© 2021 Journal of Pharmacy & Pharmacognosy Research, 9 (2), 143-152, 2021
ISSN 0719-4250
http://jppres.com/jppres

Original Article

Antioxidant activity of fractions isolated from hemolymph of garden snail *Helix lucorum*

[Actividad antioxidante de fracciones aisladas de hemolinfa del caracol común de jardín *Helix lucorum*]

Albena Alexandrova[1,3*], Lubomir Petrov[1,3], Lyudmila Velkova[2], Aleksander Dolashki[2], Elina Tsvetanova[3], Almira Georgieva[3], Pavlina Dolashka[2]

[1]Department of Physiology and Biochemistry, National Sports Academy, 21, Acad. Stefan Mladenov, str., 1700 Sofia, Bulgaria.
[2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 9, Georgi Bonchev str., 1113 Sofia, Bulgaria.
[3]Laboratory of Free Radical Processes, Institute of Neurobiology, Bulgarian Academy of Sciences, 23, Georgi Bonchev str., 1113 Sofia, Bulgaria.
*E-mail: a_alexandrova_bas@yahoo.com

Abstract

Context: The snail hemolymph is a multi-component mixture comprising various substances with pharmacological activity.

Aims: To evaluate the antioxidant activity of hemocyanin and fractions isolated of hemolymph from garden snail *Helix lucorum*.

Methods: The antioxidant activity was tested in chemical systems generating superoxide radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$) and was presented as percent inhibition. The fractions' composition was analyzed by MALDI-TOF/TOF mass spectrometry.

Results: At concentration of 90 µg/mL the hemolymph fraction with compounds with molecular weight (MW) <100kDa demonstrated the strongest $O_2^{\bullet-}$- scavenger effect (82%), followed by the hemocyanin (58%), and hemolymph fraction with compounds with MW<1kDa (15%). The most powerful $^{\bullet}OH$ scavenger effect at concentration of 90 µg/mL showed hemocyanin (43%) followed by MW<100kDa fraction (26%). No $^{\bullet}OH$ scavenger effect was observed by the MW<1kDa fraction. The highest chelation properties (55%) were demonstrated by the MW<100kDa fraction at concentration of 130 µg/mL; both the hemocyanin and MW<1kDa hemolymph fraction showed 33% inhibitory effect at the same concentration. The analysis of the composition of the tested fractions revealed high content of hydrophobic surfaces in MW<1kDa fraction and presence of antioxidant enzymes-catalase, superoxide dismutase and glutathione peroxidase in MW<100kDa fraction that contribute to the overall antioxidant effect of *H. lucorum* hemolymph.

Conclusions: The complex antioxidant effect of hemolymph from *H. lucorum* is thought to be related to the presence of low molecular weight peptides in the MW<1 kDa fraction including glutathione and mainly to the antioxidant enzymes in the MW<100 kDa fraction.

Keywords: antioxidant activity; *Helix lucorum*; hemocyanin; hemolymph fractions; mass spectrometry.

Resumen

Contexto: La hemolinfa de caracoles es una mezcla multicomponente que contiene diversas sustancias con actividad farmacológica.

Objetivos: Evaluar la actividad antioxidante de hemocianina y fracciones aisladas de hemolinfa del caracol común de jardín *Helix lucorum*.

Métodos: La actividad antioxidante fue probada en sistemas químicos que generan radicales superóxido ($O_2^{\bullet-}$) y radicales hidroxilo ($^{\bullet}OH$) y se presentó como porcentaje de inhibición. La composición de las fracciones se analizó mediante espectrometría de masas MALDI-TOF/TOF.

Resultados: A una concentración de 90 µg/mL, la fracción de hemolinfa con compuestos con masa molecular (M) <100 kDa mostró el efecto eliminador de $O_2^{\bullet-}$ más fuerte (82%), seguido de la hemocianina (58%) y la fracción de hemolinfa con compuestos con M<1 kDa (15%). A la misma concentración de 90 µg/mL, la hemocianina mostró el efecto eliminador de $^{\bullet}OH$ más potente (43%) seguida de la fracción de M<100 kDa (26%). No se observó ningún efecto eliminador de $^{\bullet}OH$ por la fracción de M<1 kDa. Las propiedades de quelación más altas (55%) se demostraron de la fracción de M<100 kDa a una concentración de 130 µg/mL; tanto la hemocianina como la fracción de hemolinfa de M<1 kDa mostraron un efecto inhibidor del 33% a la misma concentración. El análisis de la composición de las fracciones ensayadas reveló un alto contenido de superficies hidrófobas en la fracción de PM<1 kDa y la presencia de enzimas antioxidantes-catalase, superoxide dismutase y glutatión peroxidase en la fracción de M<100 kDa que contribuyen al efecto antioxidante global de la hemolinfa de *H. lucorum*.

Conclusiones: Los datos sugieren que el efecto antioxidante complejo de la hemolinfa de *H. lucorum* está relacionado con la presencia de péptidos de bajo peso molecular en la fracción de M<1 kDa que incluye glutatión y principalmente con la presencia de enzimas antioxidantes en el M<100 kDa fracción.

Palabras Clave: actividad antioxidante; espectrometría de masas; fracciones de hemolinfa; *Helix lucorum*; hemocianina.

ARTICLE INFO
Received: August 31, 2020.
Received in revised form: November 12, 2020.
Accepted: November 12, 2020.
Available Online: November 16, 2020.

