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

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Abstract

Background. Colorectal cancer is the third most commonly diagnosed cancer. Combination therapy, the administration of a conventional chemotherapeutic agent or radiotherapy and a natural compound/natural compounds, is a novel use of natural products. 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 cell line.
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 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 24 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 the same treatment times. 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, increased the

46   degree of cell membrane damage and the amount of lipid peroxidation products, induced
47   apoptosis and reduced necrosis, after this time.
48   **Conclusions.** Antioxidants, phenols, lipid peroxidation products, anticancer peptides, restriction
49   of methionine, appropriate ratio of essential amino acids to non-essential amino acids, vitamin
50   D_3, Ca, Mg, S, Cu, Mn, Zn, Se and other bioactive compounds comprised in the extracts and their
51   additive and synergistic effects may have influenced Caco-2 cells. Natural extracts or the
52   chemical compounds contained in them might be used in the combination therapy of colorectal
53   cancer, which requires further research.

Introduction

56   Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of
57   cancer death [1]. The incidence rates of colorectal cancer are over three-fold greater in countries
58   that have completed their transition comparing to transitioning countries while the mortality rates
59   are over two-fold greater. The greatest colon cancer incidence rates were found in Europe,
60   Australia/New Zealand, Northern America, Uruguay and Eastern Asia.
61   Colorectal cancer occurrence and progression are caused by many risk factors, the main of which
62   are age, gender, family and personal history, and region [2]. The risk of this cancer can be
63   reduced through proper dietary habits and lifestyle [3].
64   The main treatment for patients suffering from a potentially curable colorectal cancer is surgery,
65   and chemotherapy and/or radiation therapy may be given before/after it, depending on the stage
66   of the disease [4]. However, this treatment is not sufficient to control colorectal cancer as 30% of
67   stage I to III patients and up to 65% of stage IV patients relapse, underlining the urgent need to
68   find more effective therapies. Adverse effects of the above treatment impair the life quality, may
69   unfavorably influence on the course, outcomes and costs of treatment [3].
70   Treatment outcomes and life quality can be improved by application of natural products derived
71   from plants, animals and microorganisms, well tolerated and less toxic than conventional
72   chemotherapeutic agents [2]. There are nine anticancer drugs from marine organisms on the
73   market and other molecules from these organisms are being examined as anticancer drugs in
74   different phases of clinical trials [5].
75   Novel use of natural products is combination therapy, the administration of a conventional
76   chemotherapeutic agent or radiotherapy and a natural compound/natural compounds [3,4].
77   Certain compounds may sensitize to cytotoxic therapy, intensify the effective concentration of a
78   drug, enhance the combined effects of both therapeutics or exert a cytotoxic effect specifically on
79   cancer cells. Moreover, combination therapy targets many signaling pathways and uses a variety
80   of mechanisms to decrease the development of anticancer drug resistance.
81   Snails can provide many bioactive compounds for the pharmaceutical and cosmetics industries,
82   applicable in the development of novel preparations with lower toxicity and subsequent effects in
83   comparison with compounds commonly used for this purpose [6]. Information on the chemical
84   composition and nutritional value of mucus, foot tissues and shells of one of the most popular
85   edible land snails of the subspecies *Helix aspersa aspersa* is presented in the article by
86   Matusiewicz et al. [7]. The use of water extracts from lyophilized mucus and foot tissues of these
87   snails reduced the viability of Caco-2 human colorectal adenocarcinoma cells.
88   *In vitro* experiment of Ellijimi et al. demonstrated that the mucus extract derived from *Helix*
89   *aspersa maxima* snails decreased the content of melanin and tyrosinase activity in B16F10
90   murine melanoma cells and IGR-39 and SK-MEL-28 human melanoma cells [8]. It decreased the
91   viability of human melanoma cells and did not influence on the viability of HaCaT human
92   keratinocytes. It induced a caspase-dependent apoptosis of human melanoma cells, inhibited their
93   migration and invasion by decreasing the production of matrix metalloproteinase-2. It strongly
94   affected the adhesion of IGR-39 cells by blocking $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin functions and by

95   decreasing the production of α_v and β_1 integrins. The mucus and its two fractions derived from
96   *Achatina fulica* snail reduced the viability of MCF-7 breast cancer cells and Vero epithelial cells
97   [9].
98   Water extract from *H. a. aspersa* snails exhibited anticancer activity against Hs578T breast
99   cancer cells [10]. It induced necrosis of cancer cells, stimulated the mRNA expression of tumor
100   necrosis factor (TNF)-α and inhibited the expression of nuclear factor kappa-light-chain-enhancer
101   of activated B cells (NF-κB), phosphatase and tensin homolog (PTEN) and tumor protein 53
102   (p53).
103   Many studies showed that mollusk and arthropod hemocyanins, oxygen-transporting hemolymph
104   glycoproteins, have significant anticancer activity which was demonstrated in both *in vitro* and *in*
105   *vivo* models. Hemocyanins have immunostimulatory activity, thanks to the structural properties,
106   they stimulate the immune system nonspecifically, they interact with macrophages, granulocytes,
107   CD4+ and CD8+ cells, induce potent cellular and humoral immune responses [11-22]. The
108   structural subunits of hemocyanins derived from the land snails *H. a. aspersa* and *Helix lucorum,*
109   the marine snail *Rapana venosa* and the mucus of *H. a. aspersa* snails reduced the viability of
110   HT-29 human colorectal adenocarcinoma cells [22]. The mucus and the α and βc subunits of *H.*
111   *a. aspersa* hemocyanin decreased the viability to the greatest extent. The half-maximal inhibitory
112   concentrations (IC_{50}) of above preparations for HT-29 cells were lower than for Balb/c3T3
113   fibroblasts. The mechanisms of their anticancer activity included apoptosis. The hemocyanins
114   isolated from the land snail *Helix pomatia* and the marine snail *Rapana thomasiana* demonstrated
115   strong anticancer and antiproliferative actions in a colon carcinoma murine model [23]. The
116   immunization with these hemocyanins resulted in prolonged survival of animals, improved
117   humoral anticancer response, inhibition of tumor growth, splenomegaly and appearance of lung
118   metastasis. Treatment with hemocyanins derived from *H. pomatia* and *R. thomasiana* in other
119   study resulted in the production of large amounts of antitumor IgGs, plasma cells as well as
120   tumor specific cytotoxic T cells, stimulation of the secretion of proinflammatory cytokines,
121   suppression of tumor size and growth, and prolongation of the life span of a colon carcinoma
122   murine model [24].
123   Considering the above anticancer properties of compounds derived from different tissues of
124   various snail species, the extracts from snail eggs, containing chemical compounds characterized
125   by potential anticancer activities, may affect the growth and development of cancer cells. The
126   purpose of the research was to evaluate the chemical composition of eggs from the popular edible
127   farm snails *Helix aspera maxima* and *Helix aspersa aspersa*, water extracts from these eggs and
128   fractions of extracts containing particles of different molecular weights. The action of extracts
129   and their fractions on the viability of Caco-2 human epithelial colorectal adenocarcinoma cell line
130   was determined. The effect of the extracts on the percentage of live and dead cells, the integrity
131   of cell membranes, the content of lipid peroxidation products in cells and the types of cell death
132   was then examined.
133

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

144 **Preparation of extracts and their fractions for the determination of antioxidant indicators,**
145 **phenols and total carbohydrates**
146 Before each analysis, lyophilized eggs derived from *H. a. maxima* and *H. a. aspersa* snails were
147 homogenized in deionized water (concentration 100 mg/mL), by vortexing. The samples were
148 left for extraction (30 min, 4 °C), subjected to centrifugation (1600× *g*, 10 min) and the
149 supernatants (extracts) were collected. Then, the extracts were filtered using polyvinylidene
150 fluoride (PVDF) syringe filters (pore size 0.22 μm, EuroClone, Pero, Italy). The extracts were
151 fractionated, based on the molecular weight, using ultra centrifugal filter devices containing
152 regenerated cellulose membranes (Merck Millipore, Burlington, MA, USA), including 3, 10 and
153 50 kDa cutoffs, in compliance with the manufacturer's prescriptions regarding g-force and
154 centrifugation time. Four fractions were obtained: >50 kDa (>50 K), 10-50 kDa (10–50 K), 3–10
155 kDa (3–10 K) and <3 kDa (<3 K).
156 **Determination of antioxidant indicators**
157 *Ferric-reducing antioxidant power*
158 Ferric-reducing antioxidant power of extracts and four fractions: >50 K, 10-50 K, 3-10 K and <3
159 K of extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was determined
160 using the modified Oyaizu method [25,26]. It involves reduction of Fe^{3+}, being in stoichiometric
161 excess over antioxidants, because of electron donation by them. The absorbance increase is noted
162 as the reduction capability is higher. Properly diluted extracts and fractions (2.5 mL) were mixed
163 with 0.2 M sodium phosphate buffer pH 6.6 (2.5 mL) and 1% potassium ferricyanide (2.5 mL).
164 The samples were incubated at 20 °C for 20 min and then 10% trichloroacetic acid (TCA, 2.5 ml)
165 was added. The probes were centrifuged (3000× *g*, 5 min) and the supernatants (0.4 mL) were
166 combined with deionized water (0.4 mL) and 0.1% ferric chloride (160 μL). The absorbance was
167 measured at 700 nm, using microplate reader (Infinite M200, Tecan, Männedorf, Switzerland).
168 The standard curve was generated by applying various concentrations, ranging 0-100 μM, of (±)-
169 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), a water-soluble analog of
170 vitamin E. The assay was conducted in three replicates (*n* = 3).
171 **2.2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS·⁺) scavenging**
172 **activity**
173 The relatively stable ABTS·⁺ discolors when is reduced. To determine ABTS·⁺ scavenging
174 activity of extracts and four fractions of extracts from *H. a. maxima* and *H. a. aspersa* eggs, the
175 procedure of Sun at al. with modifications was used [26,27]. This method is based on the addition
176 of antioxidants to ABTS·⁺ solution and spectrophotometric determination of the remaining
177 ABTS·⁺. To make ABTS reagent, 7 mM ABTS (5 mL) was combined with 140 mM $K_2S_2O_8$ (88
178 μl). To generate free radicals, the mixture was put in the dark (16 h, room temperature (RT)). The
179 reagent was diluted using the absolute ethanol, to get the absorbance of 0.70 ± 0.02 at 734 nm
180 (Infinite M200 microplate reader, Tecan, Männedorf, Switzerland). ABTS·⁺ scavenging activity
181 was assayed by combining of ABTS reagent (0.9 mL) with properly diluted extracts or fractions
182 of extracts from snail eggs (0.1 mL). After the incubation of samples (6 min, RT), the absorbance
183 was registered. The standard curve was obtained using TROLOX (see Ferric-reducing
184 antioxidant power section), *n* = 3.
185 **2.2-diphenyl-1-picrylhydrazyl radical (DPPH·) scavenging activity**
186 To evaluate DPPH· scavenging activity of extracts and fractions of extracts from *H. a. maxima*
187 and *H. a. aspersa* eggs, the method of Li et al. with some modifications was utilized [26,28]. The
188 procedure is based on donation of hydrogen atom or electron by antioxidants to the unpaired
189 electron of DPPH·, the absorbance falls proportionally to rise of DPPH non-radical form. 0.2 mM
190 DPPH· in absolute methanol was mixed with properly diluted extracts and fractions of extracts
191 from eggs (2:1, v/v). After incubation of the samples (30 min, without access to light), they were
192 centrifuged (15000× *g*, 10 min). Thereafter, the supernatant absorbance was registered at 517 nm

193   (Infinite M200 microplate reader, Tecan, Männedorf, Switzerland). The standard curve was
194   obtained as in Ferric-reducing antioxidant power section, $n = 3$.
195   Determination of phenols
196   The method of determination of total phenols, the Folin-Ciocalteu method, is based on the
197   reading of the absorbance of the complex arising from the reduction of Folin-Ciocalateu reagent,
198   i.e. salts of hetero polyacids, phosphomolybdic and phosphotungstic [26,29]. Over the reaction
199   Mo (VI) ions are reduced to Mo (V) ions, resulting in a blue color of $[PMoW_{11}O_{40}]^{4-}$. To evaluate
200   the concentration of phenols, properly diluted extracts and fractions of extracts derived from snail
201   eggs (0.5 mL) were mixed with Folin-Ciocalteu reagent (diluted 1:10 in deionized water, 2.5 ml).
202   After incubation for 2 min, the samples were mixed with 7.5% Na_2CO_3 (2 mL) and incubated in a
203   water bath (50 °C, 10 min). Then, the absorbance was read at 760 nm (Spectronic 20D cuvette
204   spectrophotometer, Milton Roy, Rochester, NY, USA). The standard curve was obatained using
205   various quercetin levels (0-100 μg/mL), $n = 2$.
206   Determination of total carbohydrates, crude protein and crude fat
207   Concentration of total carbohydrates in extracts and fractions of extracts from *H. a. maxima* and
208   *H. a. aspersa* eggs was measured using the phenol-sulfuric acid method [26,30]. The absorbance
209   was registered at 490 nm and glucose was utilized as the standard, $n = 3$.
210   Content of crude protein in lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was
211   determined by the Kjeldahl method, according to AOAC International [7,31], $n = 2$.
212   Crude fat was extracted from lyophilized snail eggs by applying petroleum ether using the
213   Soxhlet method [7,31], $n = 2$.
214   Determination of lipid peroxidation products
215   Level of lipid peroxidation products – thiobarbituric acid reactive substances (TBARS) in
216   extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was determined using the
217   procedure of Uchiyama and Mihara [7,32]. Extracts were acquired after homogenization of egg
218   lyophilizates in radioimmunoprecipitation assay (RIPA) buffer and centrifugation (1600× g, 10
219   min). The absorbance was recorded at 532 nm (Infinite M200 microplate reader, Tecan,
220   Männedorf, Switzerland). TBARS were expressed as the equivalents of malondialdehyde (MDA)
221   and its precursor - 1.2.3.3-tetraethoxypropane (TEP) was used as the standard, $n = 6$.
222   Determination of glutathione
223   Widespread in cells thiol tripeptide, glutathione (GSH), constitutes nearly 97% of non-protein
224   thiol compounds. GSH is evaluated quantitatively by assay of non-protein -SH groups in the
225   samples deproteinized by TCA. The method for the evaluation of non-protein -SH groups
226   consists in the Ellman's method, reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by thiol
227   compounds and formation of colorful 2-nitro-5-mercaptobenzoic acid, having an absorbance
228   maximum at 412 nm [26,33]. Lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails were
229   subjected to homogenization in 0.1 M phosphate buffer pH 7.4 and centrifugation (1600× g, 10
230   min). In order to deproteinize, to the supernatants (1.5 ml) was added 50% TCA (78.96 μl) and
231   samples were subjected to centrifugation (3000 rpm, 5 min). Then, deproteinized supernatants
232   (25 μl) were mixed with 0.2 M phosphate buffer pH 8.0 (200 μl) and with $6 × 10^{-3}$ M DTNB (25
233   μl), directly on a 96-well plate. The absorbance was read by applying microplate reader (Infinite
234   M200, Tecan, Männedorf, Switzerland). The standard curve was generated using different
235   contents (0–75 nmol/mL) of GSH in 2.5% TCA, $n = 6$.
236   Determination of astaxanthin and vitamins A, D_3, E and C
237   Determination of the contents of astaxanthin and vitamins A, D_3 (cholecalciferol) and E in
238   lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails was preceded by liquid extraction with
239   the application of ultrasound. The concentrations of above compounds were measured using
240   high-performance liquid chromatography coupled with UV-Vis detection (HPLC/UV-Vis), based
241   on the standard curves. The analysis of astaxanthin was performed using the Altus A-10 system

