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Antibacterial activity of bioactive fractions from mucus and hemolymph of different snails species and crab

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This study aims to evaluate the antibacterial activity of six samples contained biologically active fractions isolated from various molluscs species (garden and marine snails) and one representative of the arthropods (*Carcinus aestuarii*, also known as Mediterranean green crab). The minimum inhibitory concentration (MIC) values for *Escherichia coli* and *Brevindomonas diminita* were in ranges of 145-682.5 μg/ml and 290-431.5 μg/ml, respectively. The dissociated hemocyanin from *Carcinus aestuarii* (CaH) showed the strongest MIC (145-290 μg/ml) and effective concentrations (EC_{50} values 75.46-112.2 μg/ml) against all Gram-negative bacterial strains. Two of the tested samples – protein fraction from *Helix aspersa* mucus with Mw above 20 kDa and peptide fraction from *Helix lucorum* hemolymph with Mw below 10 kDa, demonstrated selective antibacterial activity against *E. coli* or *B. diminuta*, respectively. The results in this study showed that the bioactive fractions isolated from mucus and hemolymph of different snail species and isoforms of *C. aestuarii* hemocyanin could be considered as new natural antibacterial agents with potential biomedical application. Further studies are needed to confirm the antibacterial activity with a wider range of bacterial strains.

Key words: antibacterial activity; *Helix aspersa*; *Helix lucorum*; *Rapana venosa*; *Carcinus aestuarii*; minimum inhibitory concentration

INTRODUCTION

Increasing antimicrobial resistance to conventional antibiotics has become a global health threat in the last few decades. Bacteria and other microbes develop mutations that protect them against antibiotics and other antimicrobial drugs, meaning that infections will become more difficult, even impossible to treat. The 700,000 or more deaths that antimicrobial resistance now causes every year could grow to 10 million by 2050. New antibacterial agents are urgently needed – for example, to treat carbapenem-resistant gram-negative bacterial infections as identified in the World Health Organization (WHO) priority pathogen list. It is worth noting that for *E. coli*, *K. pneumonia* and other representatives of the family *Enterobacteriaceae*, the proportion of bacteria resistant to commonly used specified antibacterial drugs exceeded 50% in many WHO regions [1].

Antimicrobial peptides are important in the first line of the host defence system of many animal species [2]. Their value in the innate immunity lies in their ability to function without either high specificity or memory. In recent years, it has been widely recognized that many organisms use antimicrobial peptides (AMPs) as part of their host defence system against the invasion of microorganisms [3, 4]. Glycoprotein "hemocyanin" and antimicrobial peptides from the hemolymph and mucus are important component of the innate immunity [5]. The hemolymph from snail contains bioactive compounds as glycans, peptides, glycopeptides, and proteins. Many of them have been discovered in recent years [6]. One of them is a hemocyanin, an oxygen-transport glycoprotein found in the hemolymph of both mollusc and arthropod [7]. Moreover, the mucus of land snails is a rich source of peptides and proteins with broad-spectrum antibacterial activity. The following compounds are known to have activity against a large spectrum of microorganisms including bacteria, filamentous fungi, viruses, protozoan and metazoan parasites [8, 9].

Phyla Mollusca and Arthropoda constitute a large reservoir for pharmacologically active compounds. Mucus from *H. aspersa* and hemolymph from *H. lucorum* and *R. venosa*, as well hemocyanin from *C. aestuarii* are a complex mixture of bioactive components with promising application in treatment of pathogenic bacteria [9-

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12]. Dolashki *et al.* (2020) found that three peptide fractions from garden snail *Cornu aspersum* (also recognised as *Helix aspersa)*, those with Mw <3 kDa, Mw 3–5 kDa and Mw 5–10 kDa, have antibacterial activity against the Gram+ bacterial strain *Brevibacillus laterosporus*, and compounds with Mw 10–30 kDa revealed a very high inhibition effect against *E. coli* [10]. Researches indicated that the mucus fraction with Mw between 30 and 100 kDa from the common brown snail *H. aspersa* had a strong antibacterial effect against several strains of *Pseudomonas aeruginosa* and a weak effect against *Staphylococcus aureus* [13]. Mucus from the African giant land snail *Achatina fulica* also inhibited the growth of *S. aureus*, but the broad spectrum of activity reported by other workers was not observed [13].

Furthermore, it has been reported that several peptides from the hemolymph of the garden snails *H. lucorum* and *H. aspersa* exhibit a broad spectrum of antimicrobial activity against *S. aureus, Staphylococcus epidermidis, E. coli, Helicobacter pylori* and *Propionibacterium acnes* [5, 12].