AUTHOR INFO
ORCID: 0000-0002-7007-3665 (AA)

INTRODUCTION

Snails have been used since ancient times for healing purposes (Bonnemain, 2005). A number of studies have shown that snail fluids, mucus and hemolymph, as well as various proteins derived from them, have antibacterial, (Dolashka et al., 2015; Cilia and Fratini, 2018) antiviral (Dang et al., 2015) and antifungal activity (Ulagesan and Kim, 2018). Some studies indicated that although in small quantities (about 0.1%), the snail mucus contains low molecular weight substances with antioxidant properties such as uric acid, uronic acid, ubiquinone, lipoic acid, vitamin C and E, phenolic compounds, selenium, their derivatives or combinations thereof (Wang et al., 2010).

In recent years, mucus from the garden snail *Helix aspersa* Müller, 1774 has been found to contain enzymatic antioxidants as superoxide dismutase and glutathione-S-transferase (Brieva et al., 2008); catalase activity has also been measured (Kostadinova et al., 2018). The hemocyanin, isolated from *H. aspersa* showed a good radical scavenging activity and a strong chelating effect on copper ions (Raynova et al., 2015). The antioxidant potential of snail extracts makes them an interesting object of study, since oxidative stress is related to many pathological conditions and diseases: cardiovascular (atherosclerosis), neurodegenerative (Alzheimer's disease, Parkinson's disease, among others), pulmonary (asthma, respiratory stress syndrome, pulmonary fibrosis), renal, metabolic (diabetes mellitus), neoplastic (Halliwell and Gutteridge, 2015).

A large number of studies confirm the beneficial effect of exogenous antioxidants from plant or animal origin in maintaining the organism's oxidative balance and a healthy state. The complex nature of natural products (including snail mucus and hemolymph) makes difficult to identify the mechanisms and components responsible for their biological effects. Therefore, fractioning the biological fluids into different fractions based on the molecular masses of the substances contained therein is a good strategy. Thus, the purpose of the present work is to detect the antioxidant potential of fractions with molecular weight (MW) below 1 kDa and below 100 kDa from hemolymph and hemocyanin from the garden snail *Helix lucorum* L. (family *Helicidae*). This would allow the creation and development of new remedies for the prevention and treatment of diseases with oxidative etiology.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents were analytical or HPLC grade and purchased from Merck KGaA (Darmstadt, Germany).

Isolation of the bioactive compounds

Adult specimens of free-living *Helix lucorum* L. with weight about 20 g were collected in the vicinity of Sofia, in Bulgaria (GPS coordinates 42°38'08.7"N 23°21'52.5"E, 42.635746, 23.364573), and maintained in an active state in large plastic boxes at 20°C, from 9:00-11:00 h in May 2019. The hemolymph was gathered from the collected *H. lucorum* after cutting the foot muscles. The crude hemolymph was filtrated and centrifuged at 10 000 rpm and 4°C for 20 min to remove of rough particles and hemocytes as described by Velkova et al., (2010) and Georgieva et al. (2020). Three fractions were separated from the purified hemolymph: MW<100 kDa fraction, MW<1 kDa fraction and fraction above 100 kDa (hemocyanin) by ultrafiltration (using Millipore™ Ultrafiltration Membrane Filters from 100 kDa and 1 kDa).

Firstly, the obtained supernatant was divided into two fractions containing compounds with MW<100 kDa and with MW>100 kDa by ultrafiltration on 100 kDa membrane (Millipore™ Ultrafiltration Membrane Filters, Regenerated cellulose). The low molecular fraction, containing compounds with MW<1 kDa was obtained from MW<100 kDa fraction by additionally ultrafiltration on 1 kDa membranes (Millipore™ Ultrafiltration Membrane Filters Ultracel®, Regenerated cellulose membrane). The MW>100 kDa fraction, which contains mostly hemocyanin was subjected

of ultracentrifugation at 22 000 rpm and 4°C for 5 hours with rotor Kontron-Hermle A8.24 (centrifuge Centrikon). After removal of the supernatant, the sediment containing the total hemocyanin was solubilized at a concentration of about 10% in 50 mM Tris buffer (pH 7.5) containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$.

Antioxidant activity analysis

Dry snail preparations (MW<1 kDa fraction) were dissolved in water to obtain solutions at a concentration of 1 mg/mL. The protein concentration of the liquid snail preparations (MW<100 kDa fraction and hemocyanin) was quantified by the method of Lowry et al. (1951) and the samples were diluted to concentration of 1 mg protein/mL. These solutions than were used in the experiments at the final concentrations of 7, 16, 32, 48, 63, 91 µg/mL for evaluation of superoxide anion radical scavenging effect and 24, 47, 69, 90, 130 µg/mL for evaluation of both hydroxyl radical scavenging effect and chelating effect of the preparations.

Chemical systems for the generation of reactive oxygen species were used to determine the antioxidant activity of the fractions.

Superoxide anion radical generation system

The superoxide anion radicals ($O_2^{\bullet -}$) were generated photochemically in medium containing: 50 mM phosphate buffer, pH 7.8; 1.17×10^{-6} M riboflavin; 0.2 mM methionine; 2×10^{-5} M KCN and 5.6×10^{-5}M nitrobluetetrazolium (NBT) (Taniguchi and Gutteridge, 2000). The $O_2^{\bullet -}$ reduced the NBT producing blue formazan. The decrease in the color intensity at 560 nm in the presence of the tested substances was a measure of their antioxidant capacity and was expressed as a percentage inhibition relative to the control (medium without preparation).

