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A ^{1}H NMR based study of metabolites profiling of garden snail *Helix lucorum* hemolymph

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Nuclear Magnetic Resonance (NMR) was used to study the presence and functional role of the metabolites in hemolymph from land snail *Helix lucorum*. Fourteen metabolites were unambiguously identified by ^{1}H, 1D TOCSY, 2D J-resolved, 2D COSY, and 2D HSQC NMR spectra with water suppression. The type and concentration of metabolites in the two low molecular weight fractions (<1kD and <3kD) are very similar. Metabolites with known antioxidant, antibacterial, and antimicrobial activities have been detected by NMR metabolic analysis and tandem mass spectrometry of hemolymph samples from *H. lucorum*.

Key words: hemolymph; *Helix lucorum;* ^{1}H NMR; metabolites

INTRODUCTION

In recent years, the study of bioactive substances from invertebrates is extremely relevant, as it may lead to the discovery of new therapeutic agents. The mucus and hemolymph of mollusc contain an abundance of hemocytes and humoral factors which represent the first line of immune defense [1]. Various bioactive components, such as peptides and proteins with antimicrobial activity, dissolved in the hemolymph and mucus of garden snails can find application in medicine [2-9].

Molluscan hemolymph is a unique kind of body fluid which is a complex mixture that contains diverse biochemically and pharmacologically active components and in many respects is analogous to human blood, although there are several crucial differences [10]. The object of the present study is metabolites from the hemolymph of garden snails *H. lucorum*. The main protein in the hemolymph of *H. lucorum*, as well as other Mollusca organisms, is hemocyanin (over 90%) [11]. Molluscan hemocyanins are giant extracellular oxygen carriers in the hemolymph with different complex quaternary structure, usually with molecular weights between 4 and 9 MDa [12]. Hemocyanins from *H. lucorum* and *H. aspersa* have promising potential for the development of novel antitumor, antibacterial, and immunotherapeutic agents [11-15]. Moreover, several peptides with antimicrobial activity and molecular masses below 10 kDa have been identified and characterized from the hemolymph of *H. lucorum* snail [16].

Recently, the antioxidant capacity of different fractions from the hemolymph of *H. lucorum* snail was evaluated [17]. The complex antioxidant effect of *H. lucorum* hemolymph with MW < 100 kDa is thought to be related to the presence of both antioxidant enzymes and peptides (with MW < 1 kDa) [17].

In the last years, the metabolic profiles of the hemolymph from *Mytilus galloprovincialis* and two mucus fractions of *H. aspersa*, as well as metabolic profiles of isolated organs in the snail *H. aspersa* maxima (kidney, heart, digestive gland, and pulmonary membrane) were determined using ^{1}H NMR spectroscopy [18-20]. However, the presence and functional role of serum metabolites have been insufficiently studied. The present study is the first report of low molecular weight metabolites identified in the hemolymph of garden snail by NMR spectroscopy. Knowledge of the active ingredients and their action in the hemolymph of *H. lucorum* is valuable for their potential application in food supplements and/or pharmacy.

EXPERIMENTAL

Hemolymph collection and preparation of different fractions

The hemolymph was extracted from the foot of garden snail *H. lucorum* in 50 mM Tris-HCl buffer, pH 7.5. After homogenization, filtration, and centrifugation at 10000 rpm at 4 °C for 30 min to remove rough particles and haemocytes, the crude

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hemolymph extract was obtained. It was separated into two base fractions by ultrafiltration on membranes 10 kDa (Millipore™ Ultrafiltration Membrane Filters, Regenerated cellulose): below 10 kDa and above 10 kDa. The fraction below 10 kDa was subject to an additional separation in two fractions with low molecule masses – below 1 kDa and below 3 kDa, using the same method but using membranes with a different pore size (1 and 3 kDa Millipore™ Ultrafiltration Membrane Filters, Regenerated cellulose). Fractions from the hemolymph of *H. lucorum* containing compounds with molecule masses below 1 kDa and below 3 kDa were concentrated by lyophilized. The dried fractions were analyzed by NMR spectroscopy to determine the metabolic profile.