The present study aimed to evaluate the antibacterial activity of six fractions isolated from mucus and hemolymph of three snail species - *Helix aspersa, Helix lucorum, Rapana venosa* and a crab species *Carcinus aestuarii* against Gram-negative bacteria for potential biomedical application.

EXPERIMENTAL

Sample collection and preparation of extracts

In this study, six samples were prepared and tested, as shown in Table 1.

S1 contained *C. aestuarii* hemocyanin (CaH), dissociated in subunits (isoforms) with molecular mass ~75 kDa. The native CaH was obtained from hemolymph of Mediterranean crab *C. aestuarii* as described [14]. The native protein was dissociated into its subunits, by dialysis for 24 h against 100 mM sodium bicarbonate buffer, pH 9.5, containing 20 mM EDTA and 1 M urea. Before the antimicrobial assay, the dissociation buffer was replaced with 50 mM Tris-HCl buffer, pH 7.6.

The mucus was collected from garden snails *H. aspersa* grown in Bulgarian farms using patented technology, as described [10]. Fractions S2 and S3 were obtained by separation of the purified mucus extract by ultrafiltration on polyethersulfone membrane filters with pore size 20 kDa and 2 kDa (Microdyn Nadir^{TM}from STERLITECH Corporation, MWCO 20 kDa Goleta, CA, USA; Sartorius Stedim Biotech, 2 kDa, Göttingen, Germany).

Table 1. List of fractions isolated from mucus and hemolymph of different snail species and hemolymph of crab *C. aestuarii*.

Sample	Purified fractions and compound mixtures (μg/ml)	Source
S1	Dissociated hemocyanin (CaH) (9 290)	*Carcinus aestuarii*
S2	Fraction from mucus with Mw above 20 kDa (2 730)	*Helix aspersa*
S3	Fraction from mucus with Mw 2-20 kDa (775.1)	*Helix aspersa*
S4	Fraction from hemolymph with Mw below 10 kDa (1 726)	*Helix lucorum*
S5	Fraction from hemolymph with Mw 10-50 kDa (1 305)	*Rapana venosa*
S6	Hydrolysate of hemocyanin subunit βc – HaH with Mw below 30 kDa	*Helix aspersa*

The hemolymph from garden snails *H. lucorum* and marine snail *R. venosa* were obtained as described previously in [15, 6]. Fractions S4 and S5 were obtained after ultrafiltration of *H. lucorum* hemolymph and respectively *R. venosa*, using membrane filters from 10 kDa, 50 kDa and 100 kDa NMW (EMD Millipore Corporation, regenerated cellulose, Billerica, MA, USA).

S6 was obtained after proteolytic hydrolysis of structural subunit βc of *H. aspersa* hemocyanin (βc – HaH). The subunit βc – HaH (Mw ~400 kDa) was obtained after dissociation of native hemocyanin HaH as described [16]. After trypsin digestion of βc–HaH (ratio 1:200, trypsin:hemocyanin), at temperature 37ºC for 8 hours, the proteolytic mixture was subjected to membrane ultrafiltration by Amicon® Ultra-15 centrifugal tube with 30 kDa membrane. So, the S6 contains peptides and polypeptides from βc – HaH with MW below 30 kDa.

Antibacterial activity assay

Bacterial strains

Gram-negative strains *E. coli* DSM 1607, *E. coli* DSM 1116 and *B. diminuta* DSM 1635 were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and used for the antibacterial screening. Strains were selected according to the list for antibiotic susceptibility

testing. Bacterial strains were subcultured in 10 ml nutrient broth for 24 h at 37 ºC (*E. coli* DSM 1607, *E. coli* DSM 1116) and for 24 h at 28 ºC (*B. diminuta* DSM 1635) and kept frozen at −80 °C in broth media supplemented with 25% (v/v) glycerol as a stock culture. Before the antimicrobial assays, they were inoculated into the nutrient broth and were incubated at 37 °C (*E. coli* DSM 1607, *E. coli* DSM 1116) and at 28 °C (*B. diminuta* DSM 1635) for 24 h.

Microdilution assay

Minimum inhibitory concentrations (MIC) was determined by using the broth microdilution method according to Clinical Laboratory and Standards (CLSI) guidelines [17]. Briefly, the bacterial suspension cultured to the logarithmic phase was diluted to 0.5 Mcfarland Standard (approximately 1.5×10^8 CFU/mL) and then diluted 150 times to 1×10^6 CFU/mL using nutrient fractions. A 50 µl volume of serial twofold dilutions of BACs with nutrient broth was dispensed in 96-well microtiter plates. Subsequently, an equal volume of adjusted inoculum (1×10^6 CFU/mL) was added to each well of the Microtiter plates up to a final volume of 100 µL. The nutrient media with bacterial culture without bioactive fractions was used as a negative control, and in the positive control bioactive fractions were replaced by aminoglycoside antibiotics gentamicin (GEN) – (stock solution 40 mg/ml) and tobramycin (TOBR) (stock solution 40 mg/ml). The growth was observed and the optical density was read at 600 nm spectrophotometrically (Varioskan™ LUX multimode microplate reader, Thermo Fisher Scientific) for the development of turbidity. After incubation for 24 h at 37 ºC, MIC was defined as the lowest concentration of the bioactive fractions that prevents visible growth of a microorganism. Effective concentration of each sample was determined that reduced the growth of the tested bacteria by 50% (EC_{50}). The growth of bacteria was expressed as a percentage of the highest optical density observed in the inoculum. EC_{50} was determined using dose response curve in GraphPad Prism 9 statistics software.