Hydroxyl radical generation system

Hydroxyl radicals (•OH) were generated in systems containing:

A) A 10 mM potassium phosphate buffer, pH 7.4; 0.1 mM $FeSO_4$, 0.5 mM H_2O_2 and 2 mM deoxyribose (DR).

B) A 10 mM potassium phosphate buffer, pH 7.4; 0.1 mM EDTA-Fe^{2+} complex, 0.5 mM H_2O_2 and 2 mM deoxyribose (DR).

The variants in absence and in presence of EDTA, were used to determine the chelation or •OH- scavenger potential of the preparations (Miles and Grisham, 1994).

After incubation at 37°C for 30 minutes in the presence of increasing concentrations of the tested fractions, to the samples was added 0.6 mL of a mixture of 2.8% trichloroacetic acid, 0.1 mL of 5 N HCl, and 0.2 mL of thiobarbituric acid (2% w/v in 50 mM NaOH). The samples were incubated at 100°C for 15 min. After cooling, the absorbance of the resulting colored compound (TBARS, thiobarbituric acid reactive substances) was read at 532 nm. The reduced formation of TBARS is a measure of the antioxidant capacity or chelation effect of the tested substances and was expressed as a percentage of inhibition relative to the control.

Mass spectrometric analysis

The MW<1 kDa and MW<100 kDa fractions from the hemolymph were analyzed by MALDI-TOF/TOF mass spectrometry on an AutoflexTM III. High Performance MALDI- TOF/TOF System (Bruker Daltonics) uses a 200 Hz frequency-tripled Nd–YAG laser operating at a wavelength of 355 nm. Samples were prepared by mixing of 2.0 µL of the fraction with 2.0 µL of matrix solution (10 mg/mL of α-cyano-4-hydroxycinnamic acid) in 50% acetonitrile containing 0.1% trifluoroacetic acid or synaptic acid). Only 1.0 µL of the mixture was spotted on a stainless steel 192-well target plate. The mixture of angiotensin I, Glu-1-fibrinopeptide B, ACTH (1–17), and ACTH was used for calibration of mass spectrometer. The MS/MS spectra were carried out in reflector mode with external calibration using fragments of Glu-fibrino-peptide B.

The MW<1 kDa fraction also was analyzed by COMPACT UHPLC-QqTOF Systems (Bruker Daltonics, Germany).

Statistical analysis

All measurements were made in triplicate and averaged. Data were analyzed using Excel 2019 spreadsheet program (Microsoft Corporation, USA) and expressed as the mean ± SEM. Statistical significance of differences were determined using one-way analysis of variance (ANOVA). The level of significance was set at $p<0.05$. IC_{50} values were derived by a sigmoidal dose-response (variable slope) curve using GraphPad Prism 7.0 software (GraphPad software Inc., San Diego, CA, USA).

RESULTS

Antioxidant activity

Activity of preparations as scavengers of superoxide radicals

The activity of the preparations as scavengers of $O_2^{\bullet-}$ is presented in Fig. 1. The MW<100 kDa fraction had the greatest statistically significant ($p<0.05$) $O_2^{\bullet-}$-scavenger effect (82% inhibition of NBT reduction at the highest concentration tested), followed by the hemocyanin with about 20% lower effect (58% inhibition of NBT reduction) and the MW<1 kDa fractions of hemolymph showed less effect (about 42%) at the highest concentration. The MW<1 kDa fractions and the hemocyanin differed significantly ($p<0.05$) only at the highest concentration tested. The calculated IC_{50} for the MW<100 kDa fractions of hemolymph was lowest (21.89 µg/mL), compared to those of MW<1 kDa fractions of hemolymph (24.99 µg/mL) and hemocyanin (37.03 µg/mL).

Activity of preparations as scavengers of hydroxyl radicals

The activity of the preparations as scavengers of $^{\bullet}OH$ is presented in Fig. 2. Under conditions of Fe^{2+} chelation with EDTA, the binding of the metal to the detector DR molecules is prevented and $^{\bullet}OH$ generated in the reaction of Fe^{2+}-EDTA with H_2O_2 (Fenton reaction) can attack both the DR and the tested preparations (Miles and Grisham, 1994). The latter, by reacting with the radicals, neutralize them and reduce their reaction with DR, and thus evaluate their $^{\bullet}OH$ scavenger effect.

In this experimental setting, the highest $^{\bullet}OH$ scavenger effect showed the hemocyanin - 43% inhibition of DR degradation at the highest concentration tested. The MW<100 kDa fraction had significantly lesser effect of 26% inhibition of DR degradation at the same concentration. The MW<1 kDa fractions at all concentrations tested showed negative values. It is possible that these fractions are rich in carbohydrates and their reaction interferes with TBARS assay, being a substrate of the reaction (Du and Bramlage, 1992).

Chelation potential of the preparations

The experimental design used in this study is based on the ability of DR to chelate iron ions and in the presence of H_2O_2 to generate $^{\bullet}OH$, which reacting with DR produce TBARS. Thus, if the preparations tested have a greater chelation capacity for the iron ions than DR, they have a protective effect by taking the free radical attack on themselves.

The chelating potential of the preparations is presented in Fig. 3. The results demonstrated that the MW<100 kDa fraction had the greatest statistically significant ($p<0.05$) chelation effect (with 55% inhibition of DR degradation at the highest tested concentration). The effect of this preparation was nearly twice as much effect as the effects of the others at all concentrations. The hemocyanin and the MW<1 kDa fraction had significantly different inhibitory effect up to concentration of 90 µg/mL. However, these two preparations showed equal effects at concentrations of 90 and 130 µg/mL as the inhibition at the highest concentrations tested was about 33%. The calculated IC_{50} were also significantlly different, as follow: 16.3 µg/mL for MW<100 kDa fractions of hemolymph, 18.21 µg/mL for MW<1 kDa fractions of hemolymph and 88.75 µg/mL for hemocyanin.