^{1}H NMR spectroscopy

The recently developed protocol for the determination and assignment of metabolites in mucus from *H. aspersa* by NMR spectroscopy [19] was adopted to study the low molecular weight fractions of hemolymph from *H. lucorum* (<1kD and <3kD). Details of the NMR spectroscopy experiments are presented in the Supp. info.

Assignment of metabolites and database search

After processing with Bruker TOPSPIN 3.5 program the 1D ^{1}H NMR spectra were analysed preliminary with Bayesil web system [21]. The assignment of the metabolite resonances was then manually checked and refined by comparison between experimental 1D NMR spectra and spectra of single metabolites downloaded from "BioMagResBank" [22]. In the case of overlapped multiples (Figure S2) JRES spectra with water suppression were very useful in the assignment of resonances. For identification of metabolites with close resonances or in cases of overlap of several multiplets 1D selective TOCSY experiments were used (Figure S3). For several samples, simultaneous suppression of the signals of water, Tris, and Acetic acid was utilized using the 3 channels of the spectrometer. In Figure S4 a typical ^{1}H 1D NOESY NMR spectrum of lyophilized hemolymph from *H. lucorum* (< 1kD) is presented.

RESULTS AND DISCUSSION

The crude hemolymph extract was collected from the foot of garden snail *H. lucorum* and three fractions were prepared: fraction containing compounds with molecular masses (Mw) below 10 kDa, a fraction containing compounds with Mw below 3 kDa, and a fraction containing compounds with Mw below 1 kDa. After lyophilization and concentration, the two low Mw fractions were analyzed by NMR spectroscopy to determine the metabolic profile.

^{1}H NMR of hemolymph from H. lucorum and identification of the main metabolites

Nuclear magnetic resonance (NMR) in combination with mass spectrometry was found to be an analytical approach suitable for metabolic profiling of garden snail mucus samples [19]. ^{1}H NMR spectra of hemolymph from *H. lucorum* (< 1kD or < 3kD) are presented in Fig. 1 and S1.

The spectra are dominated by the sharp resonances of low molecular weight metabolites in the aliphatic region between 4.5 and 0.5 ppm; while, the aromatic region is almost devoid of signals, or contains very low intensity ones. Overall, 1D-NMR spectra showed very good chemical shift reproducibility due to the addition of a concentrated phosphate buffer to the sample. The metabolic stability was assessed in preliminary experiments, where noesy1d NMR spectra of the freshly prepared sample of lyophilized hemolymph from *H. lucorum* (< 3kD) were analyzed immediately and after 24 h (sample kept at 298 K). It was found that there is not any visible change in the spectra and there are not any ongoing biochemical processes.

Five metabolites were unambiguously identified by signals in the HSQC (Table 1). The presence of these metabolites was confirmed by analysis of the COSY spectrum, as well. Nine additional metabolites were unambiguously identified during inspection and database-matching of the 1D-spectra. In addition, a number of resonances remain unassigned or ambiguously assigned, so they are not included in Table 1. The metabolites having the highest concentration in hemolymph were osmolytes (betaine and glycine) and nutrients (glucose and amino acids such as alanine). In addition to the resonances of assigned metabolites, several others could not be unambiguously assigned. In general, the resonances of unassigned species have low signal intensities.

