https://doi.org/10.31925/farmacia.2024.4.20 **ORIGINAL ARTICLE**

ISOLATION, IDENTIFICATION AND REDOX-MODULATION CAPACITY OF HEMOLYMPH'S SUBUNITS FROM *RAPANA VENOSA* INHABITING THE BULGARIAN BLACK SEA

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Abstract

This study aimed to evaluate the radical scavenging potential and metal-chelating activity of fractions from haemolymph with molecular weight (MW) of 1 - 10 kDa, 10 - 50 kDa and 30 - 100 kDa, and hemocyanin subunits RvH1 and RvH2, isolated from *Rapana venosa*, originating from the Black Sea. The compounds were tested *in vitro* as inhibitors of superoxide ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$) generation, against DPPH radicals, as well as for their iron reduction and chelation capacity and peroxidase activity. We showed a good antioxidant effect of *R. venosa* haemolymph fractions (especially 30 - 100 kDa) in contrast to hemocyanin subunits. The mechanisms underlying these properties can be assumed to be due at least in part to high proline content, reducing capacity by one-electron transfer, and peroxidase activity.

Rezumat

În cadrul studiului ne-am propus evaluarea potențialului de captare a radicalilor și activității de chelare a metalelor din fracțiile de hemolimfă cu greutate moleculară (MW) de 1 - 10, 10 - 50 și 30 - 100 kDa și din subunitățile de hemocianină RvH1 și RvH2, izolate din *Rapana venosa*, originară din Marea Neagră. Compușii au fost testați *in vitro* ca inhibitori ai generării de radicali superoxid ($O2^{\bullet-}$) și hidroxil ($^{\bullet}OH$) împotriva radicalilor DPPH, precum și pentru capacitatea lor de reducere și chelare a fierului și activitatea peroxidazică. S-a demonstrat un efect antioxidant bun al fracțiilor de hemolimfă a *R. venosa*, spre deosebire de subunitățile de hemocianină. Se poate presupune că mecanismele care stau la baza acestor proprietăți se datorează cel puțin parțial conținutului ridicat de prolină, capacității de reducere prin transferul unui electron și activității peroxidazei.

Keywords: hemocyanin subunits, redox-modulation activity, *Rapana venosa*, hemolymph

Introduction

Rapana venosa (Valenciennes, 1846) is a marine snail introduced into the Black Sea in the early 1940s. As a voracious predator characterized by high fertility, growth rate and tolerance to unfavourable environmental conditions, it displaces the native bivalve fauna, occupying a significant place in the Black Sea ecosystem [1]. Being a rich source of macro- and micronutrients with beneficial health effects, *R. venosa* has become a valuable food with increasing economic importance in the Black Sea region [2]. In addition to its nutritional value, it has been found that fractions isolated from the haemolymph of this marine snail have antibacterial [3], antifungal [4], antiproliferative [5] and antitumor [6] effects. They are probably due to the presence in the tested fractions of specific proteins, causing direct and indirect effects [3, 6]. Methanol extracts of dry *R. venosa* soft tissues have been shown to have radical-scavenging capacity using synthetic radicals such as 1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2 amino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals, cupric-reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) [1]. It has been suggested that this antioxidant activity is due to the significant amount of total phenolics.

The antioxidant potential of *R. venosa* arouses interest due to the possibility of its use for the prevention and therapy of pathological conditions associated with oxidative stress (OS). Many diseases: neurodegenerative (Alzheimer's disease, Parkinson's disease, etc.), cardiovascular (atherosclerosis), pulmonary (asthma, respiratory stress syndrome, pulmonary fibrosis), renal inflammation, metabolic (diabetes mellitus, neoplastic) have oxidative aetiology and application of phytocomplexes from

plant or substances with animal origin have beneficial healthy effects [7].

Natural products like *R. venosa* haemolymph are complex mixtures containing various bioactive compounds. However, too often, the complexity of their composition makes it difficult to identify and understand the mechanisms by which the components contained in them are responsible for realizing their pharmacological effects. Therefore, biological fluids' fractionations based on the molecular masses of the substances they contain can be helpful as part of a promising therapeutic strategy. Furthermore, the antioxidant properties may allow the development of remedies for preventing and treating diseases with oxidative aetiology. So, in this work, we aimed to isolate, identify and evaluate the redox-modulating potential of three haemolymph fractions with molecular weight (MW) of 1 - 10 kDa, 10 - 50 kDa and 30 - 100 kDa, as well as two hemocyanin subunits RvH1 and RvH2 from *R. venosa* collected from the Bulgarian Black Sea.

