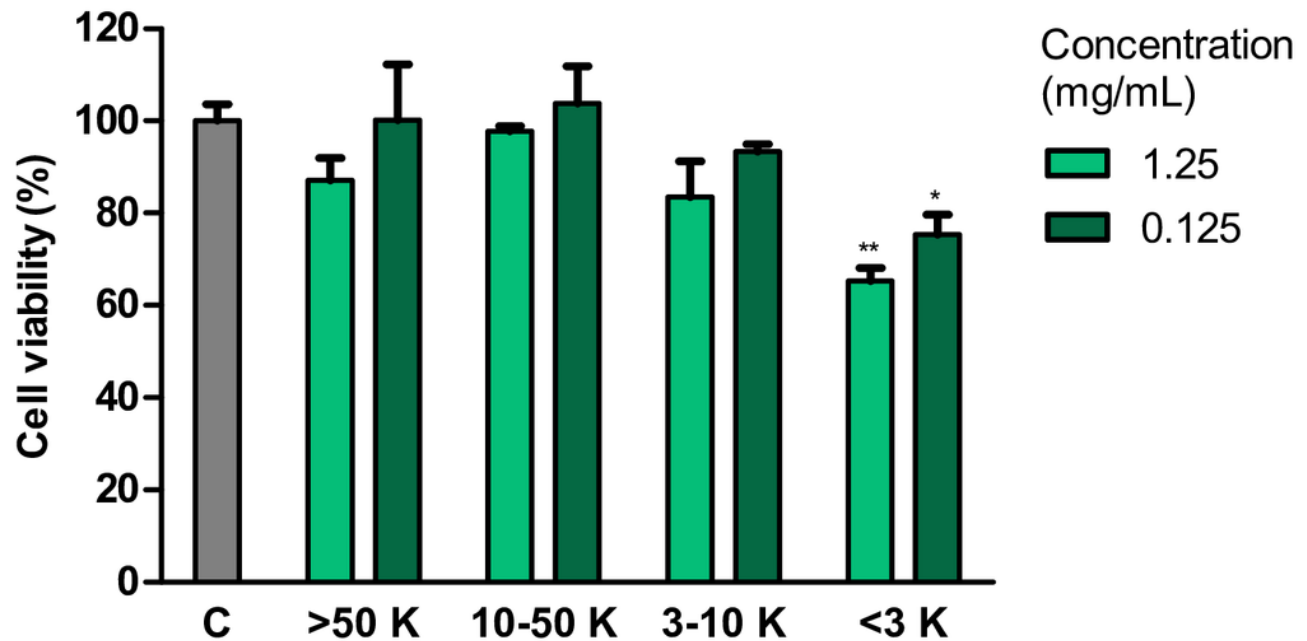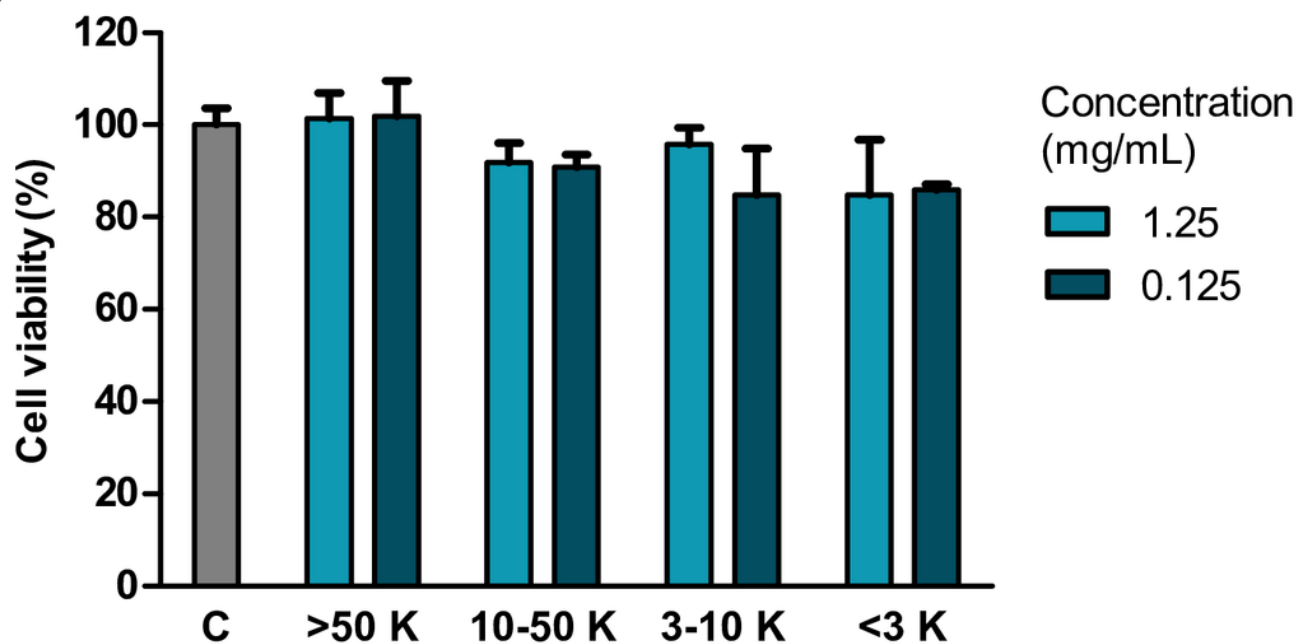
Figure 4