Metabolic pathway and activity

The detected metabolites in hemolymph from *H. lucorum* are known to take part in various metabolic pathways in humans, mammals, and

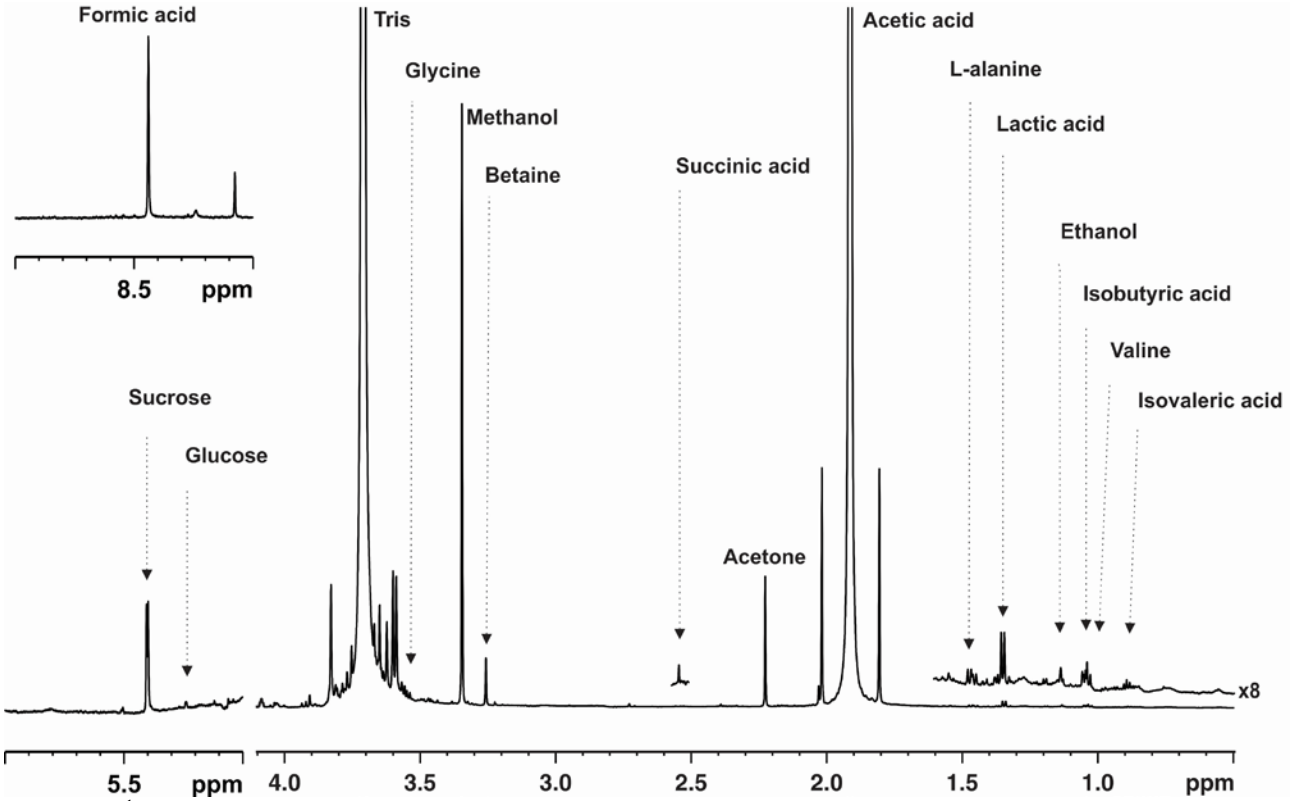

Fig. 1. [1]H-NMR spectrum of lyophilized hemolymph from *H. lucorum* (< 1 kD). 1D-noesy pulse sequence, with pre-irradiation of water, pH=7.35, 298.0 K), with resonance assignment. The spectral regions devoid of signals, that contain the residual water signal and that of reference TSP have been cut away.

Table 1. List of the metabolites identified by NMR in lyophilized hemolymph from *H. lucorum*, reported as the average from 5 controls.