Materials and Methods

Isolation and processing of the biological material

The haemolymph was obtained from the marine snail *R. venosa* collected from their natural habitats in the Bulgarian Black Sea. The foot muscles were cut, filtrated and centrifuged for 20 min at 10,000 rpm and 4°C to remove rough particles and haemocytes [6]. The fractions 1 - 10 kDa, 10 - 50 kDa and 30 - 100 kDa were isolated after ultrafiltration of the crude haemolymph extract using membrane Millipore Ultra-filtration Membrane Filters. The fraction above 100 kDa was ultracentrifuged for 180 min at 22,000 rpm and 4°C using a Kontron-Hermle A8.24 rotor (centrifuge CENTRIKON). The sediment containing the native hemocyanins (RvH) was dissolved in 50 mM Tris buffer (pH 7.5) up to a concentration of 10% and was purified by gel filtration on the Sephadex G-200 column. The RvH was dialyzed against 0.13 M glycine/NaOH buffer, pH 9.6. The separation of the structural sub-units RvH1 and RvH2 was carried out by ion-exchange chromatography using a 16/10 Q Sepharose High-Performance column equilibrated with 50 mM Tris/HCl buffer and 10 mM EDTA (pH 8.5) with a linear gradient of 0.0 – 0.5 M NaCl by FPLC system as described previously [6].

Protein fractions analysis

Protein fractions were analysed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the Laemmli method with modifications [8] and visualized by staining with Coomassie Brilliant Blue G-250. Molecular weights of standard proteins from Bio-Rad between 10 - 250 kDa were used.

DPPH analysis

The DPPH analysis was conducted after Brand-Williams *et al.* [9]. The tested fractions of haemolymph and hemocyanin subunits were added to the freshly made 0.1 mM DPPH in methanol solution at a ratio of 1:1. The mixture was incubated for 30 minutes in the dark, and the absorbance at 517 nm was read. As a control sample, methanol was added to the DPPH solution instead of the tested substances. The antioxidant activity was computed as follows:

Inhibition of DPPH radical (%) = [(A517 control - A517 probe)/A517 control] x 100.

FRAP analysis

FRAP analysis was performed according to Benzie *et al*. [10] with some modifications. The following solutions were prepared: (i) 0.03 M acetate buffer, pH 3.6; (ii) 1.0 mM 2,3,5-triphenyltetrazolium chloride (TPTZ), in 40 mM HCl and (iii) 1.5 mM $FeCl_3$ x 6 H_2O and were mixed in a ratio 10:1:20. Each sample (50 µL) was added to of the reaction mixture (1.5 mL) and the test blank was prepared with H_2O. The absorbance was measured at 593 nm after 4 minutes of incubation at 37°C. Trolox was used to create a calibration curve in the following concentrations: 4, 8, 16 and 32 µM. Micromoles Trolox/mg protein was used to express the antioxidant activity.

Iron Chelation

The method of Dinis *et al*. [11] for analysing iron chelation capacity was used. The reaction mixture, consisting of 0.74 mL of 0.1 M acetate buffer (pH 5.25), 0.02 mL of 2 mM ferrous sulphate solution in 0.2 M hydrochloric acid and 0.2 mL of a sample containing various concentrations of the tested fractions were mixed for 10 - 15 seconds. After adding 0.04 mL of ferrozine solution (5 mM), the samples were incubated at room temperature in the dark for 10 minutes, and the mixture's absorbance at 562 nm was read.

Activity (%) = [(Ac - As)/Ac] x 100,

where, Ac is the absorbance of the control solution (without biological material) and As is the absorbance of the sample solution, was used to calculate the iron chelating capacity.

Superoxide anion radicals generating systems

The superoxide anion radicals ($O_2^{\bullet-}$) were generated photochemically in a medium containing 50 mM potassium phosphate buffer, pH 7.8; 1.17 x 10^{-6} M riboflavin; 0.2 mM methionine; 2 x 10^{-5} M KCN and 5.6 x 10^{-5} M nitro-blue tetrazolium (NBT) [12]. The reduction of NBT by $O_2^{\bullet-}$ to a blue formazan product, in the absence (control) and presence of increasing concentrations of the tested substances, was measured at 560 nm. The antioxidant capacity of the three haemolymph and the two hemocyanin subunits RvH1 and RvH2 against $O_2^{\bullet-}$ was expressed as a concertation of tested substances that give 50% inhibition of NBT reduction.