Percentage of live and dead Caco-2 cells after treatment for (a) 12 h, (b) 24 h, (c) 48 h and (d) 72 h with extracts from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa* (25 mg/mL).

C – control cells (treated with deionized water). Error bars indicate standard error of the mean. Statistically significant effect: * represents values that differ from control at $p < 0.05$, ** represents values that differ from control at $p < 0.01$. $n = 5$.
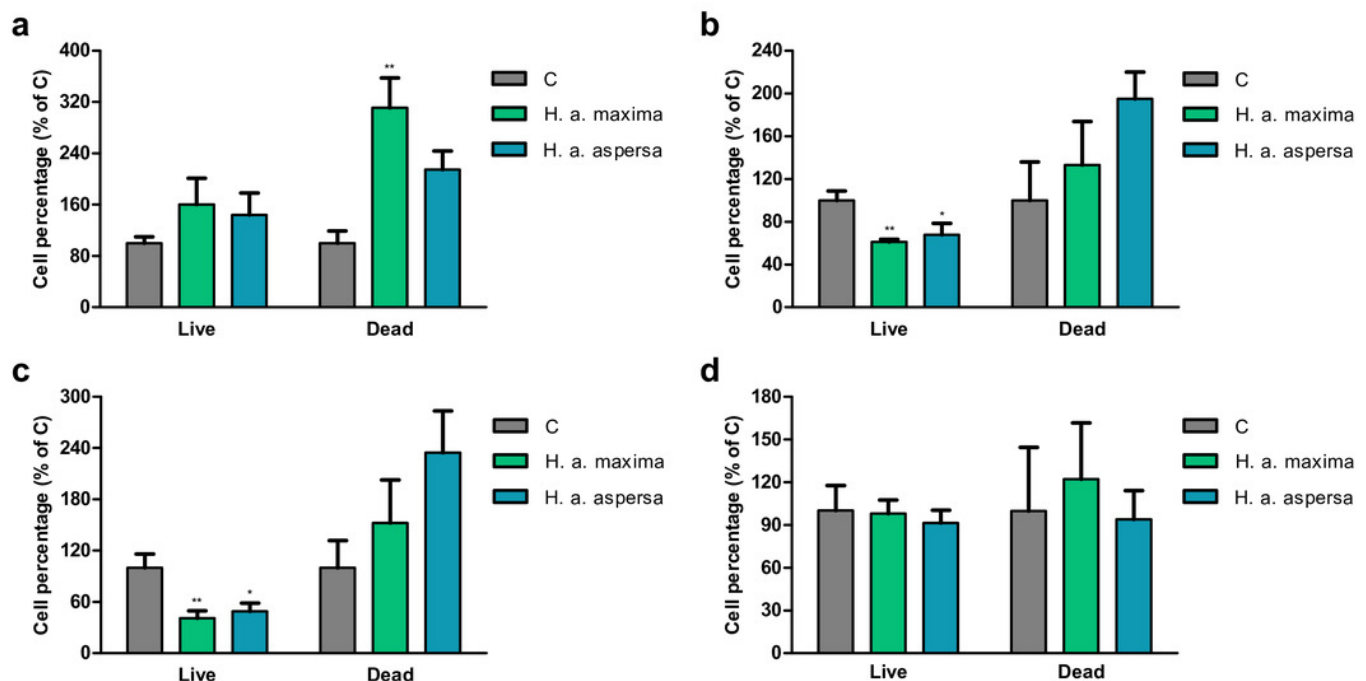
Figure 5

Integrity of membranes of Caco-2 cells after treatment for (a) 24 h and (b) 72 h with extracts from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*, at different concentrations.