RESULTS AND DISCUSSION

Six different fractions were isolated and purified from garden snails *H. aspersa* and *H. lucorum*, marine snail *R. venosa* and marine crab *C. aestuarii*, according to Table 1. Two fractions from mucus of garden snail *H. aspersa* (with Mw above 20 kDa and with Mw 2-20 kDa, respectively) and two fractions from hemolymph of *H. lucorum* (Mw below 10 kDa) and *R. venosa* (Mw 10-50 kDa) were analysed by MALDI-TOF/MS/MS (date not shown). MALDI-MS analyzes prove the presence of peptides rich in important amino acids such as glycine, proline, tryptophan, etc., which are characteristic of peptides with antibacterial activity against Gram-positive and Gram-negative bacteria.

The pharmacological potential of natural products has great attention due to the bioactivity nature of those products and several medicines prepared mainly from plant or animal origin are used by people. Recently, the marine and arthropodan organisms attract the attention of researchers due to the presence of pharmacologically active drugs. Several bioactive compounds have been isolated and characterized and some of them are active substances in the drugs [18, 19, 10, 15].

Even though molluscs are widely used for various studies in research institutions, the antibacterial nature of the substances is not well characterized. Therefore, from representatives of molluscs and arthropods were isolated fractions that were tested for antibacterial activity against three strains of Gram-negative bacteria (*E. coli* DSM 1607, *E. coli* DSM 1116 and *B. diminuta* DSM 1635). We have purified several fractions with bioactive compounds from Artropods: hemolymph of crab *C. aestuarii* - dissociated hemocyanin CaH (S1), from Molluscs: mucus from *H. aspersa* with Mw above 20 kDa (S2), mucus from *H. aspersa* with Mw 2-20 kDa (S3); hemolymph from *H. lucorum* with Mw below 10 kDa (S4), hemolymph from *R. venosa* with Mw 10-50 kDa (S5) and hydrolysate of *H. aspersa* hemocyanin - subunit βc – HaH with Mw below 30 kDa (S6).

Mucus peptides with an MW below 5 kDa include many important amino acids such as glycine, proline and tryptophan. They were determined by MALDI-MS analyzes (data not shown). These amino acids are associated with established antibacterial activity in many peptides.

The tested concentrations of the above-mentioned fractions for antibacterial screening are shown in Table 2.

The values of minimum inhibitory concentration (MIC) of all fractions isolated from crab and snail species are presented on Fig. 1. S1 had strong antibacterial activity on all of the tested microorganisms (*E. coli* DSM 1607, *E. coli* DSM 1116, *B. diminuta* DSM 1635).

Table 2. Range of tested concentrations (μg/ml) of the bioactive fractions for antibacterial activity.

Sample	*E. coli* DSM 1607	*E. coli* DSM 1116	*B. diminuta* DSM 1635
S1	4645-18.14	4645-18.14	4 645- 18.14
S2	1365-170.625	1365-170.625	1365-170.625
S3	385-48.438	Not tested	385-48.438
S4	863-107.875	863-107.875	863-53.9
S5	652.5-81.563	652.5-81.563	652.5-81.563
S6	600-37.5	600-37.5	600-37.5

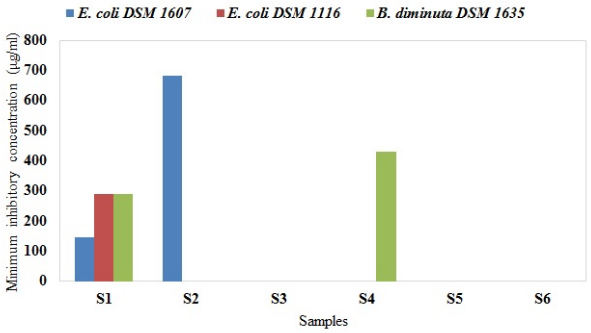

Fig. 1. Minimum inhibitory concentration (MIC) of the samples against the tested bacterial strains.