Hydroxyl radicals generating systems

Hydroxyl radicals ($^{\bullet}OH$) were generated in a system containing 10 mM potassium phosphate buffer, pH

7.4; 0.1 mM EDTA-Fe^{2+}, 0.5 mM H_2O_2 and 2 mM deoxyribose [13]. Samples with increasing concentrations of the tested substances were incubated for 30 min at 37°C to obtain the thiobarbituric acid-reactive substances (TBARS) resulting from the deoxyribose degradation that serves as a measure to produce •OH. The reaction was stopped by adding 0.2 mL 2.8% trichloroacetic acid, 0.1 mL 5 N HCl and 0.2 mL thiobarbituric acid (2% w/v in 50 mM NaOH). After that, the samples were incubated at 100°C for 15 min for colour development. The absorbance was read at 532 nm, and the antioxidant capacity against •OH of the tested fractions was expressed as a percentage of the control.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured using a kit CGP 1, purchased from Sigma-Aldrich Co. LLC, USA. The absorbance was measured at 340 nm, and the enzyme activity was calculated as U/mg protein using a molar extinction coefficient of 6.22 x 10^6 $M^{-1}.cm^{-1}$.

Statistical analysis

All measurements were made in triplicate. Descriptive statistics, Shapiro-Wilks test of normality and One-Way ANOVA with Tukey post hoc test were applied using GraphPad Prism 7.0. The figures presented the data as the mean ± standard error of measurement (SEM).

Results and Discussion

Protein fractions analysis

It is known that the haemolymph of marine snail *R. venosa* contained above 90% hemocyanin with molecular mass (MW) of around 8000 kDa characterized previously [4, 6]. In addition to hemocyanin, the *R. venosa* haemolymph contains other proteins. We isolated two protein subfractions from the 10 - 100 kDa fraction as described previously [4]. Several protein bands were observed in the fractions, as in the fraction with MW 10 - 100 kDa they were at ~ 24 kDa, between ~ 37 - 50 kDa and ~ 65 kDa; in the fraction with MW10 - 50 kDa at ~ 14 kDa, ~ 18 kDa, between 37 - 40 kDa, ~ 50 kDa, while for the fraction with MW 30 - 100 kDa three leading protein bands were established at ~ 50 kDa, ~ 65 kDa and ~ 100 kDa (Figure 1).

$O_2^{\bullet-}$ scavenger effect

The highest $O_2^{\bullet-}$ scavenger effect had the fraction with MW 30 - 100 kDa followed by the fraction with MW 10 - 50 kDa and those with MW 1 - 10 kDa that demonstrated 50% inhibition of NBT reduction at 28.4 µg/mL, 37.3 µg/mL and 65.9 µg/mL respectively. The RvH1 and RvH2 were less potent, inhibiting NBT reduction by 50% at about 100 µg/mL (Figure 2).

•OH scavenger effect

The most potent •OH scavenger effect showed the fractions with MW 1 - 10 kDa and MW 10 - 50 kDa with more than 70% inhibition of TBARS formation at the highest concentrations tested (25 mg/mL, final concentration). The RvH1 and RvH2 had a significantly lower effect - below 50% inhibition at the same concentration (Figure 3).

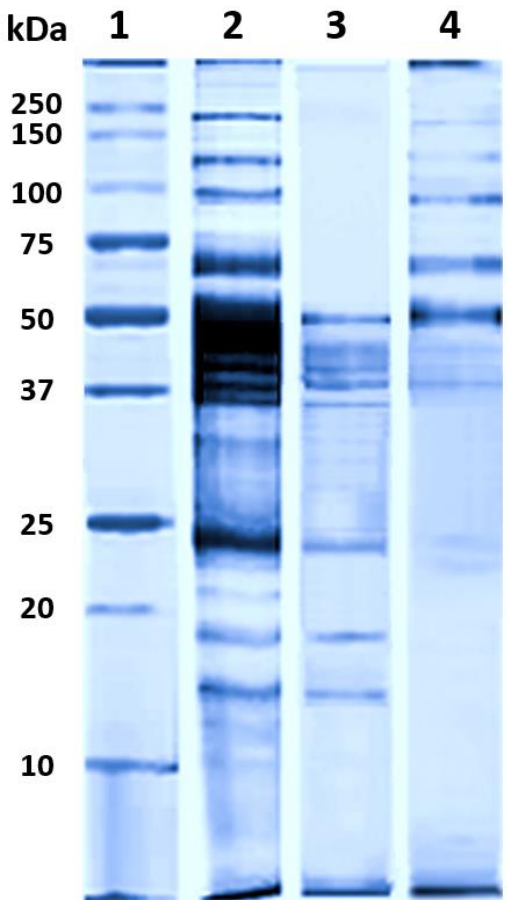

Figure 1.