242 (PerkinElmer, Waltham, MA, USA) and LiChroCART 250-4, C18 column (Merck & Co., Inc.,
243 Kenilworth, NJ, USA). The analyses of vitamins A and E were carried out by Shimadzu
244 HPLC/UV-Vis system (Kyoto, Japan) and LiChroCART 125-4, C18 column (Merck & Co., Inc.,
245 Kenilworth, NJ, USA). The analysis of vitamin D_3 was done using the Altus A-10 system
246 (PerkinElmer, Waltham, MA, USA) and LiChroCART 125-4, C18 column (Merck & Co., Inc.,
247 Kenilworth, NJ, USA). The analyses were carried out in a private analytical laboratory (Olsztyn,
248 Poland), $n = 3$.
249 The analysis of vitamin C, as the sum of ascorbic acid and dehydroascorbic acid, in lyophilized
250 snail eggs was carried out using HPLC/UV-Vis, in the laboratory which is accredited by the
251 Polish Centre for Accreditation [34]. In order to determine the total content of vitamin C in the
252 sample extracts, the reduction of dehydroascorbic acid to ascorbic acid was performed with
253 dithiothreitol. The analysis was carried using a chromatograph with Waters® 2487 Dual
254 Wavelength Absorbance Detector (Waters Corp., Miliford, MA, USA) with a Symmetry C18
255 column, 100 Å, 5 µm, 4.6 mm x 150 mm (Waters Corp., Miliford, MA, USA), column
256 temperature: 25 °C, injection volume: 25-30 µl. The analysis was carried out at 245 nm
257 wavelength and 0.8 mL/min mobile phase flow, $n = 2$.
258 **Analysis of molecular weights of proteins and glycoproteins**
259 For the preparation of protein extracts, lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails
260 were homogenized in phosphate-buffered saline (PBS) with inhibitors of proteases and
261 phosphatases (Sigma-Aldrich, St. Louis, MO, USA), in the ratio 119 mg lyophilizate/1 ml PBS,
262 and then centrifuged (1600× *g*, 10 min). Extracts were submitted for sodium dodecyl sulfate-
263 polyacrylamide gel electrophoresis (SDS-PAGE), with the application of a 5% stacking gel and
264 10% resolving gel, by the Laemmli method [7,35] with modifications. The samples (30 µL) were
265 subjected to denaturation and reduction using the Laemmli sample buffer (Bio-Rad Laboratories,
266 Hercules, CA, USA) with β-mercaptoethanol (30 µl) and then heating (95 °C, 5 min). Each
267 sample (20 µL) and a protein marker (5 µL, ColorBurst™ Electrophoresis Marker, Sigma-
268 Aldrich, St. Louis, MO, USA) were loaded onto two gels and resolved by electrophoresis (Mini-
269 PROTEAN® Tetra Vertical Electrophoresis Cell System, Bio-Rad Laboratories, Hercules, CA,
270 USA).
271 To analyze the molecular weights of proteins, protein bands separated on the first gel were fixed,
272 stained (QC Colloidal Coomassie Stain, Bio-Rad Laboratories, Hercules, CA, USA) and
273 destained in accordance with the manufacturer's procedure (Bio-Rad Laboratories, Hercules, CA,
274 USA).
275 Sugar moieties of glycoproteins separated on the second gel were detected by applying the
276 commercial kit, according to the manufacturer's procedure (Pierce Glycoprotein Staining Kit,
277 Thermo Fisher Scientific, Waltham, MA, USA). Glycols present in glycoproteins are oxidized to
278 aldehydes when treated with periodic acid and are stained magenta.
279 The gels were visualized by applying Azure c400 imaging system (Azure Biosystems, CA,
280 USA).
281 **Analysis of amino acids**
282 The concentrations of the amino acids with the exception of tryptophan (Trp) in lyophilized eggs
283 of *H. a. maxima* and *H. a. aspersa* snails were assayed by ion-exchange chromatography with
284 spectrophotometric detection (IEC-VIS) [36]. The analysis was performed using an automatic
285 amino acid analyzer AAA 400 and an ion-exchange column (Ingos, Prague, Czech Republic).
286 The level of Trp was determined using high-performance liquid chromatography with
287 fluorescence detection (HPLC-FLD, Agilent 1100 Series, Agilent Technologies, Santa Clara, CA,
288 USA) [7,37]. Zorbax® ODS C18, 4.6 mm ID × 250 mm (5 µm) column (Agilent Technologies,
289 Santa Clara, CA, USA) was used.

290   Analysis of amino acids was carried out in the laboratory accredited by the Polish Centre for
291   Accreditation, *n* = 2.
292   The amino acid score (AAS), chemical score (CS) and essential amino acid index (EAAI) were
293   calculated using the equations below [7,38,39]:

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

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

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

294   where aa - the amount of amino acid per examined protein (%); AA (standards) - the amount of
295   amino acid per protein in the Food and Agriculture Organization of the United Nations (FAO)/the
296   World Health Organization (WHO) reference pattern for preschool children, 2–5 years (%); AA
297   (egg) - the amount of amino acid per protein in whole egg protein reference pattern (%); n - the
298   number of amino acids; A, B, C, . . . , H - the amount of essential amino acids per examined
299   protein (%); AS, BS, CS, . . . , HS - the amount of essential amino acids per protein in reference
300   pattern (%).
301   **Analysis of minerals**
302   The contents of Ca, P, Na, K, Mg, Cu, Fe, Mn and Zn in lyophilized eggs of *H. a. maxima* and *H.*
303   *a. aspersa* snails were assayed by inductively coupled plasma—atomic emission spectroscopy
304   (ICP-AES, iCAP 6500, Thermo Fisher Scientific, Waltham, MA, USA) [40]. The concentrations
305   of Ni, Cr, Mo, B, Co, Se, V and Sn in snail eggs were determined by mass spectrometry with
306   ionization in inductively coupled plasma (ICP-MS, Varian 820-MS, Varian, Inc., Palo Alto, CA,
307   USA) [41]. The contents of S, Cl, Si, I and F in eggs were evaluated by wavelength-dispersive X-
308   ray fluorescence (WDXRF, Axios, PANalytical, Almelo, the Netherlands) [42]. The analyses
309   were carried out in the laboratories accredited by the Polish Centre for Accreditation, *n* = 3.
310   **Preparation of extracts and their fractions for cell culture tests**
311   Before cell culture tests, lyophilized eggs derived from *H. a. maxima* and *H. a. aspersa* snails
312   were homogenized in deionized water, at the concentration of 25 mg/mL ((tests described in
313   Effect of extracts on cell viability (MTT test), Effect of extracts on the percentage of live and
314   dead cells (trypan blue test), Effect of extracts on the integrity of cell membranes and Effect of
315   extracts on the types of cell death sections)) or 2.5 mg/mL (Effect of extracts on the content of
316   lipid peroxidation products section). The homogenates were left for extraction (30 min, 4 °C),
317   centrifuged (1600× *g*, 10 min) and the extracts were collected.
318   For the experiment described in Effect of fractions of extracts on cell viability (MTT test)
319   section, the extracts at the concentration of 100 mg/mL were prepared, filtered and fractionated to
320   obtain four fractions: >50 K, 10–50 K, 3–10 K and <3 K, as in Preparation of extracts and their
321   fractions for the determination of antioxidant indicators, phenols and total carbohydrates section.
322   Extracts or fractions were sterilized (PVDF syringe filters, pore size 0.22 μm, EuroClone, Pero,
323   Italy) under the biological safety cabinet (TopSafe™ 1.2, class II, BIOAIR, Pavia, Italy). Then,
324   for some tests, appropriate dilutions were prepared with sterile deionized water.
325   **Caco-2 cell culture**
326   Human epithelial colorectal adenocarcinoma (Caco-2) cell line (ECCC, 55 passage, Sigma-
327   Aldrich, St. Louis, MO, USA) was cultivated in polystyrene plates designed for adherent cell
328   culture ((for the experiments described in Effect of extracts on cell viability (MTT test), Effect of
329   fractions of extracts on cell viability (MTT test) and Effect of extracts on the integrity of cell

330   membranes sections - in 96-well plates, at an initial density of 1×10^4 cells/100 µl, for the
331   experiment presented in Effect of extracts on the percentage of live and dead cells (trypan blue
332   test) section – in 24-well plates, at a density of 5.94×10^4 cells/594 µl and for the experiments
333   described in Effect of extracts on the content of lipid peroxidation products and Effect of extracts
334   on the types of cell death sections - in 6-well plates, at a density of 0.75×10^5 cells/1.5 mL)) in
335   Minimum Essential Medium (MEM) comprising 2 mM L-glutamine, 10% fetal bovine serum
336   (FBS), 1% non-essential amino acids (NEAA) and 1% antibiotic-antimycotic (all solutions were
337   purchased from Thermo Fisher Scientific, Waltham, MA, USA) [7,26]. The cells were kept at 37
338   °C in 5% CO_2 and 95% relative humidity in a CO_2 incubator (INCO 108 med, Memmert GmbH +
339   Co. KG, Schwabach, Germany) for 24 h. After incubation and reaching about 70% confluency,
340   they were starved in MEM with 1% FBS and 1% antibiotic-antimycotic (Thermo Fisher
341   Scientific, Waltham, MA, USA) overnight.
342   **Effect of extracts on cell viability (MTT test)**
343   90 µL of new medium (MEM with 1% FBS and 1% antibiotic-antimycotic; Thermo Fisher
344   Scientific, Waltham, MA, USA) and 10 µL of extracts from lyophilized eggs of *H. a. maxima* and
345   *H. a. aspersa* snails, at the concentrations of 25, 25×10^{-1}, 25×10^{-2}, 25×10^{-3}, 25×10^{-4} and $25 \times$
346   10^{-5} mg/mL were added to the cells. Equal volumes of deionized water (sterile) were introduced
347   into the control cells. Additional controls were also included. After 24 h and 72 h of incubation in
348   a CO_2 incubator (INCO 108 med, Memmert GmbH + Co. KG, Schwabach, Germany; 37 °C, 5%
349   CO_2 and 95% relative humidity), the MTT (methylthiazolyldiphenyl-tetrazolium bromide) test
350   was done using the method of Tada et al. with modifications [7,26,43]. Yellow MTT solution is
351   converted to water-insoluble, dark blue MTT formazan, by mitochondrial dehydrogenases of
352   living cells. 15 µl of MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) in PBS (5 mg/mL) was
353   added to the cells and they were incubated (37 °C, 4 h). Then, 100 µl of lysis buffer (10% SDS in
354   0.01 M HCl) was added and plates were incubated overnight at 37 °C. The absorbance was
355   measured at 570 nm (Infinite M200 microplate reader, Tecan, Männedorf, Switzerland), $n = 6$.
356   **Effect of fractions of extracts on cell viability (MTT test)**
357   90 µL of new medium and 10 µL of four fractions: >50 K, 10–50 K, 3–10 K and <3 K of extracts
358   from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails, at the concentrations of 1.25 and
359   0.125 mg/mL were introduced into the cells. The same controls as in Effect of extracts on cell
360   viability (MTT test) section were applied. After 24 h incubation, the MTT test was done, as in
361   Effect of extracts on cell viability (MTT test) section. $n = 4$.
362   **Effect of extracts on the percentage of live and dead cells (trypan blue test)**
363   The trypan blue test is based on the fact that live cells have intact cell membranes which exclude
364   dyes such as trypan blue, while dead cells take up the dyes [44]. After mixing the cell suspension
365   with trypan blue, live cells have clear cytoplasms, while dead cells have blue ones. 535 µL of
366   new medium and 59 µL of extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa*
367   snails, at the concentration of 25 mg/mL were added to the cells. Equal volumes of sterile
368   deionized water were introduced into the control cells. After 24 h and 72 h of incubation in a CO_2
369   incubator (INCO 108 med, Memmert GmbH + Co. KG, Schwabach, Germany; 37 °C, 5% CO_2
370   and 95% relative humidity), the trypan blue test was done. Initially, the cells were trypsinized,
371   washed in cold PBS and centrifuged. The supernatants were removed and the cell pellets were
372   resuspended in PBS (100 µl). Then, 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO, USA; 50
373   µl) were mixed with the cell suspensions (50 µl). After incubation for 5 min, the samples (10 µl)
374   were loaded into a Bürker chamber. Cells in the entire chamber were photographed using a DMi8
375   inverted light microscope with a MC190 HD camera, employing the LAS V4.10 software (Leica,
376   Wetzlar, Germany). The results were expressed as the percentage of live and dead cells in the
377   groups treated with the extracts relative to the control group, $n = 5$.
378   **Effect of extracts on the integrity of cell membranes**