C – control cells (treated with deionized water), LDH – lactate dehydrogenase. Error bars indicate standard error of the mean. Statistically significant effect: * represents values that differ from control at $p < 0.05$, ** represents values that differ from control at $p < 0.01$. $n = 4$.
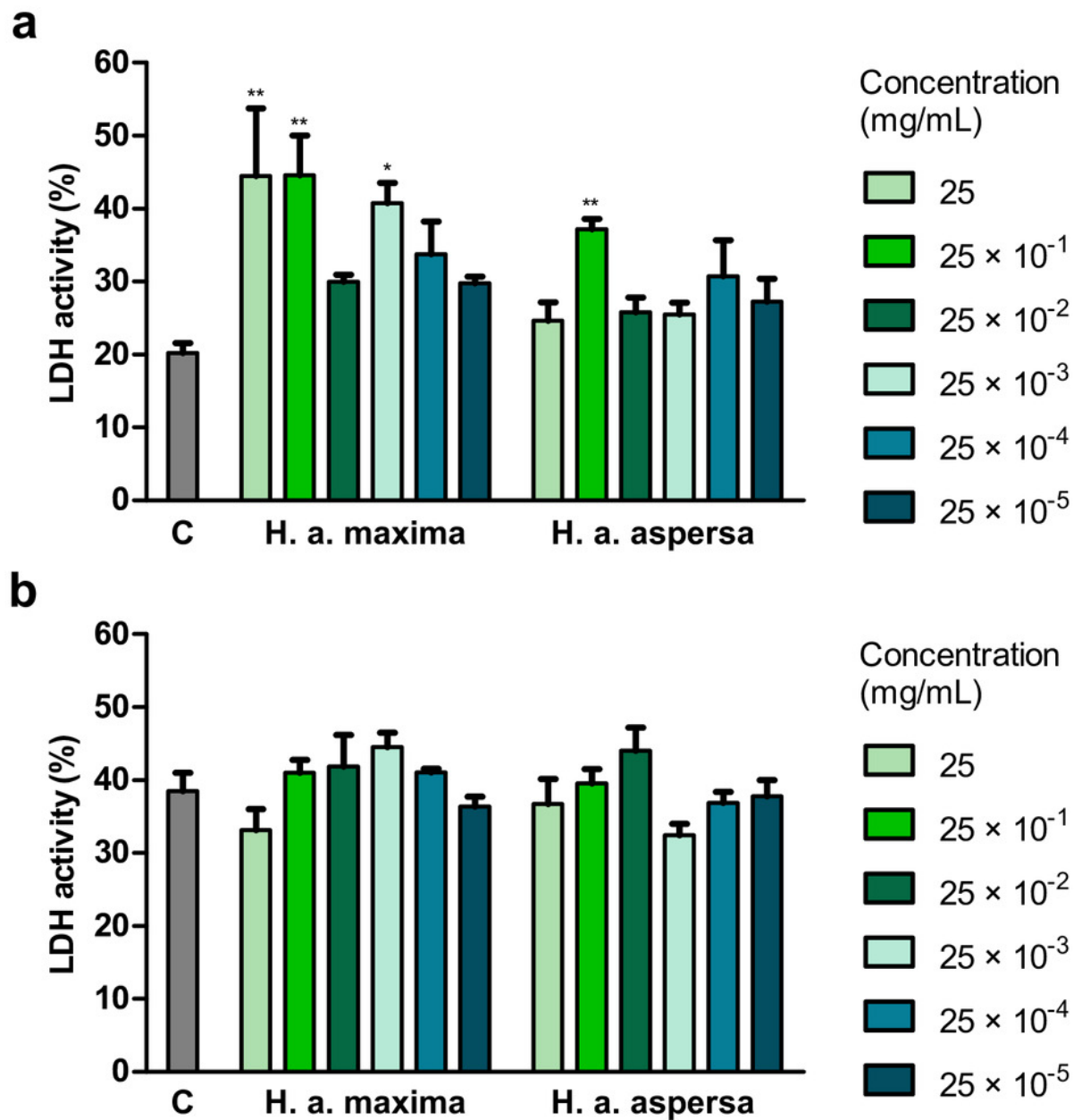
Figure 6