Metabolite	Mass	^{13}C Chemical Shift [a,b]	^{1}H Chemical Shifts (Multiplicity, Coupling Constants) [a,c]	Concentrations (<1kD) Sample (mM)	Concentrations (< 3kD) Sample (mM)
Acetic acid	60.05	23.45	1.92(s)	374.74	311.80
L-alanine	89.09		1.49(d, J=7.2)	0.21	0.11
Betaine	117.15	53.31	3.27(s)	0.16	0.14
Ethanol	46.07	62.80	3.67(q, J=7.1), 1.19(t, J=7.1)	0.20	0.22
Formic acid/formate	46.03		8.46(s)	1.20	1.41
Glycerol	92.09		3.55(dd), 3.64(dd)	7.53	0.09
Glycine	75.07	62.73	3.56(s)	0.33	
Glucose	180.16		5.22(d)	0.67	
Isobutyric acid	88.11		1.05(d, J=7.2)	0.14	0.08
Isovaleric acid	102.13		0.90(d, J=6.5), 1.95(m), 2.04(d, J=7.4)	0.01	0.06
Lactic acid	90.08	20.23	4.12(q, J=7.0), 1.33(d, J=7.0)	0.59	0.45
Succinic Acid	118.09		2.40(s)	0.18	0.15
Sucrose	342.30		5.41(d, J=3.9), 4.22(d), 4.06(t), 3.48(t)	0.97	0.47
Valine	117.15		0.99(d, J=7.0), 1.02(d, J=7.0), 3.57(dt)	0.04	
TSP			-0.0159	0.186	0.186

[a] [1]H chemical shifts (in ppm) are referenced to 0.186 mM TSP at −0.0159 ppm [23]. [b] Determined from HSQC (in ppm). [c] Only chemical shifts of easily distinguished signals in the 1H-NMR spectra are reported. Chemical shifts that are not detectable and/or not distinguishable in either 1D or 2D NMR spectra are not provided.

plants. The available literature data will be discussed here in conjunction with the detected concentrations of these metabolites (Table 1).

Among the detected metabolites, the concentration of acetic acid was the highest one in the [1]H-NMR metabolic profile of hemolymph from

H. lucorum. This high concentration of acetic acid is not a surprise: it is produced by Gram-negative bacilli and Gram-positive cocci [24] and it is found in *Akkermansia*, *Bacteroidetes*, *Bifidobacterium*, *Prevotella*, and *Ruminococcus* [24, 25]. The concentration of other microbial metabolites, such as formic acid, lactic acid, and succinic acid, is moderate. In tissue extracts of many invertebrate species, including *A. subfuscus* and *H. aspersa*, the presence of lactate as a metabolite has been found [20, 26]. In mollusks, succinic acid is the major end product, while lactic acid is a minor product of anaerobic glycolysis. Lactate is an important end product in terrestrial and freshwater gastropods but not in marine species [27].

The concentration of betaine is also moderate. Betaine is known to function as a methyl donor and to facilitate the necessary chemical processes. Its origin is either from food or from oxidation of choline. Betaine insufficiency is associated with metabolic syndrome, lipid disorders and diabetes, and may have a role in vascular and other diseases [28]. Cytosine as a pyrimidine base is a major building block of nucleic acids. Cytosine is unstable and could be converted to uracil by spontaneous deamination. Not surprisingly, the nucleotide of cytosine is the prime mutagenic nucleotide in leukemia and cancer [29].

In the tested hemolymph fractions, only three amino acids (alanine, glycine, and valine) were found at relatively low concentrations. As a non-essential amino acid alanine is produced in the body from either the conversion of the carbohydrate pyruvate or the breakdown of DNA and the dipeptides carnosine and anserine. It is one of the most important amino acids released by muscle, functioning as a major energy source. It also participates in the metabolism of sugars and organic acids and improves the immune system through its contribution to the formation of antibodies. Glycine controls the release of oxygen during cell formation and strengthens the immune system. Glycine takes part in the production of DNA, phospholipids, and collagen, and in the release of energy in the body [30]. The detected valine along with leucine and isoleucine are essential amino acids. Therefore, valine must be ingested, usually as a component of proteins [29].