12.0% SDS-PAGE analysis, visualized by staining with Coomassie G-250, position: 1) molecular weights of standard proteins from Bio-Rad; 2) 10 - 100 kDa fraction form *R. venosa* haemolymph; 3) 10 - 50 kDa fraction from *R. venosa* haemolymph; 4) 30 - 100 kDa fraction from *R. venosa* haemolymph

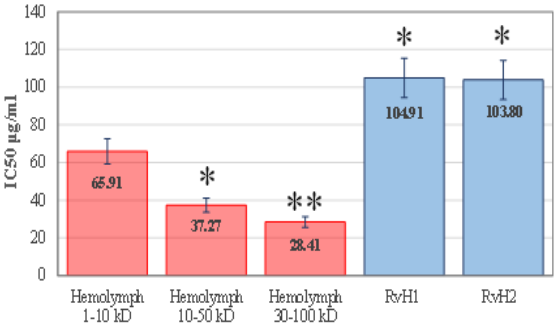

Figure 2.

Superoxide radical scavenger effect of the haemolymph fractions with MW 1 - 10, 10 - 50 and 30 - 100 kDa and the hemocyanin subunits RvH1 and RvH2, isolated from *R. venosa*; * significant difference *vs.* haemolymph 1 - 10 kD at $p < 0.05$; ** significant difference *vs.* haemolymph 1 - 10 kDa at $p < 0.01$

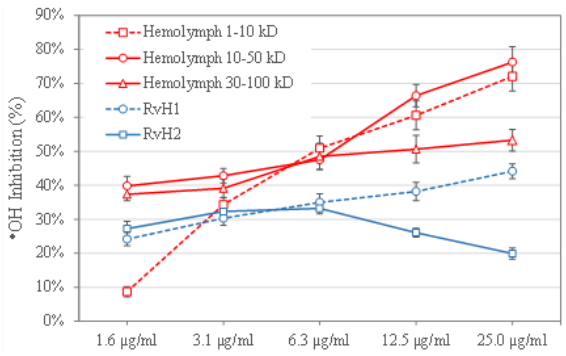

Figure 3.
Hydroxyl radical scavenger effect of the haemolymph fractions with MW 1 - 10, 10 - 50 and 30 - 100 kDa and the hemocyanin subunits RvH1 and RvH2, isolated from *R. venosa*

Since the hemocyanin subunits RvH1 and RvH2 did not demonstrate good antioxidant properties, the subsequent analyses were performed with only the haemolymph fractions 10 - 50, 30 - 100 and 50 - 100 kDa.

DPPH analysis

The DPPH test showed that fraction of 50 - 100 kDa had a higher radical inhibitory potential (15.6 ± 2.3%), compared to 10 - 50 kDa (11.0 ± 2.3%) and 30 - 100 kDa (10.8% ± 2.1%) fractions (Figure 4). The difference between fractions was not significant.

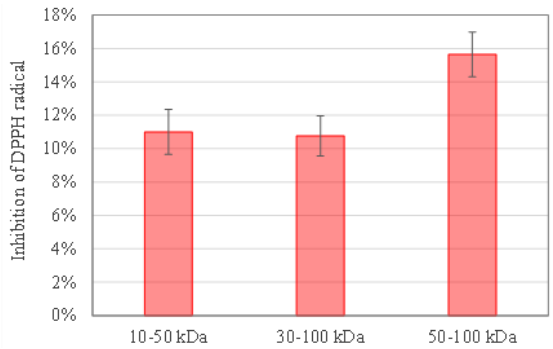

Figure 4.
DPPH inhibition by different fractions of *R. venosa* haemolymph

FRAP analysis

The FRAP test showed that fraction of 30 - 100 kDa had the highest reducing power (1.94 ± 0.043 µmol Trolox/mg protein) compared to 10 - 50 kDa (1.63 ± 0.040 µmol Trolox/mg protein) and 50 - 100 kDa (1.48 ± 0.05 µmol Trolox/mg protein) fractions (Figure 5).

Iron Chelation

All three fractions of *R. venosa* haemolymph did not demonstrate any iron chelation effect.

Glutathione peroxidase activity

The glutathione peroxidase activity of 8.52 ± 0.310 U/mg/pr showed only the 30 - 100 kDa fraction.