379   Cell membrane damage results in the release into the medium of the cytosolic enzyme, lactate
380   dehydrogenase (LDH). This enzyme can be quantified by applying a coupled enzymatic reaction.
381   It is a catalyst for the transformation of lactate to pyruvate by reduction of NAD^+ to NADH.
382   Thereafter, diaphorase utilizes NADH in order to reduce INT tetrazolium salt to red formazan
383   that content is measured (490 nm). The LDH test was carried out as recommended by the
384   commercial kit manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). 100 µL of new
385   medium and 10 µL of extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails, at
386   the concentrations of 25, 25×10^{-1}, 25×10^{-2}, 25×10^{-3}, 25×10^{-4} and 25×10^{-5} mg/mL were
387   added to the cells. Equal volumes of sterile deionized water were added to the control cells.
388   Additional controls were also included. After 24 h and 72 h of incubation in a CO_2 incubator
389   (INCO 108 med., Memmert GmbH + Co. KG, Schwabach, Germany; 37 °C, 5% CO_2 and 95%
390   relative humidity), LDH activity was evaluated and expressed as % of maximum LDH activity
391   (after cell lysis), $n = 4$.
392   **Effect of extracts on the content of lipid peroxidation products**
393   1.5 mL of new medium and 150 µL of extracts from eggs of two snail subspecies, at the
394   concentration of 2.5 mg/mL were introduced into the cells (each of the extracts was added to 18
395   wells). Equal volumes of sterile deionized water were introduced into the control cells. After 24 h
396   incubation (CO_2 incubator INCO 108 med., Memmert GmbH + Co. KG, Schwabach, Germany;
397   37 °C, 5% CO_2 and 95% relative humidity), the cells were trypsinized, washed in cold PBS (two
398   times) and centrifuged. The pellets were resuspended in RIPA buffer (130 µl) and incubated (30
399   min, 4 °C) to lyse cells. Lysates were centrifuged ($14000 \times g$, 10 min., 4 °C), supernatants were
400   collected, frozen in liquid nitrogen and stored at -80 °C. Then, concentration of TBARS in the
401   supernatants was determined as in Determination of lipid peroxidation products section. The total
402   protein concentration in the supernatants was also determined, by the Bradford method, using
403   bovine serum albumin as the standard [45]. The results were expressed in µg/mg total protein, $n =$
404   3.
405   **Effect of extracts on the types of cell death**
406   1.5 mL of new medium and 150 µL of extracts from lyophilized eggs of *H. a. maxima* and *H. a.*
407   *aspersa* snails, at the concentration of 25 mg/mL were added to the cells. Equal volumes of
408   sterile deionized water were added to the control cells. After 24 h of incubation in a CO_2
409   incubator (INCO 108 med, Memmert GmbH + Co. KG, Schwabach, Germany; 37 °C, 5% CO_2
410   and 95% relative humidity), the types of cell death were determined by flow cytometry, in
411   accordance with the procedure of the commercial kit with Alexa Fluor® 488 Annexin V and
412   propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA). The last one, a
413   fluorescent dye, binds to the nucleic acids staining dead cells and Annexin V conjugated to Alexa
414   Fluor® 488 fluorophore binds to phosphatidyl serine on the external surface of the apoptotic cell
415   membrane. Live cells do not stain, Annexin V-stained cells are considered early apoptotic,
416   PI/Annexin V-stained cells - late apoptotic and PI-stained cells - necrotic. The cells were
417   trypsinized and washed two times in cold PBS. Then, they were centrifuged, the supernatants
418   were removed and the pellets were resuspended in Annexin-binding buffer (100 µl). Alexa Fluor®
419   488 Annexin V (5 µl) and PI working solution (1 µl) were introduced into the cell suspensions.
420   After incubation of the cells for 15 min (RT), Annexin-binding buffer (400 µl) was introduced,
421   the samples were mixed by pipetting and kept on ice. The cells were assayed using BD
422   FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), fluorescence
423   emission intensity was registered by FL1 channel for Alexa Fluor® 488, at 530 nm and FL2 for
424   PI, at 575 nm, with excitation at 488 nm. 10000 events were registered per sample. Flowing
425   Software 2.5.1 (Perttu Terho, Turku, Finland) was used to generate the plots, $n = 5$.
426   The cells were photographed using a DMi8 inverted light microscope with a MC190 HD camera,
427   employing the LAS V4.10 software (Leica, Wetzlar, Germany).

Statistical analysis

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

Results

Antioxidant indicators, content of phenols and total carbohydrates

In the case of eggs of both snail subspecies, the fraction containing particles >50 kDa (K) was marked by the greatest ferric-reducing antioxidant power, several times higher than the value for the extract and fraction with particles 10-50 K (Table 1). The lowest ferric-reducing antioxidant power was shown for fractions containing particles <10 K. The significant subspecies-fraction interaction demonstrated that the fractions comprising particles >50 K were characterized by the greatest ABTS·$^+$ scavenging activity. The lower ability to reduce ABTS·$^+$ was exhibited by extract from eggs of *H. a. maxima*, followed by extract from eggs of *H. a. aspersa* and fractions containing particles 10-50 K. The lowest ABTS·$^+$ scavenging activity was noted in fractions with particles <10 K.

The fractions with particles >50 K had the highest DPPH· scavenging activity. A lower DPPH· scavenging activity was noted for extracts. Fractions containing particles <50 K did not show the ability to reduce the DPPH·.

The statistically significant interaction demonstrated that the content of phenols was the highest in the fractions with particles >50 K. This content was lower in the extracts and the extract from *H. a. maxima* eggs comprised more phenols than the extract from *H. a. aspersa* eggs. Phenol concentration in fractions comprising particles <50 K was slight or no such compounds were detected.

The concentration of total carbohydrates in the lyophilized eggs from *H. a. maxima* was 17.97 ± 0.89 %, while in the lyophilized eggs from *H. a. aspersa* - 14.01 ± 0.33 %. Significantly the highest concentration of total carbohydrates was observed in the fractions containing particles >50 K. Fractions with particles <50 K contained few carbohydrates.

Content of crude protein, crude fat, lipid peroxidation products, glutathione, astaxanthin and vitamins

The data showed that lyophilized snail eggs comprised mainly crude protein and its content was similar in the eggs of both subspecies (Table 2). Furthermore, the presence of lipid peroxidation products – TBARS was noted in extracts from eggs. The content of GSH was statistically significantly higher in the eggs of *H. a. maxima* than in *H. a. aspersa*. The presence of crude fat, astaxanthin and vitamins A, C and E was not detected in the eggs of both subspecies. The lyophilized eggs of *H. a. aspersa* contained significantly more vitamin D$_3$.

Analysis of molecular weights of proteins and glycoproteins

Profiles of proteins and peptides derived from extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* separated by SDS-PAGE are shown in Fig. 1A. The results indicate that the egg proteins and peptides had molecular weights in the range of protein standards: from 8 kDa to 220 kDa. Proteins of molecular weight >50 kDa predominated. The comparison of the protein and peptide profiles shows that *H. a. maxima* eggs (panel (b)) contained more components of low molecular weights compared to *H. a. aspersa* eggs (panel (c)). Protein and peptide bands around

477 and below 8 kDa can be observed in the case of both analyzed extracts, although in the case of *H.*
478 *a. maxima* they occured in a greater amount.
479 The eggs of *H. a. maxima* (Fig. 1B, panel (b)) and *H. a. aspersa* (Fig. 1B, panel (c)) contained
480 glycoproteins of molecular weights ranging from 8 kDa to 220 kDa. Glycoproteins >50 kDa
481 predominated, especially in the range from 50 kDa to 100 kDa and the profile of glycoproteins
482 was similar in case of both subspecies.
483 **Analysis of amino acids**
484 The amino acid profiles of lyophilized eggs of *H. a. maxima* and *H. a. aspersa* are presented in
485 Table 3. Eighteen amino acids were identified in eggs, eight of which are essential amino acids
486 (EAA) for humans – leucine (Leu), lysine (Lys), phenylalanine (Phe), valine (Val), threonine
487 (Thr), isoleucine (Ile), methionine (Met) and tryptophan (Trp) [46]. The content of individual
488 amino acids, except for Thr and Trp, in the crude protein of the eggs of two snail subspecies
489 differed statistically significantly. AAS, CS and EAAI for snail eggs are shown in Table 4. AAS
490 for amino acids of snail eggs was >1.00, except AAS for Lys and Leu for *H. a. aspersa* eggs. The
491 first limiting amino acid, according to AAS, was His (histidine) for *H. a. maxima* eggs and Lys
492 for *H. a. aspersa* eggs. The contents of Phe + Tyr (tyrosine), Val and Ile in eggs were the highest
493 compared to FAO/WHO standard protein. CS for amino acids of snail eggs was <1.00, except CS
494 for Phe + Tyr and Lys for *H. a. maxima* eggs. The first limiting amino acid, according to CS, was
495 Met + Cys for eggs of both snail subspecies. The concentration of Met in eggs was low compared
496 to most amino acids (Table 3). The EAAI (FAO/WHO reference amino acid pattern) for snail
497 eggs was >100 and the EAAI (whole egg reference amino acid pattern) was <100 (Table 4). This
498 index had higher value in the case of *H. a. maxima* eggs.
499 Taking into account non-essential amino acids (NEAA), snail eggs had the highest content of
500 glutamic acid (Glu), aspartic acid (Asp) and serine (Ser) (Table 3).
501 Snail eggs were characterized by a high total concentration of delicious amino acids (DAA) -
502 Glu, Asp, Ala (alanine) and Gly (glycine). Gly content was the lowest in this group of amino
503 acids.
504 The concentrations of total amino acids (TAA), EAA, half-essential amino acids (HEAA), NEAA
505 and DAA were higher in the crude protein of *H. a. maxima* eggs than of *H. a. aspersa* eggs.
506 EAA/TAA and EAA/NEAA ratios were higher for *H. a. maxima* eggs and DAA/TAA ratio was
507 higher for *H. a. aspersa* eggs.
508 **Analysis of minerals**
509 The content of macroelements and microelements, except for Mg, Mn and Se, in the lyophilized
510 eggs of *H. a. maxima* and *H. a. aspersa* snails was statistically significantly different (Table 5).
511 The concentration of macroelements (descending order) in the eggs of both snail subspecies was
512 as follows: Ca, P, Na, K, Mg, S, Cl. The concentration of microelements (descending order) in *H.*
513 *a. maxima* eggs was as follows: Cu, Ni, Si, Fe, Mn, Cr, Mo, B, Zn, Co, V, Se, I, Sn and in *H. a.*
514 *aspersa* eggs: Si, Cu, Fe, Mn, Ni, B, Zn, Mo, Cr, Co, Se, I, V, Sn. No F was detected in the eggs.
515 **Effect of extracts on cell viability (MTT test)**
516 The influence of various concentrations of water extracts from lyophilized eggs of *H. a. maxima*
517 and *H. a. aspersa* snails on the viability of Caco-2 cells was determined using the MTT test,
518 based on the mitochondrial oxidative activity in live cells. Treatment with an extract from *H. a.*
519 *maxima* eggs (at concentrations of 25, 2.5×10^{-1} and 2.5×10^{-4} mg/mL) and an extract from *H. a.*
520 *aspersa* eggs (25, 2.5×10^{-1} and 2.5×10^{-3} mg/mL), for 24 h, resulted in a statistically significant
521 reduction in the viability of Caco-2 colon cancer cells compared to control cells, treated with
522 deionized water (Fig. 2a). Treatment with extracts from eggs of both snail subspecies, at different
523 concentrations, for 72 h, did not statistically significantly affect the viability of Caco-2 cells
524 compared to control cells (Fig. 2b).
525 **Effect of fractions of extracts on cell viability (MTT test)**

526 Treatment with fraction of an extract from *H. a. maxima* eggs containing particles of a molecular
527 weight <3 kDa (1.25 and 0.125 mg/mL), for 24 h, resulted in a statistically significant reduction
528 of the viability of Caco-2 cells compared to control cells (Fig. 3a). Treatment with fraction of an
529 extract from *H. a. aspersa* eggs containing particles <3 kDa (concentrations as above), for the
530 same time, resulted in a reduction of the viability of Caco-2 cells compared to control cells, but
531 this effect was not statistically significant (Fig. 3b).
532 **Effect of extracts on the percentage of live and dead cells (trypan blue test)**
533 The effect of extracts from eggs of *H. a. maxima* and *H. a. aspersa* on the percentage of live and
534 dead cells was evaluated by the trypan blue assay, in which live cells, having intact cell
535 membranes, exclude trypan blue, while dead cells take up this dye and have blue cytoplasms.
536 Treatment with extracts from eggs of both snail subspecies (25 mg/mL), for 24 h, resulted in a
537 statistically significant reduction in the percentage of live Caco-2 cells as compared to control
538 cells, treated with deionized water and did not significantly affect the percentage of dead cells
539 (Fig. 4a). Treatment with these extracts for 72 h did not significantly affect the percentage of live
540 and dead Caco-2 cells as compared to control cells (Fig. 4b).
541 **Effect of extracts on the integrity of cell membranes**
542 Treatment with an extract from *H. a. maxima* eggs (25, 25×10^{-1} and 25×10^{-3} mg/mL) and an
543 extract from *H. a. aspersa* eggs (25×10^{-1} mg/mL), for 24 h, statistically significantly increased
544 the degree of damage to Caco-2 cell membranes, the activity of the cytosolic enzyme - LDH,
545 released from damaged cells, compared to control cells, treated with deionized water (Fig. 5a).
546 Treatment with extracts from the eggs of both snail subspecies ($25 - 25 \times 10^{-5}$ mg/mL), for 72 h,
547 did not significantly affect the activity of released LDH (Fig. 5b).
548 **Effect of extracts on the content of lipid peroxidation products**
549 Treatment of cells with extracts from the eggs of both snail subspecies (2.5 mg/mL), for 24 h,
550 increased the amount of lipid peroxidation products – TBARS compared to control cells, treated
551 with deionized water, but this effect turned out to be statistically insignificant (Fig. 6).
552 **Effect of extracts on the types of cell death**
553 Treatment of Caco-2 cells with extracts from eggs of both snail subspecies (25 mg/mL), for 24 h,
554 caused the induction of apoptosis and reduction of necrosis - an increase in the percentage of
555 early apoptotic cells (effect statistically significant for *H. a. aspersa* egg extract) and a decrease
556 in the percentage of necrotic cells compared to control cells, treated with deionized water (Fig.
557 7).
558

Discussion
560 High concentrations of reactive oxygen species (ROS) in cells may lead to oxidative stress,
561 damage to the cell membranes, proteins and DNA [47]. Antioxidants are necessary in prevention
562 of the formation and inhibition of the activity of ROS [48]. Some antioxidants and a greater total
563 antioxidant capacity are related to decreased risk of colorectal cancer [47,49,50]. These
564 compounds may be beneficial in the initiation and progression of cancer [48]. Their action may
565 include a positive effect on cell proliferation, apoptosis, metastasis and drug resistance. In the
566 current research, higher antioxidant activity, expressed as ABTS·$^{+}$ scavenging activity, and
567 phenol content in the extract from lyophilized eggs of *H. a. maxima* than of *H. a. aspersa* were
568 noted. Antioxidants including phenols were concentrated in fractions >50 K. The differences in
569 the antioxidant potential of the fractions determined with the use of various methods result from
570 the complex kinetics of the contained antioxidants and the time they reach stable endpoints [51].
571 Ferric-reducing antioxidant power and ABTS·$^{+}$ scavenging activity are based on electron
572 donating capacity of bioactive compounds, and DPPH· scavenging activity is based on electron
573 and hydrogen atom transfer [52]. ABTS·$^{+}$ is more reactive than DPPH·. Ferric-reducing
574 antioxidant power differs from the two methods used to determine the concentration of