Recently, various amino acids (glycine, valine, lysine, threonine, asparagine, tyrosine, and histidine) were detected in hemolymph serum of *M. galloprovincialis* by ^{1}H NMR spectroscopy [18]. Contamination with Cu^{2+} leads to increased levels of glucose and amino acids, including lysine, threonine, serine, glutamine, and alanine [18]. A number of free amino acids (for example, Ala, Arg, Glu, Gly, Leu, and Ile), including taurine, have been found previously in various marine mollusks and gastropods [31]. Free taurine, betaine, and glycine seem to be present as metabolites in all marine invertebrates. The concentrations of the following metabolites are also moderate: ethanol, glucose, and sucrose. Ethanol is also found as a metabolite in hemolymph serum from the marine mollusk *M. galloprovincialis* [18] as well as in other invertebrates *Lumbricus rubellus* (Hoffmeister) and *Eisenia andrei* (Savigny) [26]. Ethanol is metabolized in the body to acetyl CoA, an intermediate in glucose metabolism that can be used for energy in the citric acid cycle or biosynthesis [29]. The identified metabolite glucose is a major source of energy for living organisms. The carbohydrate metabolism of gastropods includes D-glucose as the most common monosaccharide, identified in the hemolymph and tissues of many gastropods. Renwrantz et al. detected in hemolymph filtrates of the snail *Helix pomatia* the presence of glucose, galactose, fucose, and mannose [32, 33]. Glycogen and galactogen are the main storage polysaccharides. The presence of sucrose is detected in the hemolymph of snail *Littorina littorea* and *H. aspersa* mucus [19, 33].

In mollusc, classical vertebrate metabolic pathways such as glycolysis, Krebs cycle, and the respiratory chain have also been described [34]. In living organisms, succinic acid is known to be in the form of an anion, succinate, which has many biological roles as a metabolic intermediate. Succinate is known as an intermediate in the Krebs cycle, described in both vertebrates and molluscs [34]. The increase of this metabolite may be due to a decrease in Krebs cycle activity. Succinate is also the end product of the glucose-succinate and aspartate-succinate pathways, which assume anaerobic metabolism in invertebrates and may be associated with the lactate pathway described in molluscs, like the blue mussel *Mytilus edulis* [35].

The ^{1}H NMR detected concentration of glycerol in hemolymph from *H. lucorum* is relatively high in the lyophilized sample (<1 kDa). Glycerol and fatty acids are the basis of the structure of fats. Glycerol can be transformed into glucose, thus providing energy for cellular metabolism [29].

Metabolites with antibacterial activity

Metabolites with known antibacterial activity (acetic, citric, lactic, tartaric, isovaleric acids, and

glycerol) have been recently detected by NMR metabolic analysis of mucus samples from *H. aspersa* [19], which encouraged us to look for metabolites with the same effect in hemolymph from *H. lucorum*.

Some of the detected metabolites such as acetic acid, citric acid, lactic acid, and tartaric acid exhibit inhibitory effect against *Salmonella typhimurium* [36]. Moreover, it is found that 0.5% lactic acid could completely inhibit the growth of various bacteria as *Salmonella enteritidis, Escherichia coli, and Listeria monocytogenes* [37]. The results suggested that the antimicrobial effect is due to physiological and morphological changes in bacterial cells caused by treatment with lactic acid [37]. A study [38] has proved that low concentrations of acetic acid, act as a local antiseptic agent in problematic infections caused by organisms, such as *Proteus vulgaris, Acinetobacter baumannii*, or *Pseudomonas aeruginosa*. Additionally, it was found that pyruvic or succinic acid provides an effective reduction of Salmonella, after treatment of chicken meat [39]. Recently antimicrobial effects of glycerol monolaurate (monolaurin), ethanol, and lactic acid have been determined, either alone or in combination, against *L. monocytogenes* as well as their minimal inhibitory concentrations [40].