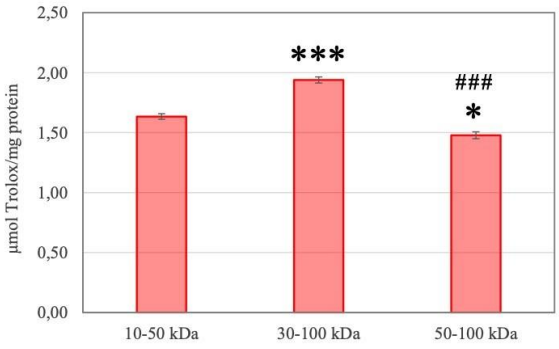

Figure 5.
Reduction of Fe^{3+} in complex with TPTZ by *R. venosa* haemolymph fractions. * - $p < 0.05$ *vs.* 10 - 50 kDa; *** - $p < 0.001$ *vs.* 10 - 50 kDa; ### - $p < 0.001$ *vs.* 30 - 100 kDa

In recent years, there has been a trend of renewed interest in natural products to prevent pathological conditions and therapy. This tendency is determined by their beneficial effects, tolerability by the human organism and the possibility of overcoming multidrug resistance due to their complex composition, and in this way, avoiding combined therapy with several synthetic drugs. Natural products with antioxidant activity are of particular interest, as oxidative stress (OS) causes many diseases [7]. In order to elucidate the biological effect of natural products and their extracts, nowadays, the identification and isolation of the most active ingredients and the mechanism of their action is sought. Studies on medicinal plants' phytochemical composition, pharmacokinetic and pharmacodynamic profile, molecular mechanisms and interactions with other substances are widely presented [14-17]. However, unlike plant products, those of animal origin are relatively less studied. Thus, the present work evaluates the antioxidant effect of three fractions of haemolymph and the two subunits of hemocyanin from *R. venosa*.

The superoxide and hydroxyl radicals are generated naturally in organisms, but when they are generated in excess or reduced antioxidant protection of cells is present, they lead to OS induction. Therefore, the $O_2^{-\bullet}$ and $^{\bullet}OH$ scavenger activity of fractions of *R. venosa* haemolymph and subunits of *R. venosa* hemocyanin were analysed herein. The results obtained in this study showed a higher antioxidant effect on the haemolymph fractions compared to the hemocyanin subunits (RvH1 and RvH2) in both radical-generating systems (Figure 1 and Figure 2). Haemocyanins, dissolved in the *R. venosa* haemolymph, were found to be distinguished by high carbohydrate content [18, 19]. Although there is some evidence that glycoproteins may have antioxidant activity [20], likely the specific monosaccharide composition of the hemocyanin sub-units tested in this study does not contribute to the presence of significant antioxidant properties. On the other hand, the haemolymph of *R. venosa* is a complex

mixture of biochemically and pharmacologically active components such as peptides and proteins [3]. The better $O_2^{-\bullet}$ scavenger effect of the haemolymph fractions compared to the hemocyanin subunits is probably due to their relatively high proline content. It has been found that most of the peptides purified from the haemolymph of Rapana snails are highly cationic with a proline-rich N-terminal region [3]. Indeed, peptides rich in proline were isolated from the plasma of other marine organisms, *e.g.*, shrimp *Penaeus vannamei* and *Penaeus stylirostris* [21], and they have high homology with peptides from the haemolymph of *R. venosa* [3]. Although the protective effect of proline against $O_2^{-\bullet}$ has been debated [22, 23], a recent study showed that *in vitro* proline can eliminate these radicals *via* an electron transfer reaction [24]. In the present study, the 30 - 100 kDa fraction demonstrated the highest $O_2^{-\bullet}$ scavenger activity. This fraction also showed a statistically reliable higher effect in the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions (FRAP assay), which confirms the electron transfer reaction-based mechanism of the antioxidant action. The proline contribution to the $^{\bullet}OH$ scavenging is well established. The process is assumed to be via a proline-proline cycle without the consumption of proline [23, 25]. *In vitro* experiments have shown that at least two $^{\bullet}OH$ radicals are consumed *per* one proline molecule [25]. Proline has been proposed to be added to non-enzymatic antioxidants required to mitigate the ROS impact in microorganisms, plants and animals [26]. Furthermore, the presence of proline in peptides and proteins also contributes to the manifestation of antioxidant properties [27, 28] and has a beneficial effect on OS disorders. Proline-rich oligopeptides have neuroprotective activity against H_2O_2-induced OS [29]. It is known that not only the individual properties of amino acids, but also nearby amino acids, steric structure and molecular weight are decisive for the biological activity of peptides and proteins. In this study, low molecular weight fractions (1 - 10 and 10 - 50 kDa) of *R. venosa* haemolymph showed the highest $^{\bullet}OH$ scavenger effect (Figure 3). It was found that short-chain peptide fractions (with MW < 3 kDa), compared to those with a higher MW exhibited higher antioxidant activities [30].