575   antioxidants, because the reduction of Fe^{3+} to Fe^{2+} is monitored and free radicals are not involved
576   [53]. The content of hydrophilic and high-pigmented antioxidants is better reflected by $ABTS\cdot^+$
577   scavenging activity than $DPPH\cdot$ scavenging activity.
578   Phenols are compounds that can initiate the process of autoxidation under certain conditions, and
579   therefore they can act as prooxidants [54,55]. Such conditions include, for example, high pH,
580   high contents of transition metal ions, Cu^{2+}, Fe^{3+}, and the presence of oxygen molecules. The high
581   concentration of phenols also favors their prooxidative effect [55]. In addition, low molecular
582   weight phenols are easily oxidized and show prooxidative activity, unlike those with high
583   molecular weight, which have little or no prooxidative activity [54].
584   The direct prooxidative properties of phenols consist in the generation of an aroxyl radical or a
585   redox complex with a metal cation. The aroxyl radical is able to react with oxygen to form $O_2\cdot^-$
586   and it may form ternary compounds of DNA, copper and flavonoids [56]. Some polyphenols
587   have a prooxidative effect due to the activation of intracellular ROS production by NADPH
588   oxidase [57,58].
589   The prooxidative activity of these compounds could also be associated with transition metals, by
590   the reduction of metal ions participating in redox cycling and promotion of the production of
591   hydroxyl radicals as a result of Fenton and Fenton-like processes. Cancer cells are characterized
592   by a higher level of transition metal ions and the mobilization of such endogenous metal ions as
593   iron and copper may explain the selective toxicity of polyphenols toward cancer cells [59]. Three
594   phenolic acids in Cu^{2+} presence induced the cleavage of DNA in HL-60 human promyelocytic
595   leukemia cells [60]. Stilbene piceatannol can interact with Cu^{2+}, promoting Fenton and Haber-
596   Weiss reactions generating ROS, which induce DNA cleavage. Piceatannol is converted to *ortho*-
597   quinone, that forms DNA covalent adducts [61]. Moreover, cancer cells of many solid tumors,
598   because of the Warburg effect, are characterized by a high glycolysis level, which leads to a
599   decrease in pH, that affects the structure of DNA and exposes copper bound to chromatin to a
600   potential attack of prooxidants, such as resveratrol [62].
601   Polyphenol-induced production of ROS may play a major role in apoptosis initiation, even
602   though ROS are also generated as a consequence of it [63-66]. Evidence for a prooxidant-
603   associated cytotoxic activity of polyphenols is abundant. It is connected with the activation of
604   apoptosis as well as cell cycle arrest.
605   Emodin anthraquinone, through the production of ROS, sensitized COC1/DDP human ovarian
606   carcinoma cells, resistant to cisplatin, to cisplatin-induced apoptosis [67]. In addition, emodin
607   reduced the expression of multidrug resistance-related protein 1 through a mechanism related to
608   ROS.
609   The prooxidative activity of some polyphenols, in non-cytotoxic concentrations, may sensitize
610   cancer cells to other cancer therapies [68].
611   Epigallocatechin gallate treatment of chemoresistant HT-29 colon cancer cells was related to an
612   increased ROS production, reduced proliferation and sensitization to 5-fluorouracil [69].
613   ROS induced by phenolic compounds, with/without the presence of transition metals, may
614   interact with cell macromolecules, contributing to lipid peroxidation, protein oxidation and DNA
615   damage [70].
616   It was shown that the proapoptotic activity of curcumin on a rat model of colorectal cancer was
617   associated with increased ROS generation [71]. The increased level of intracellular ROS in
618   isolated colonocytes was accompanied by the mitochondrial membrane potential loss and
619   inhibition of one antiapoptotic pathway.
620   Epigallocatechin gallate showed cytotoxic activity against H1299 human lung cancer cells, *in*
621   *vitro* as well as in xenograft tumors [72]. Following treatment with this compound *in vitro*, a
622   dose-dependent reduction of the cell viability by intracellular and mitochondrial ROS induction
623   was noted. Dose-dependent inhibition of tumor growth was reported, accompanied by cancer cell

624 apoptosis and DNA oxidative damage. However, no increase in the level of a marker of oxidative
625 DNA damage was noted in the host organs. It was suggested that the prooxidative activity of
626 epigallocatechin gallate is also associated with an increase in the concentration of 5-fluorouracil
627 in the plasma, due to the inhibition of the major enzyme involved in its catabolism [73]. Research
628 suggests that consumption of green tea, rich in epigallocatechin gallate, may influence on the
629 pharmacokinetics of chemotherapeutic agents.
630 In the study by Kostadinova et al., the fraction of *H. a. aspersa* mucus containing particles <5
631 kDa was characterized by a greater antioxidant potential compared to the other fractions tested
632 (<10 kDa, <20 kDa and 10-30 kDa) [74]. Low molecular weight peptides and free amino acids
633 may contribute significantly to the antioxidant potential [75]. About 70% of the antioxidant
634 peptides had a molecular weight in the range 400-650 Da [76]. In two fractions of *H. a. aspersa*
635 mucus, containing molecules <1 kDa and <3 kDa, similar metabolites with antioxidant activity
636 were detected [77]. The fraction of *Babylonia areolata* snail hydrolyzate comprising the smallest
637 particles, <0.65 kDa, had the highest antioxidant potential [78]. The antioxidant activity of *H. a.*
638 *maxima* eggs was demonstrated in the study of Górka et al. [79].
639 The concentration of carbohydrates in the lyophilized eggs of the studied snail subspecies was
640 higher than in the lyophilized foot tissues of *H. a. aspersa* snails and similar to their content in
641 the lyophilized mucus of these snails [7]. Carbohydrates were also concentrated in the fraction
642 >50 K. Other authors' research shows that the shell of *H. aspersa* eggs consists of crystals of
643 calcium carbonate bound to the mucopolysaccharide matrix that are surrounded by the mucus
644 layer [80,81]. Hovingh and Linker showed that chondroitin sulfate is the major
645 mucopolysaccharide (glycosaminoglycan) present in the heart, mantle and kidney of *H. aspersa*,
646 and heparan sulfate was also present in smaller amounts in these organs [82]. Wu et al. proved
647 that chondroitin sulfate derived from sturgeon (*Acipenser*) reduced the proliferation of HCT 116
648 human colon carcinoma cells, dose-dependently, which was connected with cell cycle arrest [83].
649 Moreover, this compound induced extensive apoptosis. Chondroitin sulfate also inhibited the
650 xenograft HCT 116 tumor development in mice by inhibition of proliferation and induction of
651 apoptosis. Many anticancer drugs based on glycosaminoglycans or their mimetics have been
652 developed with promising results on animal models and in clinical trials [84]. Nicolai et al.
653 demonstrated that galactogen is the main compound contained in the eggs of *H. a. aspersa* snails,
654 and its concentration turned out to be about six times greater than that of glycogen, dominant in
655 the foot tissues [85]. In the study of Górka et al., *H. a. maxima* eggs did not contain glucose [79].
656 Our research showed that the lyophilized eggs of both snail subspecies consisted mainly of crude
657 protein and were practically fat-free (concentration of crude fat <0.2%). Protein requirement for
658 maintenance is 0.66 g/kg/d [86]. The concentration of crude protein in the lyophilized eggs of the
659 studied snails turned out to be nearly two times lower than in the lyophilized mucus of *H. a.*
660 *aspersa* and over 2.5 times lower than in the lyophilized foot tissues of these snails [7].
661 A high-protein diet, rich in good-quality proteins is an important element of prehabilitation, the
662 process of strengthening the physical condition of cancer patients before surgery [87]. In the
663 international clinical practice guidelines for sarcopenia, which affects 12-60% of colorectal
664 cancer patients, protein supplementation and a diet rich in protein are recommended for adults
665 (especially older) with sarcopenia [88]. Murine squamous cell carcinoma VII and human
666 colorectal carcinoma grew slower in mice that were administered low-carbohydrate and high-
667 protein diet comparing to a Western diet – relatively high-carbohydrate and low-protein [89].
668 This diet reduced glycolysis, on which cancer cells depend to a large extent. In turn, feeding a
669 low-protein diet decreased tumor growth in mouse cancer models [90].
670 The lyophilized mucus of *H. a. aspersa* snails comprised almost no crude fat, and the foot tissues
671 contained less than 4% [7]. The fat comprised in the lyophilized foot tissues, however, had a high
672 nutritional value. The crude protein content in lyophilized eggs of the studied snails was lower

673   than the total protein content in the dry matter of *H. a. maxima* eggs in the study of Górka et al.
674   [79]. In the studies by Maćkowiak-Dryka et al., the fat content in *H. a. maxima* eggs was 0.04%,
675   while in *H. a. aspersa* eggs was 0.03%, however, this fat turned out to have a low nutritional
676   value [91]. Other authors detected the slight presence of triacylglycerols in *H. a. aspersa* eggs
677   [85] and cholesterol in *H. a. aspersa* and *H. a. maxima* eggs [79,85].
678   The concentration of carbohydrates in *H. a. maxima* and *H. a. aspersa* eggs is greater compared
679   to caviar and the raw materials from which other caviar substitutes are obtained [92,93]. On the
680   other hand, the fat content is much lower and the fat is not a good source of polyunsaturated fatty
681   acids (PUFA).
682   In our study, in egg extracts were detected lipid peroxidation products.
683   The product of lipid peroxidation, 4-hydroxynonenal (HNE) can have a stronger cytotoxic,
684   pronecrotic and proapoptotic effect on cancer cells than on normal cells because cancer cells have
685   a lower PUFA content in the cell membrane, to protect against lipid peroxidation [94]. Moreover,
686   it influences the activity of many genes, enzymes and cytokines responsible for oxidative
687   homeostasis and growth regulation. HNE can stimulate the activity of cyclooxygenase   2 (COX-
688   2) which catalyzes the synthesis of prostaglandins and induce angiogenesis in colorectal cancer
689   [95]. In addition, overactivation of COX-2 by this compound may promote Wnt/β   catenin
690   pathway. MDA, formed by lipid peroxidation, reacts with bases of nucleic acids and forms
691   adducts that can induce genetic mutations or cause apoptosis [96]. MDA, HNE and another lipid
692   peroxidation product - acrolein are capable of covalently modifying proteins leading to their
693   structural and functional changes [97]. HNE causes DNA modifications generating cancer-linked
694   mutations.
695   4-HNE could suppress growth of Caco-2 and HT-29 cells [96]. The anticancer action might be
696   related to oxidative stress alterations, consequent apoptosis induction and telomerase activity
697   inhibition. Glutathione conjugates of HNE are used as chemotherapeutic agents in cancer models.
698   GSH, a major intracellular antioxidant, regulates cellular redox state, protects cells from lesions
699   induced by lipid peroxides, ROS, reactive nitrogen species, as well as xenobiotics [98]. GSH is
700   an important controller of cell apoptosis, ferroptosis, proliferation, differentiation and immune
701   function. Alterations in GSH antioxidant system at the molecular level and disturbances in
702   homeostasis of this compound are related to cancer initiation, progression and response to
703   treatment. Increased GSH concentrations in cancer cells are connected with cancer progression
704   and elevated resistance to chemotherapeutics. In our investigation, *H. a. maxima* eggs contained
705   more GSH than *H. a. aspersa* eggs.
706   The proteins and peptides of snail eggs had molecular weights from 8 kDa to 220 kDa, and
707   proteins >50 kDa predominated. *H. a. maxima* eggs contained more components of low
708   molecular weights, including proteins and peptides around and below 8 kDa, compared to *H. a.*
709   *aspersa* eggs.
710   Sixteen putative cationic and amphipathic anticancer peptides were predicted in two fractions of
711   *A. fulica* mucus, which decreased the viability of MCF7 and Vero cells [7,9]. Nine of the peptides
712   had a molecular weight <3 kDa, three - in the range of 3–10 kDa, two - in the range of 10–50
713   kDa and two - >50 kDa. The presence of anticancer peptides was also predicted in the mucus of
714   several mollusks, including land snails [99].
715   The eggs of both snail subspecies presented the similar profile of glycoproteins, of molecular
716   weights from 8 kDa to 220 kDa and glycoproteins >50 kDa predominated, especially in the range
717   from 50 kDa to 100 kDa.
718   The glycoprotein *Helix aspersa* agglutinin, member of the family of H-type lectins identified in
719   invertebrates, an element of the perivitelline fluid of snail eggs, may be useful in drug delivery
720   systems targeting colorectal cancer, due to binding to the surface of cancer cells [100].