Antifungal metabolic activity of betaine derivatives

In a previous study [19], it was established the mucus fraction from *H. aspersa* below 1 kDa and below 3 kDa contained metabolites with known antifungal activity (alpha-ketoisocaproic acid, betaine derivatives, and choline) which encouraged us to look for metabolites with similar effect in hemolymph from *H. lucorum*. It is known that marine gastropods possess high levels of organic osmolytes (e.g. betaine, taurine, and trehalose), but data is scarce for terrestrial snails. Our results showed that betaine is present in fractions below 1 kDa and below 3 kDa from the hemolymph of *H. lucorum* as well as in the mucus from *H. aspersa*. Betaine is not only important for maintaining the osmotic balance in cells but its derivatives showed also antimicrobial potential. In a study [20], it was demonstrated the antifungal activity and mechanism of action of a betaine derivative. It established that lauryl betaine influences ergosterol synthesis in *Cryptococcus neoformans* and *Malassezia restricta* (considered an opportunistic pathogen associated with skin disorders such as seborrheic dermatitis and dandruff).

Antioxidant metabolic activity

The antioxidant capacity of *H. lucorum* hemolymph was recently assessed [17]. 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 and mainly to the antioxidant enzymes in the MW<100 kDa fraction. The experimental results showed that the fraction with MW<1 kDa of *H. lucorum* hemolymph manifested $O^{2\bullet-}$-scavenger effect (about 42% inhibition of NBT reduction at the concentration 24.99 μg/mL) and chelating potential (about 33% inhibitory effect at concentration 90 μg/mL and 130 μg/mL). The fraction, containing compounds with MW below 1 kDa, was analyzed using COMPACT UHPLC-QqTOF Systems (Bruker Daltonics, Germany) in positive ion mode detection, by MS- and MS/MS-experiments [17]. The results from MALDI-MS analysis showed that fraction with MW below 1 kDa from the hemolymph of *H. lucorum* contained a variety of peptides, present primarily as doubly charged ions $[M+2H]^{2+}$ [17]. The molecular structure characteristics (MW, amino acid composition, amino acid sequence and molecular conformation) and hydrophobicity of the peptides are considered to be closely related to their antioxidant activity [41]. Peptides with lower MW can interact with radicals more effectively, and it is easier to exert antioxidant capacity through the intestinal barrier in vivo [42]. Table 2 illustrates primary structures of peptides in fraction below 1 kDa identified by *de novo* sequencing (MALDI-MS/MS experiment) and some other characteristics from ExPASy ProtParam tool [17]. The obtained results showed that the identified peptide structures are characterized with short chains, containing from 7 to 10 amino acid residues, with molecular masses between 757.41 Da - 1079.44 Da, mostly with hydrophobic surface and amphipathic structures. It is known, that 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 [41]. This fact is recently experimentally confirmed with the results of Alexandrova at al. for the antioxidant activity of a fraction below 1kDa [17]. The peptides presented in Table 2 are associated with the demonstrated antioxidant activity of the fraction below 1 kDa unlike the peptides from *H. aspersa* mucus with antimicrobial activity [7, 19]. Amino acid residues such as Tyr, Met, His, Lys, Trp, and Cys are often present in polypeptides with strong antioxidant

Table 2. 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), published in [17].