Concentration of thiobarbituric acid reactive substances (TBARS) produced in Caco-2 cells after treatment for 24 h with extracts from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*,

at the concentration of 2.5 mg/mL. C – control cells (treated with deionized water). Error bars indicate standard error of the mean. *n* = 3.
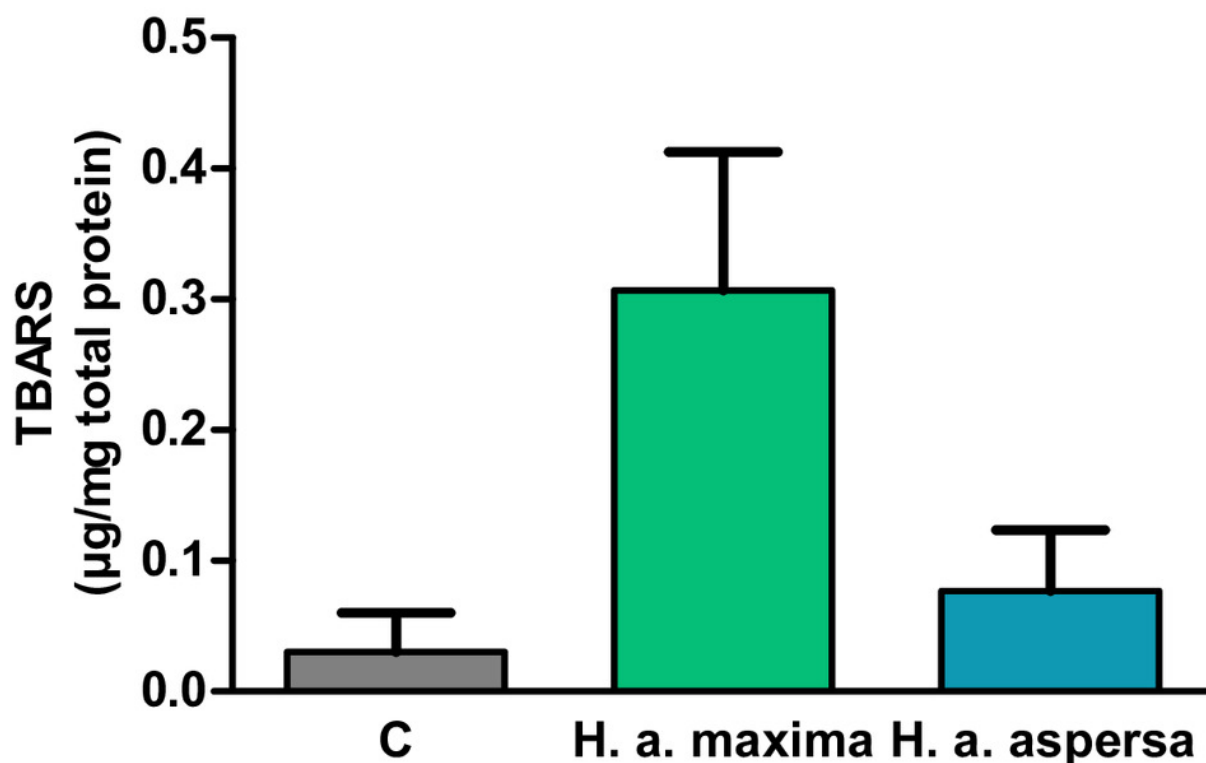
Figure 7

Types of death of Caco-2 cells after treatment for 24 h with extracts from eggs of *Helix aspersa maxima* and *Helix aspersa aspersa*, at the concentration of 25 mg/mL.

C – control cells (treated with deionized water). Error bars indicate standard error of the mean. Statistically significant effect: *** represents values that differ from control at $p < 0.001$. $n = 5$.

A

a

200 µm

C

b

200 µm

H. a. maxima

c

200 µm

H. a. aspersa

B

a

Propidium iodide

Alexa Fluor® 488 Annexin V

C

b

Propidium iodide

Alexa Fluor® 488 Annexin V

H. a. maxima

c

Propidium iodide

Alexa Fluor® 488 Annexin V

H. a. aspersa

C

Cell percentage (%)

C   H. a. maxima   H. a. aspersa

- Live cells
- Early apoptotic cells
- Late apoptotic cells
- Necrotic cells

***