Mass spectrometric analysis

The results from MALDI-MS spectrum showed that the MW<1 kDa fraction (Fig. 4) was dominated from peptides presented primarily as double charged ions $[M+2H]^{2+}$, only one single charged ion $[M+H]^{+}$ at m/z 308.046 Da and one triple

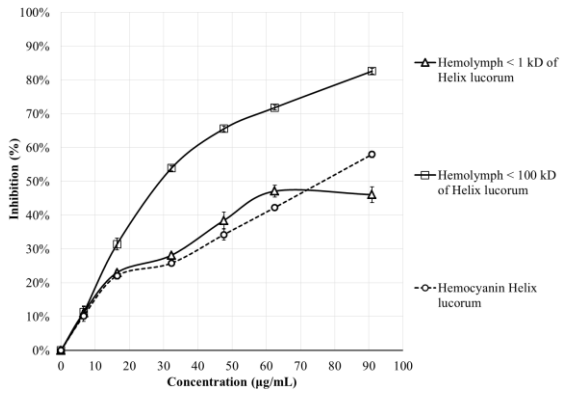

Figure 1. Activity of three different fractions isolated from hemolymph of *H. lucorum* as superoxide anion radical scavenging agents.

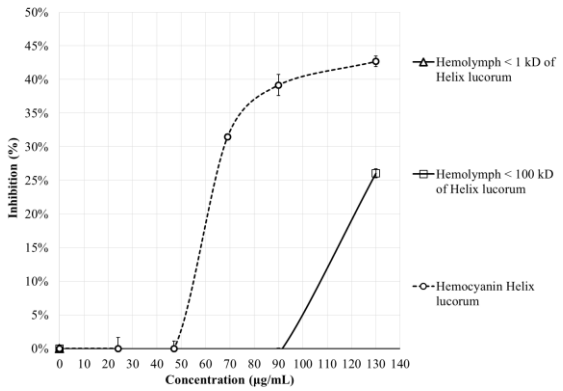

Figure 2. Activity of different fractions isolated from hemolymph of *H. lucorum* as hydroxyl radical scavenging agents.

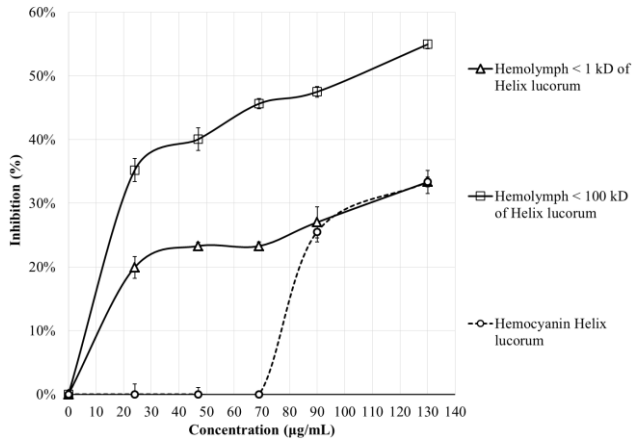

Figure 3. Chelation potential of different fractions isolated from hemolymph of *H. lucorum*.

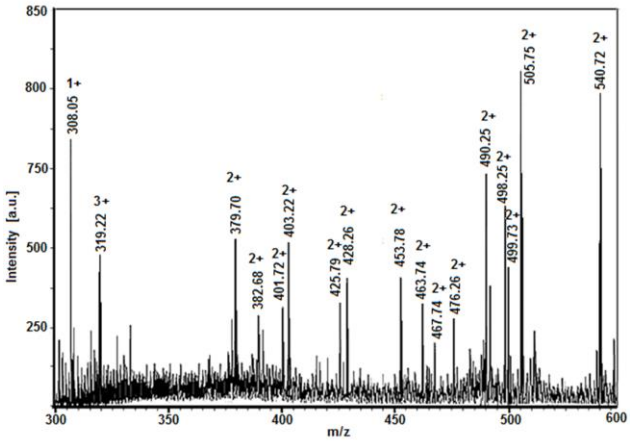

Figure 4. MALDI-MS spectrum of fraction with MW below 1 kDa from hemolymph of *H. lucorum* in the region from 300 to 550 m/z. Standard peptide solution was used to calibrate the mass scale of the COMPACT UHPLC-QqTOF Systems (Bruker Daltonics, GmbH).

charged ion $[M+3H]^{3+}$ at m/z 319.22 Da were detected.

The primary structure of the MW<1 kDa fraction peptides was identified by de novo MS/MS sequencing experiments of the protonated molecule ions. The obtained results and predicted by the ExPASy MW/pI tool program and ExPASy ProtParam tool isoelectric points (pI) and grand average of hydropathicity (GRAVY) of the peptides are shown of Table 1. Most of the peptides, identified in MW<1 kDa fraction are characterized by an amphipathic structure and display generally hydrophobic surfaces (Table 1).

The results of MALDI-TOF-MS spectrum of the MW<100 kDa fraction showed dominance of several groups of proteins with MW between 13-16 kDa, 23-25 kDa (Fig. 5A), 31-34 kDa, 49-52 and about 62 kDa (Fig. 5B).