№	Amino acid sequence of peptides	Measured mass (Da)	Calculated mass (monoisotopic) (Da)	pI	Grand average of hydropathicity (GRAVY)
1	γ- ECG (glutathione)	308.05 $[M+H]^+$	307.08	4.00	
2	VVLIKAKGK	319.22 $[M+3H]^{3+}$	954.66	10.30	0.711 (hydrophobic)
3	GIPLEMV	379.70 $[M+2H]^{2+}$	757.41	4.00	1.271 (hydrophobic)
4	SSPPFVM	382.68 $[M+2H]^{2+}$	763.36	5.24	0.586 (hydrophobic)
5	KVAPYPQ	401.72 $[M+2H]^{2+}$	801.44	8.59	-0.843 (hydrophilic)
6	VVMKELS	403.22 $[M+2H]^{2+}$	804.44	5.97	0.843 (hydrophobic)
7	GPLKIPLL	425.79 $[M+2H]^{2+}$	849.57	8.75	1.050 (hydrophobic)
8	AEPKIGKI	428.26 $[M+2H]^{2+}$	854.52	8.64	-0.312 (hydrophilic)
9	LAVSKLLY	453.78 $[M+2H]^{2+}$	905.56	8.59	1.425 (hydrophobic)
10	KWFKFGN	463.74 $[M+2H]^{2+}$	925.48	10.00	-1.000 (hydrophilic)
11	VSEGMIVSI	467.74 $[M+2H]^{2+}$	933.49	4.00	1.533 (hydrophobic)
12	GTLSSLLNF	476.26 $[M+2H]^{2+}$	950.51	5.52	0.889 (hydrophobic)
13	FLGDSTNLI	490.25 $[M+2H]^{2+}$	978.51	3.80	0.667 (hydrophobic)
14	AFQLm*KQV	490.76 $[M+2H]^{2+}$	979.52	8.80	0.450 (hydrophobic)
15	EIKLSDQY	498.25 $[M+2H]^{2+}$	994.50	4.37	-1.025 (hydrophilic)
16	ALSAWNAHE	499.73 $[M+2H]^{2+}$	997.46	5.24	-0.300 (hydrophilic)
17	HGMPLDLLD	505.75 $[M+2H]^{2+}$	1009.49	4.20	0.122 (hydrophobic)
18	STENDPSSML	540.72 $[M+2H]^{2+}$	1079.44	3.67	-0.950 (hydrophilic)

activity. The imidazole group of His is related to its metal chelation, hydrogen supply and lipid peroxidation capabilities [43]. Cysteine containing thiol can directly interact with radicals and has an important contribution to the antioxidant activity of peptides. Therefore, the presented peptide structures showed various amino acid residues, but mostly Val, Leu/Ile, Pro, Lys, Phe, Met, His, Trp, and Tyr (Table 2). We hypothesize that hydrophobic amino acids (Leu/Ile,Val, Met, Phe and Pro), aromatic amino acids (Trp, Phe and Tyr), as well as Lys and His contribute to the antioxidant activity established in the recent study of Alexandrova at al. [17]. Amino acid sequences of detected peptides in hemolymph fraction below 1 kDa from garden snail *H. lucorum* are rather different from the identified peptides in *H. aspersa* mucus [3, 7, 19]. In addition, the MALDI-MS analysis revealed tripeptide glutathione (GSH), detected as $[M+H]^+$ at m/z 308.046 Da. Glutathione also is identified in mucus fraction below 1 kDa from *H. aspersa* snails [19] and in hemolymph serum of mussels *M. galloprovincialis* [18]. GSH is the major endogenous intracellular antioxidant that is able to protect cell structures from oxidative damage by reacting directly with the reactive oxygen species and acts as a co-substrate of the antioxidant enzyme glutathione peroxidase [44].

Recently, *in vitro* tests established the antioxidant potential of lactate ions at different concentrations [45]. The results showed that lactate ion could prevent lipid peroxidation by neutralizing free radicals, such as O^{2-} and OH, but not lipid radicals. Therefore, lactate ion might be considered as a potential antioxidant agent.

Other compounds with antioxidant metabolic activity are some detected Krebs cycle intermediates, which act as energy substrates in mitochondria and manifest antioxidant neuroprotective effects on the brain, in neuronal cells, against oxidative stress [46]. It was found that pyruvate, oxaloacetate, and α-ketoglutarate, preserve HT22 cells from hydrogen peroxide-mediated toxicity. Because these intermediates did not have any toxic effects (at least up to 10 mM), they can be used in the treatment of chronic neurodegenerative diseases.

CONCLUSION

A protocol for the determination of metabolites in hemolymph from *H. lucorum* by NMR spectroscopy has been developed. The metabolic profiles of the two low molecular weight fractions (<1kD and <3kD) as well as the concentration of metabolites are very similar. A number of metabolites with known antioxidant, antibacterial, and antimicrobial activities have been detected by NMR metabolic analysis and tandem mass spectrometry of hemolymph samples from *H. lucorum*.

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Electronic Supplementary Data available here.

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