721 EAA enable endogenous synthesis of NEAA and their availability is the major limiting factor in
722 protein synthesis [101]. AAS for Lys in *H. a. aspersa* lyophilized eggs was <1.00, so this amino
723 acid concentration was lower than in FAO/WHO standard [38]. According to this score, the first
724 limiting amino acid of lyophilized *H. a. maxima* eggs was His and of lyophilized *H. a. aspersa*
725 eggs - Lys. For lyophilized foot tissues and mucus of *H. a. aspersa* more than one amino acid had
726 AAS <1.00, with Trp being the first limiting amino acid for foot tissues and Lys for mucus [7].
727 The concentrations of Phe + Tyr, Val and Ile in lyophilized eggs were dominating. In the case of
728 lyophilized foot tissues and mucus of *H. a. aspersa*, these were Ile, Thr and Val [7]. CS for amino
729 acids of lyophilized snail eggs was <1.00, except CS for Phe + Tyr and Lys for *H. a. maxima*
730 eggs, so their contents were smaller than in whole egg protein pattern. According to this score,
731 the first limiting amino acid of lyophilized eggs was Met + Cys. In the case of lyophilized foot
732 tissues and mucus of *H. a. aspersa*, CS for all amino acids was <1.00, while the first limiting
733 amino acids were for the foot tissues - Met + Cys, and for the mucus - Trp and Met + Cys [7].
734 Met content in lyophilized eggs was low comparing to most amino acids. The EAAI (FAO/WHO
735 standard) of lyophilized snail eggs was >100 and the EAAI (whole egg standard) was <100. This
736 index was greater for *H. a. maxima* eggs and significantly greater than for lyophilized foot tissues
737 and mucus of *H. a. aspersa* [7].
738 Dietary restriction of Met may be a main strategy to control cancer growth [102]. Furthermore,
739 Met restriction resulted in killing Met-dependent cancer cells co-cultured with normal cells.
740 Animal research in which diets restricted by Met were studied showed inhibited cancer growth
741 and extend healthy life-span. Met depletion in Met-dependent cancer cells can contribute to cell
742 cycle arrest in late S/G2 phase, the susceptibility of cells to death and their hypersensitivity to
743 chemotherapy.
744 Glu, Asp and Ser are NEAA found in the highest concentrations in lyophilized snail eggs. Gly
745 content was about two times lower than in the lyophilized foot tissues and mucus of *H. a.*
746 *aspersa* [7].
747 The deprivation of Gly and Ser in the diet inhibits the growth of some cancers, including
748 intestinal [103,104]. Depletion of Gly in the diet limits one route for Ser synthesis. Limiting Gly
749 and Ser may reduce the cancer's capability to cope with ROS. In addition, a combination of Gly
750 and Ser deprivation with radiation, a treatment inducing ROS may prove effective. Gly and Ser
751 deprivation limits single-carbon units for nucleotide biosynthesis and this might enhance the
752 effectiveness of drugs that target nucleotide synthesis.
753 Some amino acids had the ability to inhibit angiogenesis and thus reducing cancerogenesis [105].
754 Such activity was shown by arginine (Arg), contained in the examined lyophilized snail eggs, in
755 human colon cancer cells (SW480) and in colon cancer cells (SW480) xenograft mouse model.
756 Gly limited growth of cancer in model animals. Branched-chain amino acids (Ile, Leu, Val),
757 administered to obese, diabetic rats, showed the ability to reduce preneoplastic changes and
758 angiogenesis.
759 The ratios of EAA/TAA and EAA/NEAA for lyophilized *H. a. maxima* eggs were higher than for
760 *H. a. aspersa* eggs and these ratios were higher for the examined eggs than for the lyophilized
761 foot tissues and mucus of *H. a. aspersa* [7]. The effect of EAA and the mixture of amino acids
762 comprised 85% EAA and 15% NEAA on MCF 10A human breast epithelial cells and various
763 cancer cells (HCT 116, HeLa human cervical adenocarcinoma, Hep G2 human hepatocellular
764 carcinoma, Caco-2 and MCF7 human breast cancer cells) was examined [101]. Both EAA and
765 the mixture containing EAA and NEAA showed antioproliferative and cytotoxic activities,
766 including inhibition of proteasome activity and activation of autophagy and apoptosis. Changing
767 the ratio of EAA to NEAA may be an anticancer strategy leading to the selective cancer cell
768 death. Moreover, EAA administration, with/without the chemotherapeutic drug doxorubicin,
769 increased the mortality of various cancer cells (MCF7, HCT 116 and M14 human melanoma

770   cells) [106]. EAA increased the concentration of apoptotic markers. Higher EAA/NEAA ratio
771   may limit the survival of cancer cells and their proliferation.
772   The lyophilized *H. a. aspersa* eggs contained more vitamin D₃ than the lyophilized *H. a. maxima*
773   eggs.
774   Vitamin D₃ is produced mainly in the skin that is exposed to ultraviolet-B radiation of the sun,
775   but can also be acquired from diet and supplements [107]. In combination with vitamin D binding
776   protein in circulation, vitamin D₃ (provitamin cholecalciferol) moves to the liver and is
777   metabolized to 25-hydroxyvitamin D [25-hydroxycholecalciferol, calcidiol, calcifediol,
778   25(OH)D]. 25(OH)D is transported to the kidneys where it is metabolized to calcitriol
779   [1,25(OH)₂D] which is converted to metabolites with less activity.
780   Prevalence rates of severe deficiency of vitamin D ((25(OH)D <30 nmol/L)) of 5.9% (USA),
781   7.4% (Canada) and 13% (Europe) have been reported [108-110]. Moreover, 25(OH)D
782   concentrations <30 nmol/L are common in >20% of the inhabitants of Tunisia, India, Pakistan,
783   Afghanistan and Mongolia [111]. The prevalence of 25(OH)D concentrations <50 nmol/L
784   (deficiency) has been stated as 24% (USA), 37% (Canada) and 40% (Europe) [108-110].
785   It seems rational to suggest a vitamin D supplementation at a dose of 800–2000 IU/day (20-50
786   μg/day) [112].
787   Vitamin D and calcium affect additively incidence of colorectal adenoma, its malignant
788   transformation and progression [113]. Each 200 IU/day increase in the total intake of vitamin D
789   was connected with a 10% decrease in the colorectal adenoma risk and a 5% decrease in the
790   colorectal cancer risk. Each 400 mg/day increase in the total intake of calcium was connected
791   with a 2% decrease in the colorectal adenoma risk and a 5% decrease in the colorectal cancer
792   risk.
793   Calcitriol affects different colon cancer cell lines containing an adequate level of vitamin D
794   receptors (VDR) [114]. It inhibits proliferation of above cells through different mechanisms,
795   sensitizes them to apoptosis and promotes their differentiation. Calcitriol is an antagonist of the
796   Wnt/ß-catenin pathway which aberrant activation is the major cause of colorectal cancer
797   progression [115]. Its transcription-independent (non-genomic) activities, mediated by
798   extranuclear VDR and alternative receptors, have also been reported [114].
799   Calcitriol favors epithelial differentiation of colon cancer cells, as a result of influence on the
800   Wnt/ß-catenin pathway and gene regulatory effects [116,117].
801   Moreover, calcitriol affects the expression of cell differentiation regulators [114]. It induces
802   *CST5*/cystatin D expression, thereby increasing the production of an inhibitor of endosomal
803   cysteine proteases (cathepsins) [118]. In addition, cystatin D overexpression limits proliferation
804   and migration of colorectal cancer cells, but increases adhesion of cells to each other. Calcitriol
805   diminishes the expression of the epithelial phenotype repressor, Sprouty-2, in colorectal cancer
806   cells [119]. Calcitriol modulates the expression of histone H3 lysine-27 demethylase Jumonji C
807   domain-containing protein 3 (*JMJD3*) as well as other epigenetic regulators [120-123]. JMJD3
808   mediates the activities of calcitriol in colorectal cancer cells: increase of differentiation, decrease
809   of proliferation, gene regulation and Wnt/ß-catenin pathway antagonism.
810   Calcitriol sensitizes colon cancer cells to apoptosis induction through the increase of expression
811   of proapoptotic genes, the decrease of expression of survival genes and *via* interference with the
812   secretion of IL-1ß by macrophages [124]. VDR agonists enhance the action of chemotherapeutics
813   in animal and cell models of colorectal cancer [125].
814   Calcitriol inhibits the production of *DKK-4*, promoting chemoresistance, invasion and
815   angiogenesis in colon cancer cells [126,127]. Moreover, it modulates the angiogenic phenotype
816   [128,129].
817   Calcitriol modulates the expression of different microRNAs (miRs) of human colon cancer cell
818   line [130]. It was demonstrated that *miR*-22, induced in a VDR-dependent manner, decreases

819 proliferation, migration, EMT, invasion and growth of xenograft tumors in different cancer
820 systems including colorectal cancer [131-133].
821 Calcitriol inhibits two properties of cancer-associated fibroblasts (CAFs) from colorectal cancer
822 biopsies: the ability to alter the extracellular matrix (ECM) and the capacity to induce colon
823 cancer cell migration [134].
824 It is probable that calcitriol influences on colorectal cancer through immune cell regulation [135].
825 Vitamin D supplementation reduces inflammation in patients with colorectal adenoma [136]. The
826 influence of calcitriol on cytokines overexpressed in colorectal cancer patients is mediated by
827 inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)
828 transcription factor [137-139].
829 Calcitriol may potentiate antibody-dependent cellular cytotoxicity in colorectal cancer patients
830 receiving monoclonal antibody therapy [136,140,141].
831 Calcitriol influences on the detoxification processes in the intestine [142].
832 There is evidence of regulation between the gut microbiota and VDR/vitamin D status [143].
833 In the VITAL clinical trial, cholecalciferol (2000 IU/day), administered for over 5 years, did not
834 decrease the risk of colorectal cancer [144,145]. In another trial, vitamin D_3 (1000 IU/day),
835 administered for 3-5 years, did not decrease adenoma recurrence [144,146].
836 The large population study – ViDA [147,148] showed that supplementation with vitamin D_3 did
837 not influence on any cancer incidence.
838 The SUNSHINE trial investigated vitamin D_3 supplementation in patients undergoing
839 chemotherapy with unresectable advanced and metastatic colorectal cancer [144,149].
840 Participants receiving high-dose vitamin D_3 (firstly 8000 IU/day during 2 weeks and then 4000
841 IU/day), in comparison to the standard dose (400 IU/day), experienced a 2-month increase
842 (statistically nonsignificant) in median progression-free survival. In adjusted analyses,
843 participants receiving high-dose experienced progression and death less frequently during the
844 22.9 month-median follow-up.
845 The AMATERASU trial included participants with luminal gastrointestinal cancer, stage I to III,
846 after tumor resection [144,150]. Patients were supplemented with vitamin D_3 (2000 IU/day) or
847 placebo and a post hoc analysis showed a significant benefit from supplementation.
848 The content of Ca in the lyophilized eggs of *H. a. maxima* and *H. a. aspersa* was about 17 times
849 higher than in the lyophilized foot tissues of *H. a. aspersa* and more than 1.5 times higher than in
850 the lyophilized mucus of this subspecies [7]. The tested egg lyophilizates comprised about 2
851 times less P than the lyophilizate from *H. a. aspersa* foot tissues, while they were over 2.5 times
852 richer in this element compared to the lyophilizate from the mucus of these snails [7]. It was
853 shown that Ca possesses properties against colorectal cancer, limits proliferation, stimulates
854 differentiation and induces apoptosis [151]. Furthermore, Ca and P demonstrated a protective
855 effect at different adenoma-carcinoma sequence steps [152]. In turn, Arnst and Beck Jr showed
856 that an excess of inorganic phosphate can influence and promote the cancer phenotype [153].
857 Higher Ca, K, Mg, Mn, Zn, Se and I intakes and lower P, Na, Cu and Fe intakes may be
858 connected with lower colorectal cancer risk [154,155]. The action of the first group of elements
859 may be related to their antioxidative and other anticarcinogenic effects and the action of the
860 second group of elements - to prooxidative and other procarcinogenic effects. The concentration
861 of Na in lyophilized snail eggs was about 4 times lower than in the lyophilized foot tissues of *H.
862 a. aspersa* and several dozen times lower than in their lyophilized mucus [7]. The WHO
863 recommends a consumption of Na <2 g/day for adults [156]. As for other macroelements, the
864 content of K and Mg in lyophilized eggs turned out to be several - a dozen times lower than in the
865 lyophilized foot tissues and mucus of *H. a. aspersa*, and the content of S and Cl – several – a
866 dozen times lower than in lyophilized foot tissues of these snails [7]. The WHO recommends the
867 intake of K for adults in an amount of at least 3.51 g/day [157]. Recommended nutrient intake

868   (RNI) for Mg for males (19–65 years) is 260 mg/day and for females (19–65 years) - 220 mg/day
869   [158]. Mg and 25(OH)D may act synergistically in reducing the all-cause mortality risk in
870   colorectal cancer patients [159]. Mg plays the crucial role in biochemical reactions involved in
871   vitamin D synthesis and metabolism. In addition, every 100 mg/day increase in Mg intake was
872   connected with 13% lower colorectal adenoma risk and 12% lower colorectal tumor risk.
873   Moreover, Mg deficiency and high Ca:Mg intake have been linked to a higher colon cancer
874   incidence and mortality. Ca:Mg >2.6–2.8 may have a negative influence on colorectal adenoma
875   outcomes and Ca:Mg <1.7 may have negative consequences, and were connected with an
876   increased total mortality risk [160]. Mg deficiency may increase intracellular Ca concentration
877   promoting ROS generation and it may blunt antioxidant capacity to promote oxidative stress
878   [159]. Cell cultures and animal studies suggest Mg role in carcinogenesis through affecting cell
879   proliferation, apoptosis, differentiation, angiogenesis, inflammation and innate immunity [161].
880   In turn, Fujii et al. showed that in colorectal cancer hypomagnesemia is a predictor of the
881   effectiveness of therapy based on anti-epidermal growth factor receptor antibodies [162]. One
882   study showed the capability of S to induce apoptosis of non-small cell lung carcinoma cells that
883   are resistant to drugs [163]. Moreover, S is an element of non-enzymatic antioxidants, cancer
884   therapeutics [164]. Excess Cl by reaction with water and mineral compounds, creates
885   trihalomethane, which causes cancer [165].
886   The concentration of Cu in the lyophilized *H. a. maxima* eggs was slightly higher compared to
887   the lyophilized *H. a. aspersa* foot tissues, and in lyophilized *H. a. aspersa* eggs – slightly lower
888   [7]. Other authors showed that Cu was cytotoxic to HT-29 cells, which was connected with
889   activation of apoptosis, increased oxidative stress, alterations in β-oxidation in mitochondria and
890   changes in lipid and energy metabolism [166]. It was also demonstrated that Cu exerted
891   toxicological effect on Caco-2 cells [167]. Research on rats indicated that low intake of Cu is a
892   risk factor for the development of colon tumor, induced by 3,2'-dimethyl-4-aminobiphenyl and
893   reduced the activities of Cu,Zn-superoxide dismutase (SOD) and ceruloplasmin [168]. According
894   to other authors, progression to colon cancer in rats was related to low Zn concentration and
895   lower Cu,Zn-SOD activity in blood plasma [169]. Zn modulates folding and misfolding of p53
896   which is associated with cancer [170]. According to others, higher Cu and Zn in blood serum
897   were associated with increased risk of colorectal cancer development. Se, selenoprotein P and Se
898   to Cu ratio were connected with decreased risk [171]. In turn, Cu metabolism may be a promising
899   target for colorectal cancers harboring KRAS mutations [172].
900   The level of Ni in lyophilized *H. a. aspersa* eggs turned out to be slightly lower compared to
901   lyophilized *H. a. aspersa* foot tissues, and in lyophilized *H. a. maxima* eggs – higher [7]. Contact
902   with Ni can cause different cancers and oxidative stress and mitochondrial dysfunctions can have
903   a crucial role in its toxicity [173]. Epigenetic changes induced by Ni exposure can disrupt the
904   genome. Besides, Ni compounds can induce apoptosis in different cancer and normal cells. It was
905   demonstrated that Ni lowers the activity of different enzymes.
906   The Fe content in lyophilized eggs of both snail subspecies was lower compared to that in
907   lyophilized foot tissues and mucus of *H. a. aspersa* [7]. RNI for Fe is 19.6-58.8 mg/day for males
908   (18+) and 9.1-27.4 mg/day for females (18+), and is dependent on its bioavailability [158].
909   An excess of intestinal Fe increases the risk of developing colorectal cancer by increasing the
910   proliferation of cancer cells, contributing to colon damage induced by oxidative stress and
911   enhancing oncogenic signaling [174]. Research in rodents showed that increased dietary Fe
912   enhances colonic crypt cell proliferation and colorectal tumor development [174-176]. Fe may be
913   able to produce ROS that increase oxidative stress, which causes DNA damage, protein
914   modification and lipid peroxidation [174]. Excess oxidative stress induced by Fe can result in
915   colonic inflammation which can lead to colorectal cancer [174,177]. In turn, Fe deficiency is
916   widespread in colorectal cancer patients [174]. It can cause anemia, by limiting hematopoiesis,