DISCUSSION

The aim of this study was to assess the antioxidant potential of hemocyanin and two fractions of

Table 1. Amino acid sequences of peptides from the fraction with MW below 1 kDa of hemolymph from garden snail *H. lucorum*, identified by de novo sequencing on the COMPACT UHPLC-QqTOF Systems (Bruker Daltonics, GmbH).

№	Amino acid sequence of peptides	Mesured mass (Da)	Calculated mass (monoisotopic) (Da)	Grand average of hydropathicity (GRAVY)	pI
1	VVLIKAKGK	319.22 $[M+3H]^{3+}$	954.66	0.711 (hydrophobic)	10.30
2	GIPLEMV	379.70 $[M+2H]^{2+}$	757.41	1.271 (hydrophobic)	4.00
3	SSPPFVM	382.68 $[M+2H]^{2+}$	763.36	0.586 (hydrophobic)	5.24
4	KVAPYPQ	401.72 $[M+2H]^{2+}$	801.44	-0.843 (hydrophilic)	8.59
5	VVMKELS	403.22 $[M+2H]^{2+}$	804.44	0.843 (hydrophobic)	5.97
6	GPLKIPLL	425.79 $[M+2H]^{2+}$	849.57	1.050 (hydrophobic)	8.75
7	AEPKIGKI	428.26 $[M+2H]^{2+}$	854.52	-0.312 (hydrophilic)	8.64
8	LAVSKLLY	453.78 $[M+2H]^{2+}$	905.56	1.425 (hydrophobic)	8.59
9	KWFKFGN	463.74 $[M+2H]^{2+}$	925.48	-1.000 (hydrophilic)	10.00
10	VSEGMIVSI	467.74 $[M+2H]^{2+}$	933.49	1.533 (hydrophobic)	4.00
11	GTLSSLLNF	476.26 $[M+2H]^{2+}$	950.51	0.889 (hydrophobic)	5.52
12	FLGDSTNLI	490.25 $[M+2H]^{2+}$	978.51	0.667 (hydrophobic)	3.80
13	AFQLm*KQV	490.76 $[M+2H]^{2+}$	979.52	0.450 (hydrophobic)	8.80
14	EIKLSDQY	498.25 $[M+2H]^{2+}$	994.50	-1.025 (hydrophilic)	4.37
15	ALSAWNAHE	499.73 $[M+2H]^{2+}$	997.46	-0.300 (hydrophilic)	5.24
16	HGMPLDLLD	505.75 $[M+2H]^{2+}$	1009.49	0.122(hydrophobic)	4.20
17	STENDPSSML	540.72 $[M+2H]^{2+}$	1079.44	-0.950 (hydrophilic)	3.67

*m=M with oxidation modification.

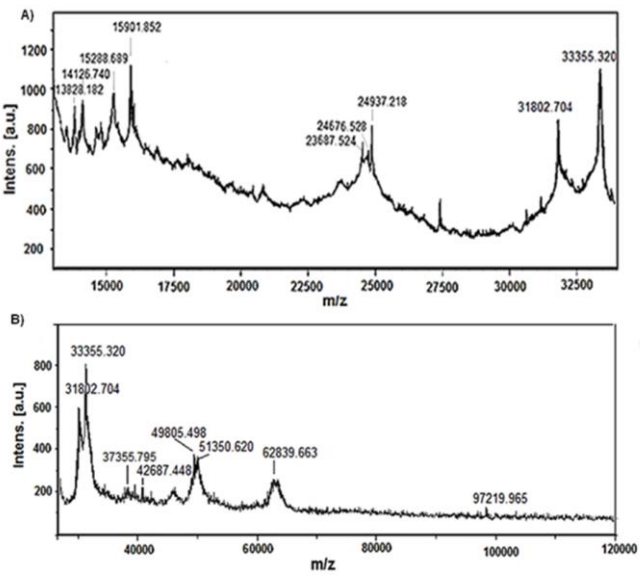

Figure 5. MALDI-TOF-MS spectrum of fraction with MW below 100 kDa from hemolymph of *H. lucorum*.

(A) proteins with molecular masses in region 13 000-34 000 Da and **(B)** in region 28 000-120 000 Da.

hemolymph from garden snail *Helix lucorum* L. and to attempt to elucidate the mechanisms responsible for this effect. From the obtained in this study data, it can be summarized that the best antioxidant effect exhibited the hemolymph MW<100 kDa fraction. This fraction suppressed

82% of the action of superoxide radicals (best $O_2^{\bullet-}$-scavenger effect compared to other tested substances) and 26% of the action of $^{\bullet}OH$ radicals (second after the hemocyanin, which suppressed by 43%) and it was the best iron ion chelator in the experimental systems used. Excess $O_2^{\bullet-}$ and $^{\bullet}OH$ radicals are dangerous for cells. The $O_2^{\bullet-}$ are not the most reactive radicals, but they are precursors to all other reactive species: H_2O_2, $^{\bullet}OH$, HOCl, and $ONOO^-$ (Halliwell and Gutteridge, 2015). Reaction between $O_2^{\bullet-}$ and NO not only produces the very reactive oxidant $ONOO^-$, but also consumes NO and thus could interrupt the NO-mediated signaling (Gadzhiev et al., 2011). The $^{\bullet}OH$ are the most reactive species, able to modify oxidatively all biological molecules in the cell disrupting in this way their function and consequently the functions of the structures in which they participate. Thus, scavenging the $^{\bullet}OH$ is crucial for cell preservation. The generation of $^{\bullet}OH$ *in vivo* requires the presence of redox active metal, most commonly iron or copper ions by the Fenton reaction mechanism (Halliwell and Gutteridge, 2015). Therefore, the ability of substances to chelate metal ions and prevent the formation of the most damaging agent in cells is essential.