S1 exhibited strongest antibacterial activity against *E. coli* DSM 1607 (MIC - 145 μg/ml) and MIC value 290 μg/ml for *E. coli* DSM 1116 and *B. diminuta* DSM 1635, respectively. The antibacterial activity of S2 was recorded only against *E. coli* DSM 1607, (MIC - 682.5 μg/ml), whereas of S4 only against *B. diminuta*, (MIC – 431.5 μg/ml). S3, S5 and S6 did not present any antibacterial activity against the Gram negative bacterial strains, even at the highest concentration tested. To compare the effectiveness of the six samples, we used positive controls with amynoglicoside antibiotics gentamycin and tobramycin. Gentamycin showed a MIC value 2 μg/ml against *E. coli* DSM 1116, 0.06 μg/ml against *E.coli* DSM 1607, and 8 μg/ml against *B. diminuta* DSM 1635. Tobramycin showed equal activity against *E. coli* DSM 1116, higher MIC value against *E. coli* DSM 1607 (0.125 μg/ml), and no activity against *B. diminuta* DSM 1635.

The MIC and EC_{50} values of the active samples S1, S2 and S4 are compared on Fig. 2.

The EC_{50} value was used to estimate the least concentration of the compound that is required to produce 50% of maximum effect and the higher the potency. Among the all active fractions against the tested bacterial strains, the value of EC_{50} was lowest for S1 (75.46 μg/ml) against *E. coli* DSM 1607. Therefore, S1 showed the highest efficacy among the tested substances against *E. coli* DSM 1607 (Fig. 2). The EC_{50} values of the fractions S2 (442.1 μg/ml) and S4 (279.9 μg/ml)) displayed low to moderate effect against *E. coli* DSM 1607 and *B. diminuta* DSM 1635, respectively (Fig. 2).

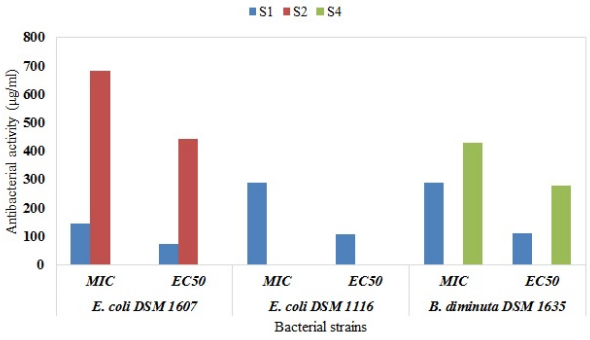

Fig. 2. The MIC and effective concentrations (EC_{50}) values of the active samples S1, S2 and S4.

Among the six tested fractions, the hemocyanin (S1) from crab *C. aestuarii* showed strong antibacterial activity against the selected Gram negative bacterial strains even at very low concentration. It was demonstrated that the grade of hemocyanin glycosylation plays an important role in its functional antibacterial properties [12, 14, 20]. Recent studies suggest that an important modification of hemocyanin, which enables it to function as a molecule involved in the immune process is its glycosylation [21-23]. Moreover, the glycosylation of hemocyanin subunits has been reported to be important for its antiviral effects [21-23] and various anti-tumour properties [24, 25]. Kizheva *et al.* (2019) demonstrated that native hemocyanin from the crab *Eriphia verrucosa* had no antimicrobial activity unlike its glycosylated structural subunits [12]. The authors found the strongest antibacterial activity of the structural subunits with highest carbohydrate content against *E. coli* and *Bacillus subtilis*. The hemocyanin of the crab *Carcinus aestuarii* contains a carbohydrate moiety that represents 1.6% of protein mass [14, 20]. Dolashka-Angelova *et al.* (2001) found that the subunit referred to as Ca2 of *C. aestuarii* hemocyanin is with highly carbohydrate content (6.3%) and contains *O*- and one *N*-linked carbohydrate chains [20]. Therefore, in this study, we tested dissociated into its subunits *C. aestuarii* hemocyanin to determine its antibacterial activity.

CONCLUSION

Our results indicate the presence of different natural antibacterial substances in the studied various molluscs species and one representative of

the arthropods. Among the six tested samples in this study, the hemocyanin (S1) of the crab *C. aestuarii* demonstrated strong antibacterial activity against tested bacteria even at very low concentration. This sample containing dissociated subunits of CaH showed considerable antimicrobial activity, which could probably add to the arsenal of antibiotics as candidates with low resistance rates. It is notable from the results that S1 showed different range of MIC values against two strains of *E. coli*. S1 showed the highest effectiveness against *E. coli* DSM 1607 according to the observed EC_{50} values. The results of this study clearly elucidate the antibacterial potential of the active fractions. Further research with a wider range of bacterial strains is needed for the support their potential biomedical application.

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