917 and attenuate immune response. This may result in a diminished immunosurveillance response
918 and changed tumor immune microenvironment, which may potentially lead to cancer progression
919 [174,178]. However, Bird et al. showed a U-shaped connection between Fe intake and
920 adenomatous polyps, demonstrating that people who ingest low (<11.6 mg/day) or high (>27.3
921 mg/day) Fe amounts had increased colorectal cancer risk, in comparison with people consuming
922 an adequate Fe amount [174,179].
923 Intake of Fe from white meat and plants, containing less heme Fe than red meat, were inversely
924 connected with colorectal cancer risk [180]. Other authors demonstrated positive and negative
925 associations between total dietary Fe and colorectal cancer risk.
926 In the colon, heme Fe is converted into the cytotoxic heme factor which causes damage to the
927 surface epithelial cells as well as reactive epithelial hyperproliferation [181,182]. Sulfate-
928 reducing and mucin-degrading bacteria intensify these actions. Colon damage is enhanced by
929 ROS production induced by heme Fe, which favors DNA, lipid and protein oxidation [181]. In
930 animal models, heme contributes to carcinogenesis by inhibiting apoptosis and exfoliation of
931 colonocytes [181,183]. Heme Fe increases the generation of N-nitroso compounds [181,184].
932 Nitrosamines, produced from the nitrites by the intestinal microflora, are carcinogenic [181].
933 Lipid peroxidation also contributes to colorectal cancer and is enhanced by heme Fe due to the
934 catalytic effect on production of aldehydes by bacteria, which in turn enhances genotoxicity.
935 The concentration of Mn in the examined lyophilized snail eggs was higher than in the
936 lyophilized *H. a. aspersa* mucus [7]. The mitochondrial Mn-SOD inhibited growth of different
937 cancer cells [185]. According to other authors, overexpression of Mn-SOD decreased HCT 116
938 cell growth by inducing cell senescence [186]. The rats that were administered low Mn in a diet
939 had 23% larger preneoplastic lesions and rats ingesting high Fe in a diet - 18% higher [187].
940 The concentration of Cr in lyophilized *H. a. maxima* eggs was higher than in lyophilized *H. a.*
941 *aspersa* foot tissues and mucus, and in lyophilized *H. a. aspersa* eggs - lower [7]. Cr (III)
942 ameliorated the healing of colitis in mice by promoting antioxidant potential, suppressing ROS
943 and inflammation [188]. In turn, Cr (VI) was cytotoxic to both cancer cells (Caco-2, HeLa, Hep2
944 human cervix carcinoma and MCF-7) and normal HEK293 human kidney (embryonic) cells
945 [189]. Chronic administration of high doses of Cr (VI) elicited gastrointestinal cancers in mice
946 [190]. Moreover, Zhang et al. demonstrated that long-term exposure of mice to Cr(VI) promotes
947 malignant tumor formation, facilitates colorectal cancer [191].
948 The Mo content in the lyophilized *H. a. maxima* eggs was higher than in the lyophilized foot
949 tissues and mucus of *H. a. aspersa*, and in the lyophilized *H. a. aspersa* eggs – lower [7]. The
950 incidence and development of induced esophageal tumors were lower in rats which were
951 administered high-Mo diet compared to animals which were fed low-Mo diet [192]. Xanthine
952 oxidase could have played an important function in inhibiting esophageal carcinogenesis caused
953 by Mo.
954 The level of B in lyophilized eggs of both snail subspecies was lower than in lyophilized *H. a.*
955 *aspersa* foot tissues and mucus [7]. Low-B diet could increase cancer risk [193]. The
956 mechanisms by which this element may affect cancer are unknown, however, evidence suggests
957 that B possesses antioxidant and anti-inflammatory activities. Possible mechanisms comprise
958 inhibition of serine protease enzymatic activity, dehydrogenase, modification of mRNA, cell
959 division and apoptosis induction.
960 The content of Zn in lyophilized eggs was lower compared to lyophilized *H. a. aspersa* foot
961 tissues and mucus [7]. RNI for Zn for males (19-65 years) is 4.2-14.0 mg/day and for females
962 (19-65 years) is 3.0-9.8 mg/day, and is dependent on its bioavailability [158]. Zn plays an
963 important role in the immune response [194]. However, excess Zn may show immunosuppressive
964 effects. Zn stabilizes the structure and regulates NF-κB and other transcription factors. NF-κB
965 induces the expression of various genes related to cell proliferation, apoptosis inhibition,

966   angiogenesis, resistance to chemotherapeutics, metastasis, promotes tumor formation and
967   enhances inflammatory reactions. Activation of NF-κB can be blocked by affecting redox status
968   of cells, the application of competitive inhibitors binding to its DNA site, such as Zn or Cr.
969   However, Zn can both inhibit and induce NF-κB. Zn could protect healthy cells from the
970   cytotoxic and genotoxic effects of H_2O_2, but enhances its toxicity in tumor tissue [194,195]. Zn,
971   as an element of CuZn-SOD, has a strong antioxidant potential [194]. CuZn-SOD and Mn-SOD
972   affects tumor formation and development [194,196,197]. Another mechanism of the antioxidant
973   activity of Zn comprises its antagonism against minerals participating in lipid peroxidation (Fe
974   and Cu) [194]. Zn also protects the protein -SH groups from oxidation by chelate formation. Zn
975   ions play an important role in the induction of metallothioneins. Their antioxidant, detoxifying,
976   regenerating and angiogenic activities can contribute to cancer development, while their anti-
977   inflammatory activity can inhibit the cancer. Zn stabilizes the zinc finger structures, which play
978   important functions in DNA replication and repair regulation, transcription, translation,
979   proliferation and maturation of cells, and apoptosis. Related to Zn deficiency, disorders in the
980   integrity of the genome, inefficient DNA repair mechanisms, loss of mechanisms that control the
981   function of DNA may result in a higher cancer initiation and progression risk [194,195]. Zn is
982   involved in many processes related to gene expression and stabilization, plays a major role in the
983   growth and division of cells and programmed death [194]. Zn ions are linked to the histone
984   deacetylases and their inhbitors. Zn has multidirectional role in the initiation and inhibition of
985   apoptosis. Endogenous Zn is likely indispensable in autophagy induction, under oxidative stress.
986   A meta-analysis of six studies showed that higher Zn intake was associated with reduced
987   colorectal cancer risk [198]. Rat studies suggest that Zn deficiency contributes to colorectal
988   cancer development and progression, linking low Zn concentration in the intestine to pre-
989   neoplastic lesion development and colon carcinogenesis [199]. Application of Zn to rats treated
990   with dimethylhydrazine (DMH) reduced tumor incidence, size and multipilicity [200].
991   Furthermore, increased Zn concentrations inhibited growth of cells that represent various colon
992   cancer stages: HCT-116, HT-29 and SW620 and induced their death [201]. The main mechanism
993   turned out to be oxidative stress that activated stress kinase signaling, mitochondria disorders and
994   plasma membrane damage. Cell death was cell line dependent; the cells showed signs of
995   apoptosis, necrosis, autophagy and mixed types of cell death.
996   The concentration of Co in lyophilized eggs of *H. a. maxima* and *H. a. aspersa* was higher in
997   comparison with lyophilized *H. a. aspersa* foot tissues and mucus [7]. High Co concentration in
998   consumed rice may be associated with an increased colon cancer risk and high Se concentration
999   with decreased risk [202].
1000   V was also present in the lyophilized eggs of both snail subspecies. Vanadium N-(2-hydroxy
1001   acetophenone) glycinate demonstrated cytotoxic action on human colorectal carcinoma, human
1002   T-cell acute lymphoblastic leukemia, human breast cancer and human astrocytoma cells, without
1003   toxic influence on normal fibroblasts [203]. It induced apoptosis of human colorectal carcinoma
1004   cells by mechanisms including DNA and mitochondrial damage, and higher ROS level.
1005   The content of Se in lyophilized snail eggs was lower compared to lyophilized *H. a. aspersa* foot
1006   tissues and mucus [7]. RNI for Se for males (19–65 years) is 34 µg/day and for females (19-65
1007   years) is 26 µg/day [158].
1008   Se is an element of selenocysteine which is part of selenoproteins (SELENO), as glutathione
1009   peroxidase (GPX) and thioredoxin reductase (TXNRD) [204]. Their important functions include
1010   maintaining cellular redox homeostasis. Other selenoproteins, as SELENOF and SELENOS, in
1011   turn, participate in the folding and degradation of proteins. There was an inverse correlation
1012   between Se intake and colorectal cancer mortality [204,205]. Elevated SELENOP level was
1013   inversely correlated with colorectal cancer risk [204,206]. It seems that suboptimal Se
1014   concentration is a cancer risk factor [204,207]. The mechanism of Se's anticancer activity may be

1015 increased expression of selenoproteins, which prevent oxidative damage to DNA [204]. GPX2 is
1016 believed to protect healthy intestinal stem cells from DNA damage. Intestinal tumors in GPX3
1017 knockout mice had higher concentration of nuclear β-catenin compared to wild-type tumors
1018 [204,208]. SELENOH downregulated colorectal cancer cells showed increased growth properties
1019 [204,209], and loss of GPX2 [204,210] and SELENOF [204,211] impaired growth of colorectal
1020 cancer cells. The probability of relapse-free survival in colorectal cancer patients was lower in the
1021 case of high expression of GPX2 in the tumor [204,210]. The same relationship was noted for
1022 high content of intratumoral SELENOF [204,212].
1023 The studies demonstrated the anticancer potential of dietary Se by inhibiting the tumor growth of
1024 colon cancer cells [213-216]. Its chemopreventive effect could be triggered by various molecular
1025 mechanisms: antioxidant protection, cell cycle suppression, apoptosis induction and protein
1026 structural modification [213,217]. A high intake of dietary Se (2.29 μg/g), included in diets
1027 supplemented with selenized chickpea sprouts, containing or not containing isoflavonoids,
1028 reduced the growth of tumor of HT-29 colon cancer cells transplanted into immunosuppressed
1029 mice and induced cancer cell apoptosis [213]. Se was hypothesized to decrease lipid oxidation via
1030 GPX.
1031 The effect of Se on colorectal cancer in mice, induced by 1,2-dimethylhydrazine, was mediated
1032 by its influence on increasing oxidative stress, MDA concentration and apoptosis, and reducing
1033 angiogenesis [218]. Depending on the concentration, Se can act as an antioxidant and a pro-
1034 oxidant in different experimental conditions [218-220].
1035 The content of Ca, Mg and Cu in the lyophilized eggs of *H. a. maxima* and *H. a. aspersa* was
1036 higher than the content of these elements in the dry matter of *H. a. aspersa* eggs in the Beeby and
1037 Richmond study [221]. The authors also detected the presence of Zn in these eggs. The
1038 concentration of Zn and Mn in the lyophilized eggs of *H. a. maxima* and *H. a. aspersa* also
1039 turned out to be higher compared to the concentration of these minerals in the dry matter of *H. a.*
1040 *maxima* eggs in the study of Górka et al. [58], and the content of Ca, Mg, Cu, Fe and Cr in the
1041 tested lyophilized snail eggs was similar to the content in the dry matter of *H. a. maxima* eggs.
1042 The extract from *H. a. maxima* eggs (25, 2.5×10^{-1} and 2.5×10^{-4} mg/mL) and the extract from
1043 *H. a. aspersa* eggs (25, 2.5×10^{-1} and 2.5×10^{-3} mg/mL) decreased the viability of Caco-2 cells
1044 after 24 h of treatment. Cell viability was reduced by fraction of an extract from *H. a. maxima*
1045 eggs containing particles of a molecular weight <3 kDa (1.25 and 0.125 mg/mL) and fraction of
1046 an extract from *H. a. aspersa* eggs comprising particles of the same molecular weight, at the
1047 same concentrations, but the effect was not statistically significant in case of the second extract.
1048 The extracts from eggs of both snail subspecies (25 mg/mL), after this treatment time, also
1049 reduced the percentage of live Caco-2 cells. In addition, the extract from *H. a. maxima* eggs (25,
1050 25×10^{-1} and 25×10^{-3} mg/mL) and the extract from *H. a. aspersa* eggs (25×10^{-1} mg/mL)
1051 increased the degree of damage to Caco-2 cell membranes. The extracts from the eggs of both
1052 snail subspecies (2.5 mg/mL), after 24 h of treatment, increased the amount of lipid peroxidation
1053 products in cells, but this effect turned out to be statistically insignificant. The extracts from eggs
1054 (25 mg/mL) caused the induction of apoptosis and reduction of necrosis - an increase in the
1055 percentage of early apoptotic cells (effect statistically insignificant for *H. a. maxima* extract) and
1056 a decrease in the percentage of necrotic cells (effect statistically insignificant). The effect of
1057 extracts from snail eggs on the cell viability and the induction of apoptosis may be assigned to the
1058 presence of antioxidants. The phenols contained in the extracts may have shown a prooxidative
1059 activity, influenced on the greater degree of lipid peroxidation in the cells treated with these
1060 extracts. The potential cytotoxic activity of phenols, related to the production of ROS may have
1061 played an important role in activating apoptosis. Lipid peroxidation products may have also been
1062 responsible for the cytotoxic, prooxidative and consequent proapoptotic effects of extracts on
1063 Caco-2 cells. Decreased cell viability may be attributed to anticancer peptides, especially those of