The antioxidant capacity of the peptides is closely related to their structural characteristic, such as molecular mass, amino acid compositions, sequences (steric structure at the C- and N-termini and the neighboring amino acids of some residues), and hydrophobicities (Wang et al., 2016). It was found that short chain peptide fractions (with MW<3 kDa) exhibited the higher antioxidant activities compared to fractions with a higher MW (Esteve et al., 2015). Furthermore, peptides with a high content of hydrophobic amino acids show a better radical scavenger effect than those with a higher content of hydrophilic amino acids (Zou et al., 2016). The composition and the sequence of amino acids have the most impact on the antioxidant properties (Zou et al., 2016). Besides the hydrophobicity of peptides with high antioxidant activity, the amphiphilic nature of peptides also seems to enhance the radical-scavenging activities by increasing peptide solubility while facilitating interaction and proton exchanges with radical species. Other important factor for the antioxidant activity may be the interactions between amino acids in polypeptide chain. The oxidative sensitivity of amino acid residues to free radicals is associated with the participation of some functional groups in their side chains. The most reactive amino acids are those that in the side chain include nucleophilic sulfur-cysteine (Cys) and methionine (Met), aromatic ring-tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) or containing imidazole–histidine (His).

The amino acid sequences of peptides in the tested fraction with MW<1 kDa, indicate the presence of various amino acid residues, but mostly Val, Leu/Ile, Pro, Lys, Phe, Met, His, Trp, and Tyr. The presences in the peptide sequences of hydrophobic amino acids (Leu, Val and Phe), hydrophilic and basic amino acids (His, Pro and Lys), and aromatic amino acids (Phe and Tyr) are thought to contribute to their overall antioxidant activity (Najafian and Babji, 2015). Amino acid sequences of detected peptides with masses between 757.41 Da-1079.44 Da in hemolymph fraction below 1 kDa from the land snail *H. lucorum* are rather different from the identified peptides in *H. aspersa* mucus (Dolashka et al., 2015; Vassilev et al., 2020). The presented peptides in Table 1 are associated with demonstrated antioxidant activity of the fraction below 1 kDa unlike antimicrobial peptides from *H. aspersa* mucus (Dolashka et al., 2015; Dolashki et al., 2020; Vassilev et al., 2020). A presence of the tripeptide glutathione (GSH) (with monoisotopic mass 307.08 Da) was detected as $[M+H]^+$ at m/z 308.046 Da by MALDI-MS analysis. Recently, the glutathione also was identified in mucus fraction below 1 kDa from *H. aspersa* snails (Vasilev et al., 2020). The GSH maintains the cellular redox status and is able to protect cell structures from oxidative damage by reacting directly with the reactive oxygen species ($^{\bullet}OH$, HOCl, $ONOO^-$, $RO^{\bullet}$, $RO_2^{\bullet}$, $CO_3^{\bullet-}$, NO_2, $^1O_2$) and acts as a co-substrate of the antioxidant enzyme glutathione peroxidase (Halliwell and Gutteridge, 2015). The high antioxidant activity found in the tested MW<100 kDa fraction could be assumed that due to established components in the MW<1 kDa fraction, peptides with MW<10 kDa rich in Pro, Val,

Trp and Gly residues (Dolashka et al., 2015) and primarily to the antioxidant enzymes. Similar to other organisms, the snail enzymatic antioxidant system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Nowakowska et al., 2015). All these enzymes work together to eliminate the ROS produced in the body. It is assumed that they constitute the first line antioxidant defense with a key role in the total defense mechanisms in the biological systems (Ighodaro and Akinloye, 2018).

Preliminary results from the comparison of protein masses (Fig. 5) with the central hub UniProt Knowledgebase (UniProtKB) indicate that some of them may be related to previously discovered proteins in snails. For example: a protein represented as $[M+H]^+$ at m/z 15 288.689 Da (Fig. 5A) probably corresponds to CAT with a molecular weight of 15 372 Da, determined in *Lymnaea stagnalis* snail (C3U1W7_LYMST) (Bouetard et al., 2013). It is known that in hemolymph of snail the CAT is presented as a mono-functional, hem-containing enzyme. The SOD found in the hemolymph of the snail *L. stagnalis* with MW 15774 Da (Q7YXL9_LYMS) (Zelck et al., 2005) probably corresponds to the same enzyme represented as monomer $[M+H]^+$ at m/z 15901.852 Da (Fig. 5) and as a dimer at m/z 31802.704 Da in the hemolymph of *H. lucorum*. In addition very high similarity was found between GR (C3U1W6_LYMST) with MW of 13 977 Da in *L. stagnalis* snail (Bouetard et al., 2013) and molecular ion $[M+H]^+$ at m/z 13 828.182 Da in *H. lucorum* hemolymph, so we proposed a match between them. The molecular ion $[M+H]^+$ at m/z 24 937.218 Da probably corresponds to the GPx determined in *Haliotis discus* with MW 24945 Da (A0A0H3V5M4_HALDI) and with MW 24 809 Da (A0A0H3V5S1_HALDI) (Bathige et al., 2015), as well as in *Haliotis diversicolor supertexta* (Cai and Zhou, 2010) with MW 24 989 Da (D6PW93_HALDV). More of the enzymes belonging to the GPx family are extracellular proteins and in various gastropods (*Biomphalaria glabrata* (freshwater snail), *Aplysia californica* (California sea hare), *Reishia clavigera* (Sea snail *Purpura clavigera*), as well as abalones *H. diversicolor supertexta* and *H. discus*, with a molecular weight in the range between 20.0-27.5 kDa) are dissolved in the hemolymph (Moroz et al., 2006; Cai and Zhou, 2010; Rhee et al., 2012; Warren et al., 2013; Bathige et al., 2015).