A similar highest $O_2^{\bullet-}$ scavenger effect of the highest molecular weight fraction of haemolymph (30 - 100 kDa) observed herein (Figure 2) was established for the highest molecular weight fraction of the haemolymph of *Helix lucorum* [31]. This effect may be due to the presence of superoxide dismutase (or some structures with superoxide dismutase-mimetic functions) in this fraction. Antioxidant enzymes with molecular weights in this range have been found in the haemolymph of various mollusks, mainly terrestrial: *Lymnaea stagnalis, Haliotis discus, Haliotis diversicolor supertext, Helix lucorum* [31, 32].

Our recent study of the major proteins in the haemolymph of *R. venosa* using MALDI-MS, MS/MS analysis and bioinformatics found high homology of the MW ~ 100 kDa protein from *R. venosa* with known peroxidase-like proteins in various mollusk species such as peroxidase-like protein 2 and peroxidase-like protein 3 from *Lottia gigantea* (Uniprot ID: B3A0P3, with theoretical MW 92943 Da), peroxidase-like protein from *Margaritifera margaritifera* (Uniprot ID: H2A0M7) and peroxidase-like protein from *Mizuhopecten yessoensis* (Uniprot ID: A0A210Q736) [33]. Due to this finding, we tried to establish the glutathione peroxidase activity of the tested fractions of *R. venosa* haemolymph. Only the 30 - 100 kDa fraction showed a remarkable glutathione peroxidase activity of 8.52 ± 0.310 U/mg protein. As compared with terrestrial organisms, the reduced glutathione (GSH) concentrations and the activities of GSH-dependent enzymes: glutathione reductase, glutathione peroxidase and glutathione transferase in aquatic molluscs are much higher [34]. Glutathione peroxidase (GPx) is a cytosolic enzyme that reduces hydrogen peroxide to water. In this way, GPx limits the harmful effects of $^{\bullet}OH$ that could be generated from H_2O_2 in the presence of transition metals (Fenton reaction). Furthermore, it has been demonstrated that GPx is implicated in the mechanisms of many common and complex diseases, including cancer, diabetes and cardiovascular disease [35]. However, we can only assume that the manifested glutathione peroxidase activity of the 30 - 100 kDa fraction may have an essential role in antioxidant protection when applied externally to treat some skin diseases and wound healing. It will be interesting to explore further studies in appropriate experimental models to test this assumption.

Conclusions

In this study, we presented for the first time an assessment of the antioxidant activities of three fractions of haemolymph with a molecular weight of 1 - 10 kDa, 10 - 50 kDa and 30 - 100 kDa, as well as two hemocyanin subunits RvH1 and RvH2, isolated from *R. venosa*. Both RvH1 and RvH2 had insignificant antioxidant effects in contrast to haemolymph fractions. The highest $O2^{\bullet-}$ scavenger effect had the haemolymph fraction with MW 30 - 100 kDa followed by the MW 10 - 50 kDa fraction. The most potent $^{\bullet}OH$ scavenger effect showed the MW 1 - 10 kDa and 10 - 50 kDa fractions. Against DPPH radicals, all tested fractions had similar antioxidant potential. FRAP assay established the highest reducing effect of the 30 - 100 kDa fraction. All three haemolymph fractions did not demonstrate any iron chelation effect. Only the 30 - 100 kDa fraction showed glutathione peroxidase activity. For the first time, this study showed a good antioxidant effect of *R. venosa* haemolymph fractions (mainly 30 - 100 kDa fraction) in contrast to hemocyanin

subunits. It can be assumed that the mechanisms underlying these properties are due to the high proline content, the reducing capacity by one electron transfer and the peroxidase activity. The good redox-modulating effect of haemolymph fraction with MW especially that of 30 - 50 kDa suggests its possible application in pathological conditions with disturbed redox-status and oxidative stress aetiology. Undoubtedly, future *in vivo* research is needed to prove the positive effect of this fraction.

Conflict of interest

The authors declare no conflict of interest.

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