1064 a molecular weight <3 kDa, which requires further research. The glycoproteins agglutinins,
1065 belonging to the H-type lectins family, present in the extracts may have bound to the surface of
1066 cancer cells and facilitated the delivery of other compounds to them, which also requires further
1067 studies. The restriction of Met was most likely associated with the reduction of cell viability.
1068 Moreover, the appropriate ratio of EAA to NEAA may have also been responsible for limiting
1069 the cell viability by the extracts, their cytotoxic and proapoptotic effects. Furthermore, vitamin D
1070 may have contributed to the reduction of cell viability and apoptosis. Additionally, Mg may have
1071 had a positive effect on the synthesis and metabolism of vitamin D. Ca may have been
1072 responsible for limiting viability and inducing apoptosis. S may have contributed to the induction
1073 of apoptosis by the extracts. The cytotoxic effect on cells, related to the activation of apoptosis
1074 and the increase of oxidative stress, may have been demonstrated by Cu. Caco-2 cell growth may
1075 have been inhibited by Mn, the component of mitochondrial Mn-SOD. Zn may have been
1076 responsible for limiting cell viability. It may have contributed to the induction of oxidative stress,
1077 causing mitochondria disorders and damage to the cell membranes, to the activation of apoptosis.
1078 In turn, Se present in extracts may have increased oxidative stress, the degree of lipid
1079 peroxidation and apoptosis. Other bioactive compounds contained in the extracts and their
1080 additive and synergistic effects most likely also influenced Caco-2 cells.
1081

1082 ## Conclusions
1083 Extracts from lyophilized eggs of *H. a. maxima* and *H. a. aspersa* snails, due to the content of
1084 antioxidants which are necessary in prevention of the formation and inhibition of the activity of
1085 ROS, are able to reduce oxidative stress in the body and may be beneficial in the initiation and
1086 progression of colorectal cancer. Reduction of the viability of Caco-2 colon cancer cells after
1087 application of snail egg extracts, increase of the degree of damage to cell membranes, the amount
1088 of lipid peroxidation products generated by cells, induction of apoptosis and reduction of necrosis
1089 may be attributed to the presence of antioxidants, phenols, lipid peroxidation products, anticancer
1090 peptides, restriction of Met, appropriate ratio of EAA to NEAA, vitamin D, Ca, Mg, S, Cu, Mn,
1091 Zn and Se in them. Other bioactive compounds and their additive and synergistic effects most
1092 likely also influenced Caco-2 cells. Differences in the effects on cells of the extracts from eggs of
1093 two snail subspecies may result from differences in the content of bioactive compounds in them.
1094 It is planned to examine the composition of extracts and fractions containing molecules <3 kDa
1095 in terms of the content of peptides with anticancer properties, using mass spectrometry and
1096 bioinformatics tools, as well as other elements of the chemical composition of the extracts. It is
1097 planned to investigate the effect of extracts and <3 kDa fractions on cell metabolome and to
1098 examine other mechanisms of their anticancer activity, and to deepen research on their impact on
1099 cell death (apoptosis, necrosis, autophagy).
1100 Natural extracts from snail eggs or the chemical compounds contained in them might be used in
1101 the combination therapy of colorectal cancer, targeting many signaling pathways and using a
1102 variety of mechanisms to decrease the development of anticancer drug resistance. However, it
1103 should be determined whether the extracts/chemical compounds contained in them may sensitize
1104 to cytotoxic therapy, intensify the effective concentration of a drug, enhance the combined effects
1105 of both therapeutics or exert a cytotoxic effect specifically on cancer cells.
1106

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1109

1110 ## References

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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$\cdot^+$ - 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$^{\cdot+}$ scavenging activity (mg TROLOX/ g)	DPPH$\cdot$ 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.

1**Table 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*.

Panel (a) – molecular weights of standard proteins (Sigma-Aldrich, St. Louis, MO, USA); panel (b) – extract from eggs of *H. aspersa maxima* and panel (c) – extract from eggs of *H. aspersa aspersa*.

(A)

(B)
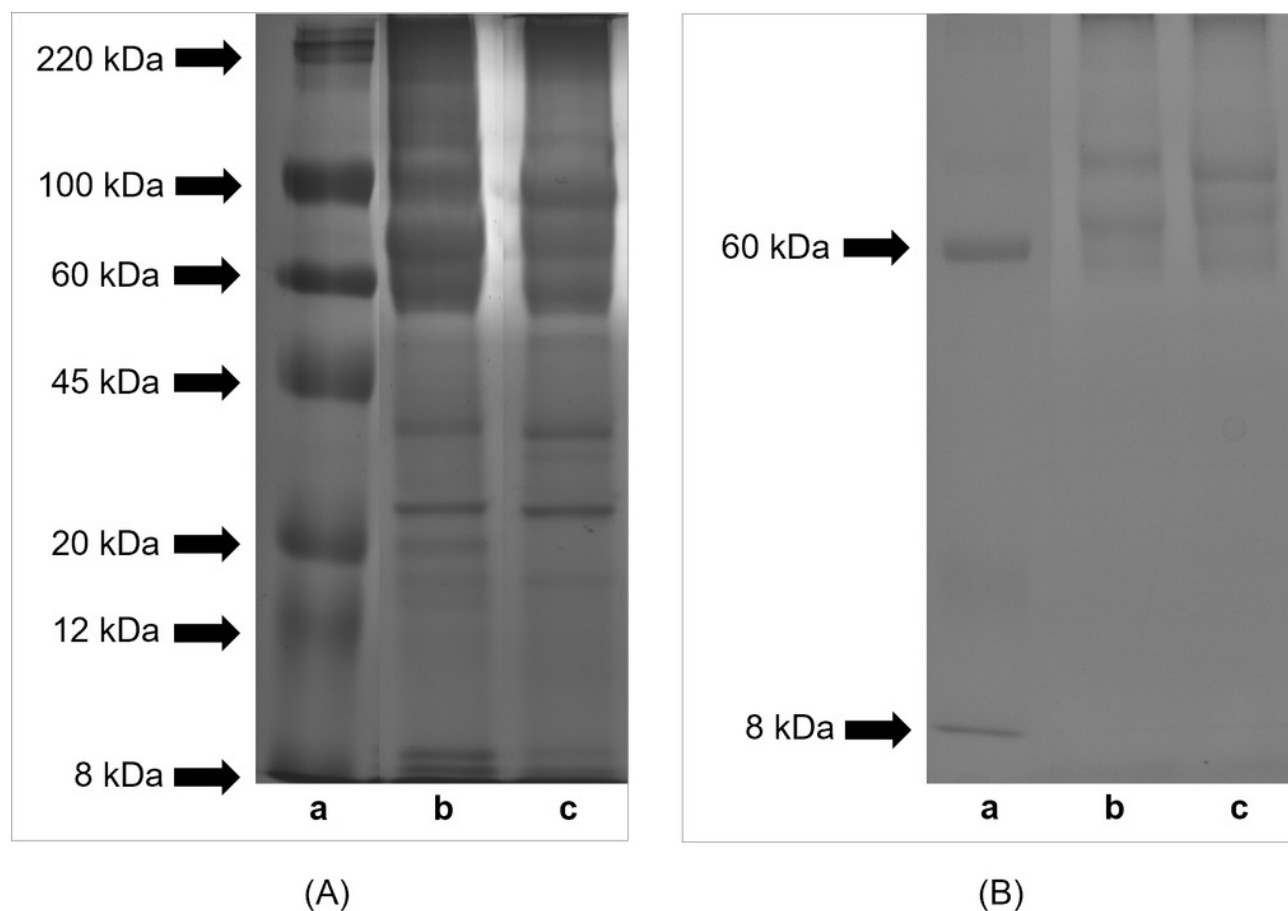
Figure 2

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 $p < 0.001$. $n = 6$.
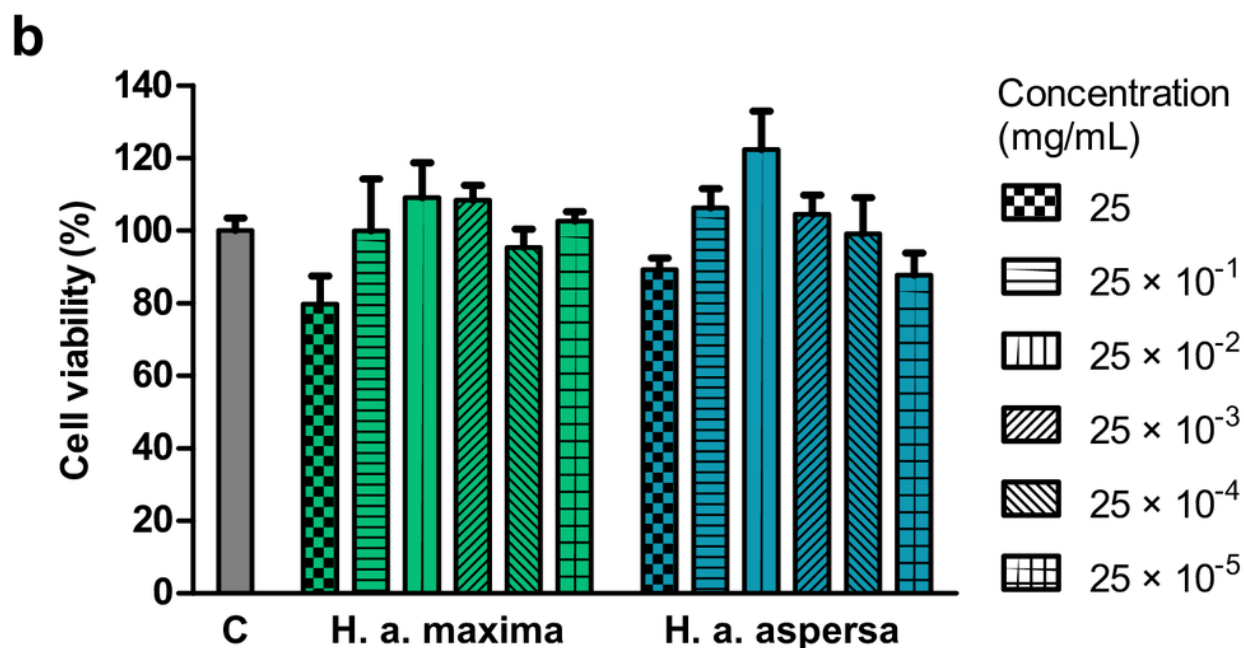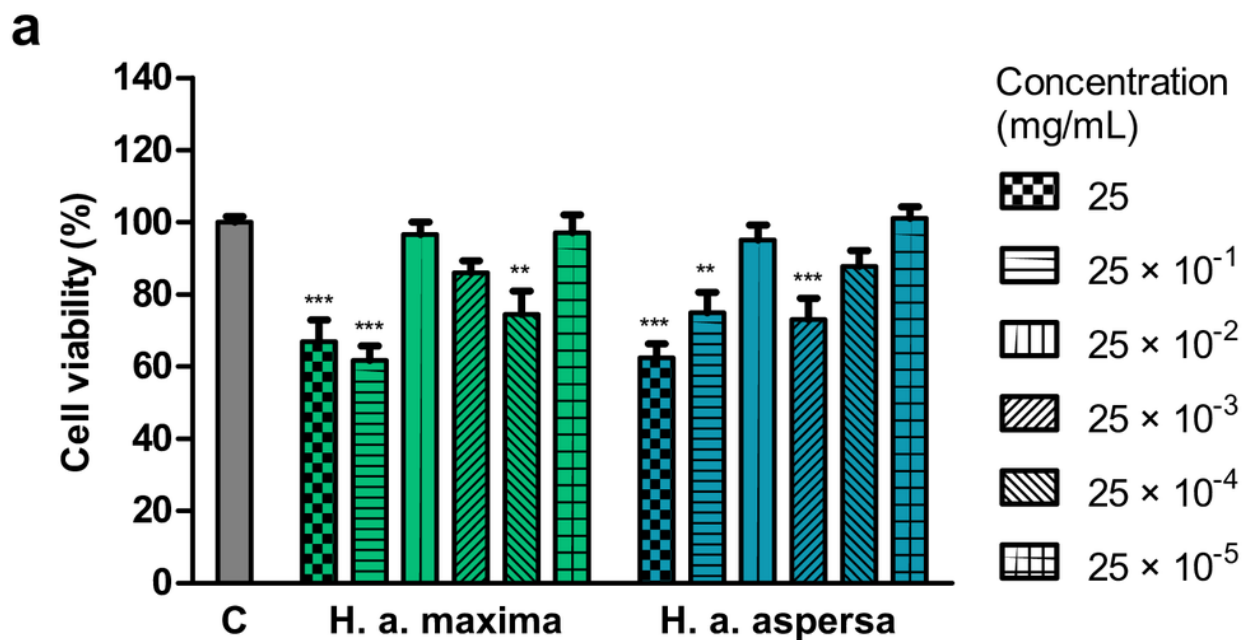
Figure 3