Hemocyanin is copper containing oxygen transporting protein freely dissolved in the hemolymph of garden snail. The structure of the *H. lucorum* hemocyanin (HlH), in contrast with other molluscan hemocyanins, is composed of three different structural subunits (isoforms) named as β_c-HlH, α_D-HlH and α_N-HlH with molecular masses ~450 kDa. Each structural subunit consists of eight globular functional units (FUs) connected by linker peptide strands, with molecular mass ~50 kDa (Velkova et al., 2010). Some of these functional units are dissolved in the hemolymph of garden snail *H. lucorum*. Each FU involves one active site with a pair of copper atoms (Cu-A and Cu-B). These copper atoms reversibly capture O_2 molecules, which bind as a peroxide ion (González et al., 2017). The native HlH is arranged by 20 subunits with 160 oxygen-binding active sites. The ability of HlH to act as a peroxidation inhibitor could be related to its metal binding capacity, which is confirmed by the absorption spectrum of native FU with bands at 280 and 345 nm, corresponding to aromatic residues and Cu II $–O_2^{2-}–$Cu II complexes at the active sites, respectively. The antioxidant activity of native HlH similar to *H. aspersa* hemocyanin (Raynova et al., 2015) probably involves both quenching of reactive oxygen species and metal ion chelation defending cellular components from oxidative stress.

CONCLUSIONS

The hemolymph of *H. lucorum* is a complex of bioactive components. A detailed study of the antioxidant activity of hemolymph fractions with MW<100 kDa and MW<1kDa was presented for the first time. This study allows distinguishing between mechanisms of action of different fractions, identifying those that are most suitable for use in pro-oxidation processes of different nature.

The complex antioxidant effect of hemolymph from *H. lucorum* is thought to be related to the

presence of both low molecular weight peptides in the MW<1 kDa fraction including glutathione and the complex of antioxidant enzymes - CAT, SOD and GPx in the MW<100 kDa fraction.

These data suggest that snail preparations may also have good antioxidant effects under oxidative stress conditions *in vivo*. Undoubtedly, further studies on animal models are needed to clarify the possible preventive properties of snail biological fluids in free radical disorders.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

ACKNOWLEDGMENTS

This work was supported by the Bulgarian Ministry of Education and Science (Grant D01-217/30.11.2018) under the National Research Programme "Innovative Low-Toxic Bioactive Systems for Precision Medicine (BioActiveMed)" approved by DCM#658/14.09.2018 and with the support of a project under contract № DN 01/14 of 19/12/16, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.

REFERENCES

Bathige SD, Umasuthan N, Godahewa GI, Thulasitha SW, Whang I, Won SH, Kim C, Lee J (2015) Two variants of selenium-dependent glutathione peroxidase from the disk abalone *Haliotis discus discus*: Molecular characterization and immune responses to bacterial and viral stresses. Fish Shellfish Immunol 45(2): 648-655.

Bonnemain B (2005) *Helix* and drugs: Snails for western health care from antiquity to the present. Evid Based Complement Alternat Med 2: 25–28.

Bouetard A, Besnard AL, Vassaux D, Lagadic L, Coutellec MA (2013) Impact of the redox-cycling herbicide diquat on transcript expression and antioxidant enzymatic activities of the freshwater snail *Lymnaea stagnalis*. Aquatic Toxicol 126: 256–265.

Brieva A, Philips N, Tejedor R, Guerrero A, Pivel JP, Alonso-Lebrero JL, Gonzalez S (2008) Molecular basis for the regenerative properties of a secretion of the mollusk; Cryptomphalus aspersa. Skin Pharmac Physiol 21(1):15–22.

Cai Z, Zhou J (2010) Embryotoxicity of phthalate esters to abalone (*Haliotis diversicolor* supertexta). Submitted (Mar-2010) to the EMBL/GenBank/DDBJ databases.

Cilia G, Fratini F (2018) Antimicrobial properties of terrestrial snail and slug mucus. J Compl Integr Med 15: 3.

Dang VT, Benkendorff K, Green T, Speck P (2015) Marine snails and slugs: a great place to look for antiviral drugs. J Virol 89(16): 8114–8118.

Dolashka P, Dolashki A, Velkova L, Stevanovic S, Molin L, Traldi P, Velikova R, Voelter W (2015) Bioactive compounds isolated from garden snails. J BioSci Biotechnol SE/ONLINE: 147–155.

Dolashki A, Velkova L, Daskalova E, Zheleva N, Topalova Y, Atanasov V, Voelter W, Dolashka P (2020) Antimicrobial activities of different fractions from mucus of the garden snail *Cornu aspersum*. Biomedicines 8(9): 315.

Du Z, Bramlage WJ (1992) Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. J Agric Food Chem 40(9): 1566–1570.

Esteve C, Marina ML, García MC (2015) Novel strategy for the revalorization of olive (*Olea europaea*) residues based on the extraction of bioactive peptides. Food Chem 167: 272–280.