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 $p < 0.01$. $n = 4$.
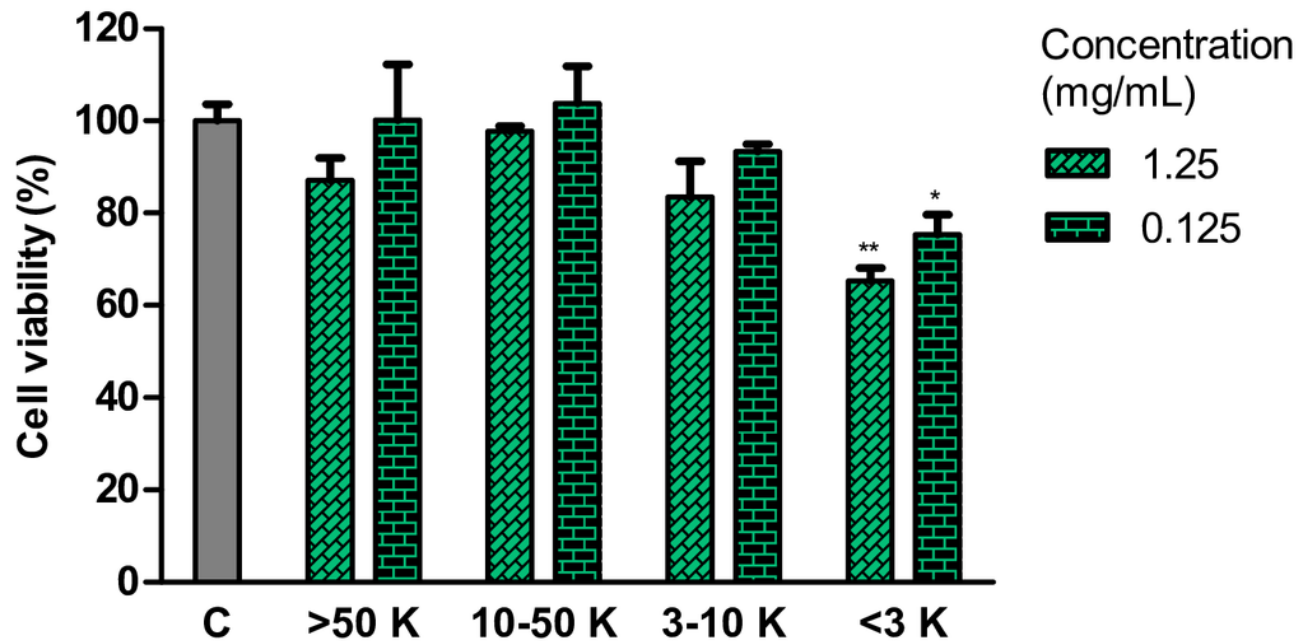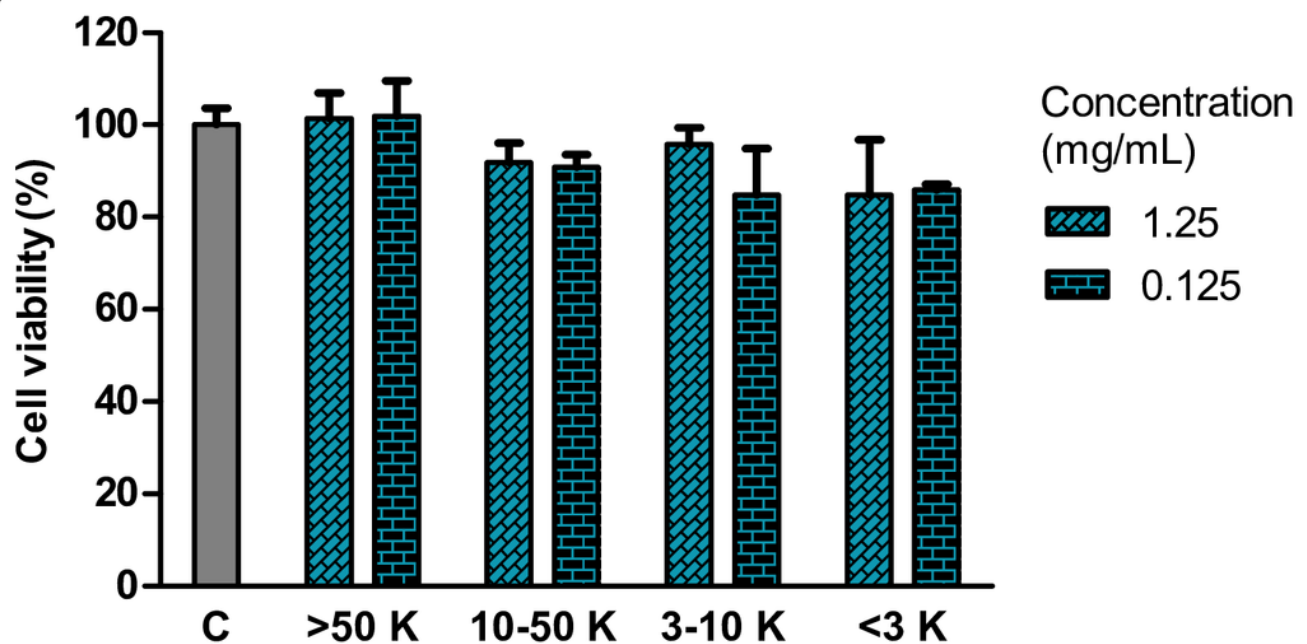
Figure 4

Percentage of live and dead Caco-2 cells after treatment for (a) 24 h and (b) 72 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 $p < 0.05$, ** represents values that differ from control at $p < 0.01$. $n = 5$.
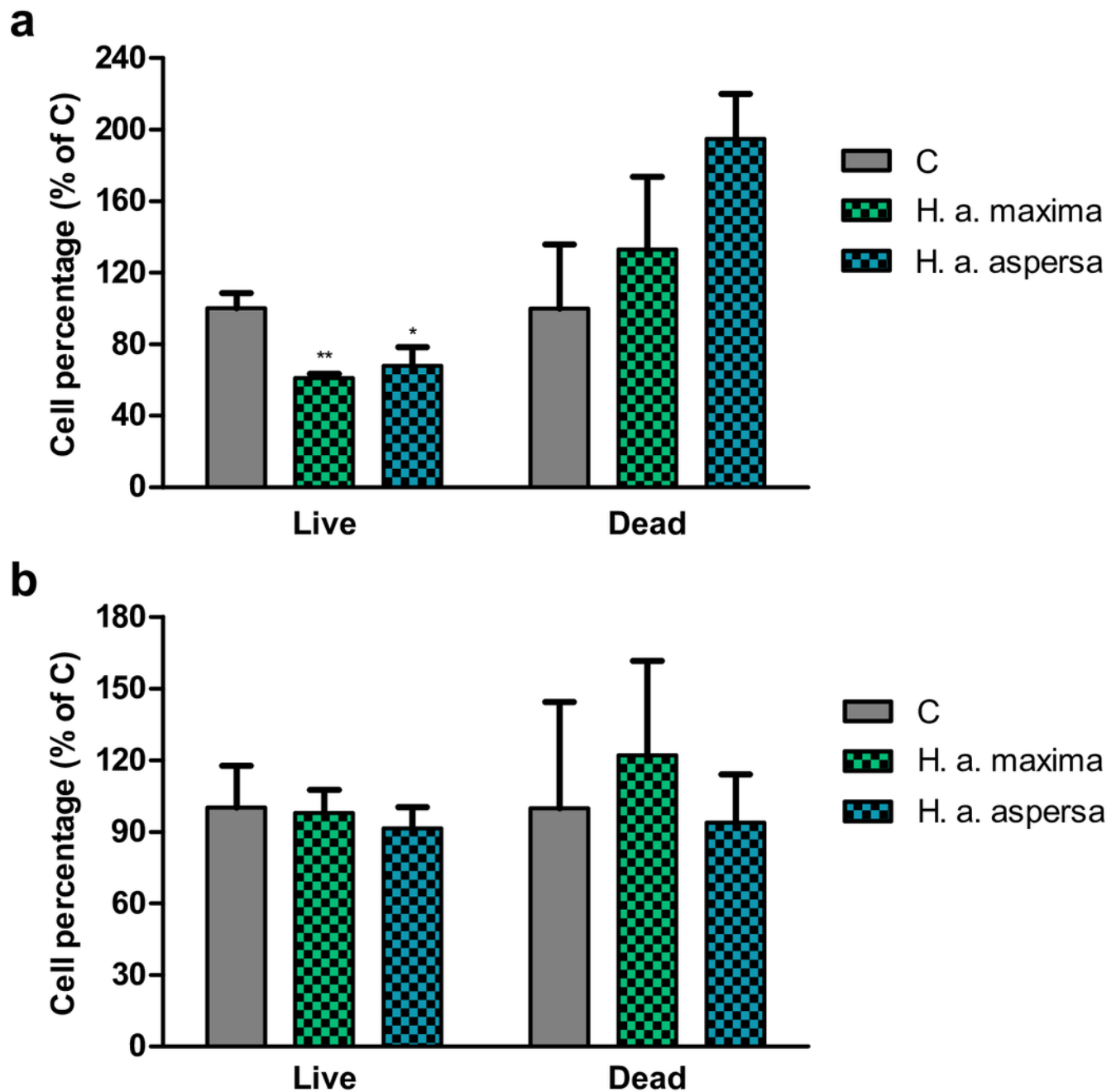
Figure 5

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 $p < 0.01$. $n = 4$.
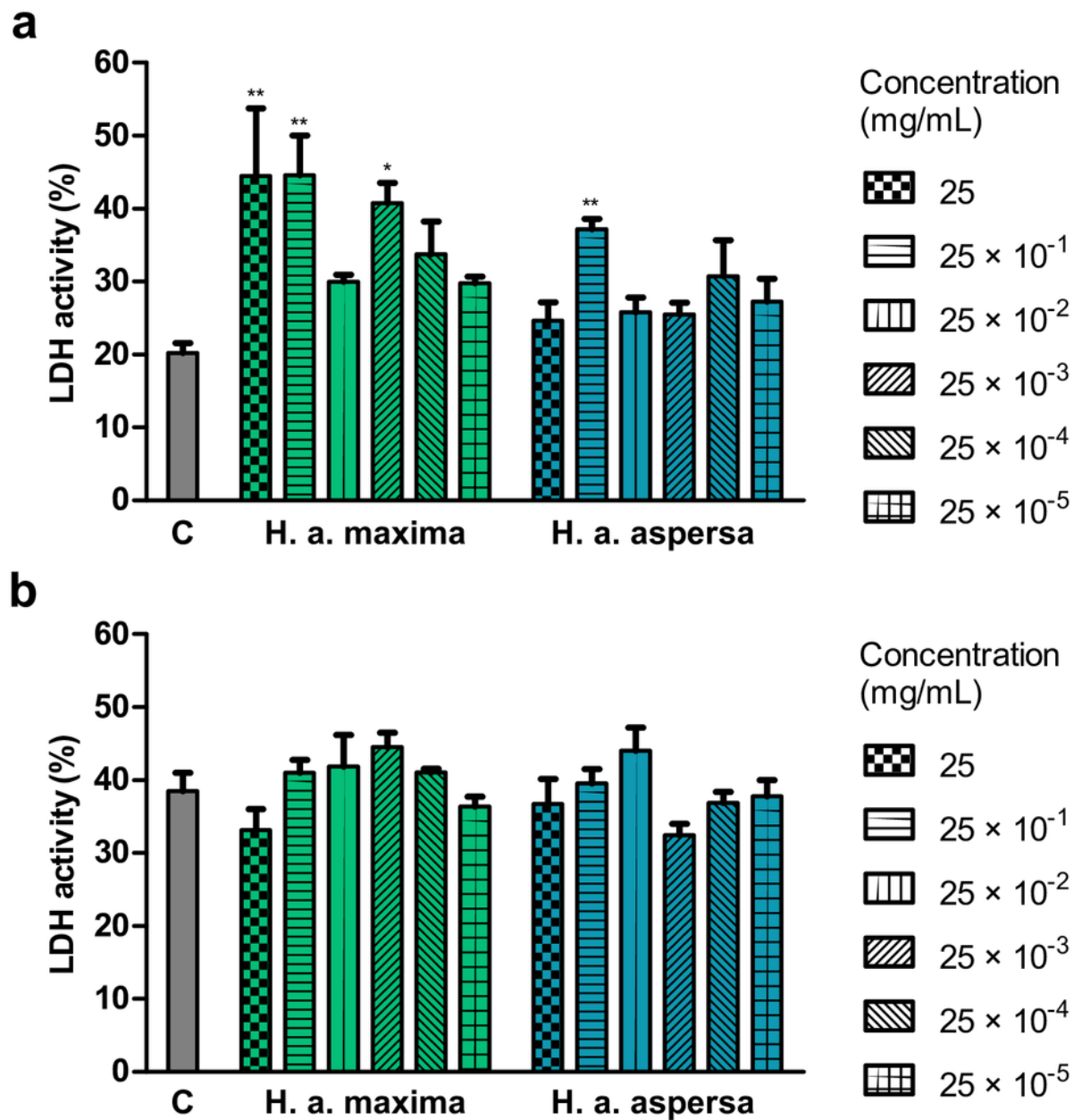
Figure 6

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.
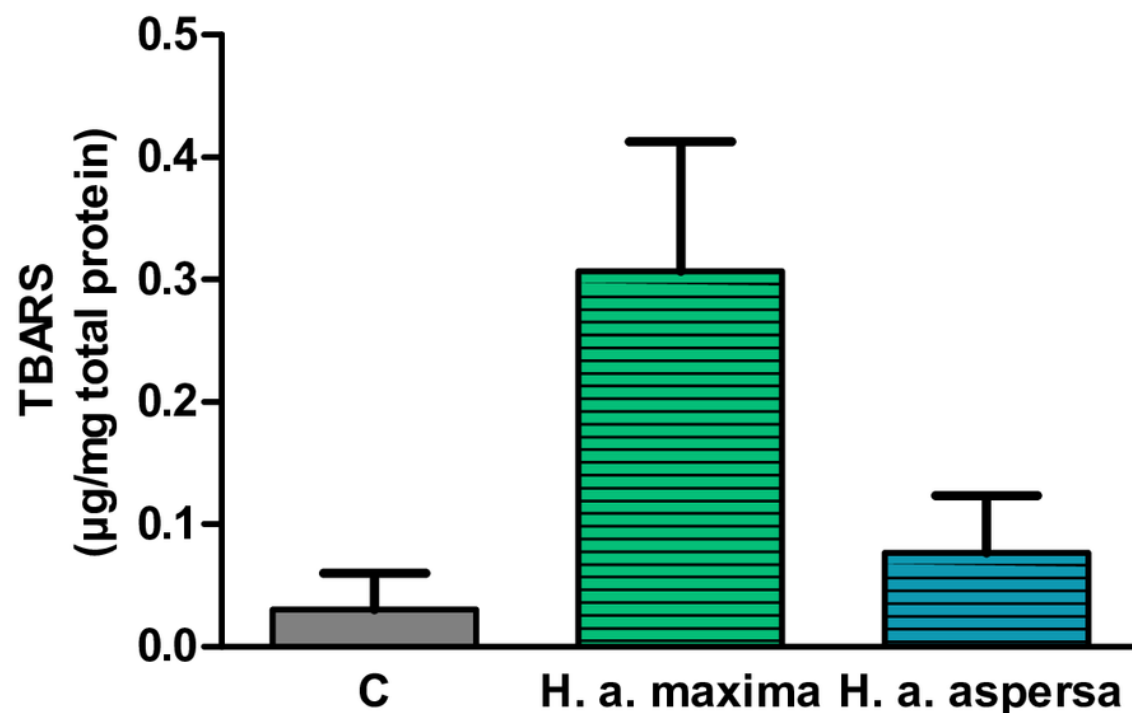
Figure 7

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

***