Gadzhiev OB, Ignatov SK, Gangopadhyay S, Masunov AE, Petrov AI (2011) Mechanism of nitric oxide oxidation reaction ($2NO+O_2 \rightarrow 2NO_2$) revisited. J Chem Theo Comput 7(7): 2021–2024.

Georgieva A, Todorova K, Iliev I, Dilcheva V, Vladov I, Petkova S, Toshkova R, Velkova L, Dolashki A, Dolashka P (2020) Hemocyanins from *Helix* and *Rapana* snails exhibit *in vitro* antitumor effects in human colorectal adenocarcinoma. Biomedicines 8(7): 194.

González A, Nova E, Del Campo M, Manubens A, De Ioannes A, Ferreira J, Becker MI (2017) The oxygen-binding properties of hemocyanin from the mollusk *Concholepas concholepas*. Biochim Biophys Acta (BBA) - Proteins and Proteomics 1865(12): 1746–1757.

Halliwell B, Gutteridge JMC (2015) Free Radicals in Biology and Medicine. 5th edn. Oxford: Oxford University Press.

Ighodaro OM, Akinloye OA (2018) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria J Med 54(4): 287–293.

Kostadinova N, Voynikov Y, Dolashki A, Krumova E, Abrashev R, Kowalewski D, Dolashka P (2018) Antioxidative screening of fractions from the mucus of garden snail *Cornu aspersum*. Bulg Chem Commun 50 (Special Issue C): 176–183.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1): 265–275.

Miles AM, Grisham MB (1994) Antioxidant properties of aminosalicylates. Meth Enzymol 234: 555–572.

Moroz LL, Edwards JR, Puthanveettil SV, Kohn AB, Ha T, Heyland A, Jezzini S (2006) Neuronal transcriptome of Aplysia: neuronal compartments and circuitry. Cell 127(7): 1453–1467.

Najafian L, Babji AS (2015) Isolation, purification and identification of three novel antioxidative peptides from patin (*Pangasius sutchi*) myofibrillar protein hydrolysates. LWT - Food Sci Technol 60(1): 452–461.

Nowakowska A, Rogalska J, Caputa M (2015) Adaptability of antioxidant defence system in *Helix pomatia* snails: effect of forced aestivation during early spring. J Mollusc Stud 82(1): 205–207.

Raynova Y, Marchev A, Doumanova L, Pavlov A, Idakieva K (2015) Antioxidant activity of *Helix aspersa maxima* (Gastropod) hemocyanin. Acta Microbiol Bulg 31(2): 127–131.

Rhee JS, Kim BM, Jeong CB, Horiguchi T, Lee YM, Kim IC, Lee JS (2012) Immune gene mining by pyrosequencing in the rockshell, *Thais clavigera*. Fish Shellfish Immunol 32: 700–710.

Taniguchi N, Gutteridge J (2000) In: Experimental Protocols for Reactive Oxygen and Nitrogen Species. Taniguchi N, Gutteridge J (eds), Oxford: Oxford press.

Ulagesan S, Kim H (2018) Antibacterial and antifungal activities of proteins extracted from seven different snails. App Sci 8(8): 1362.

Vassilev NG, Simova SD, Dangalov M, Velkova L, Atanasov V, Dolashki A, Dolashka P (2020) An ^{1}H NMR- and MS-based study of metabolites profiling of garden snail *Helix aspersa* mucus. Metabolites 10(9): 360.

Velkova L, Dimitrov I, Schwarz H, Stevanovic S, Voelter W, Salvato B, Dolashka-Angelova P (2010) Structure of hemocyanin from garden snail *Helix lucorum*. Comp Biochem Physiol. Part B: Biochem Molec Biol 157(1): 16–25.

Wang L, Yang J, Wang Y, Zhang J, Gao Y, Yuan J, Su A, Ju X (2016) Study on antioxidant activity and amino acid analysis of rapeseed protein hydrolysates. Int J Food Prop 19(9): 1899–1911.

Wang W, Yi J, Ke S, Halmela M (2010) Gastropod biological fluid, method of making and refining and use. United States Patent Application 20100233111 http://www.freepatentsonline.com/y2010/0233111.html

Warren W, Wilson RK, Hillier LW, Minx P (2013) Sequence assembly of the *Biomphalaria glabrata* genome version 4.3. Submitted (Mar-2013) to the EMBL/GenBank/DDBJ databases, 2013.

Zelck UE, Janje B, Schneider O (2005) Superoxide dismutase expression and H_2O_2 production by hemocytes of the trematode intermediate host *Lymnaea stagnalis* (Gastropoda). Develop Compar Immun 29: 305–314.

Zou TB, He TP, Li HB, Tang HW, Xia EQ (2016) The structure-activity relationship of the antioxidant peptides from natural proteins. Molecules 21(1): 72.

AUTHOR CONTRIBUTION:

Contribution	Alexandrova A	Petrov L	Velkova L	Dolashki A	Tsvetanova E	Georgieva A	Dolashka P
Concepts or ideas	x	x	x	x	x	x	x
Design	x	x	x	x	x	x	x
Definition of intellectual content				x			x
Literature search	x	x	x				x
Experimental studies	x	x	x	x	x	x	x
Data acquisition	x	x	x	x	x	x	x
Data analysis	x	x	x	x	x	x	x
Statistical analysis		x					
Manuscript preparation	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x

Citation Format: Alexandrova A, Petrov L, Velkova L, Dolashki A, Tsvetanova E, Georgieva A, Dolashka P (2021) Antioxidant activity of fractions isolated from hemolymph of garden snail *Helix lucorum*. J Pharm Pharmacogn Res 9(2): 143–152.