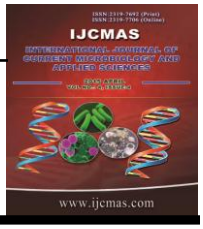
Int.J.Curr.Microbiol.App.Sci (2015) 4(4): 1061-1071

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 4 (2015) pp. 1061-1071**
http://www.ijcmas.com



Original Research Article

Antimicrobial activity of peptides from the hemolymph of *Helix lucorum* snails

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Keywords

Peptides, *Helix lucorum*, *Staphylococcus aureus*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *Escherichia coli*.

A B S T R A C T

The increasing emergence of bacterial resistance requires the development of new efficient antibiotics that can be added to the antibacterial armamentarium. Quite a series of proline-rich peptides**,** isolated from artropods and molluscs, were considered to be promising candidates for the treatment of microbial infections and suppression of microbial resistance. We here tested the antimicrobial activities of peptides isolated from the hemolymph of the molluscan garden snail *Helix lucorum*. Four peptides with molecular weights between 2 and 6**.**8 kDa were isolated and their N-terminal and amino acid sequences were determined. In the test against Gram+ (*S. aureus*, *E. faecium* and *S. epidermidis*) and Gram- (*E. coli*) bacteria only two of the peptides, tentatively named '4 and 6', exhibited inhibition effects against *S. aureus, S. epidermidis* and *E. coli*. However, Peptide 6 is the most important one of the two peptides to further investigate with respect to the structural basis of its activity. It indeed showed much higher effects, respectively of 90, 85 and 80 % on *S. epidermis, S. aureus,* and *E. coli* than Peptide 4. No activity has been detected against *E. faecium*.

Introduction

The world provides a rich source of peptides with antimicrobial, antiviral and antitumor activity, mainly isolated from insects (Chernysh, et al. 2002, Dandan, et al. 2012, Lee, et al. 2003, Kim, et al. 2013). The potential of antimicrobial peptides (AMP), both natural and synthetic, against multidrug- resistant pathogen infections has

been demonstrated in the last years (Laverty et al. 2011, Pathan et al. 2010, Peng et al. 2010, Wang et al. 2007). They have short amino acid sequences (typically ranging from 12–100 residues in length), and exhibit rapid and efficient antimicrobial toxicity against a range of pathogens in the innate immune system of both prokaryotic and eukaryotic organisms. A typical feature is that they are relatively rich in aromatic amino acids and (Pro-Arg)-fragments (Sitaram, 2006, Conti et al., 2013, Dolashka et al. 2011).

Recently, a series of active peptides and glycopeptides with different physiological functions were also extracted from marine molluscs (Charlet et al. 1996, Conti et al. 2013, El-Gamal et al. 2013, Li Rong et al. 2012, 2013). More specifically, four antimicrobial peptides with molecular masses of respectively 4464, 3158, 655, and 636 Da were identified from oyster hemolymph (Defer et al. 2013). Several cysteine-rich antimicrobial peptides such as the defensins A and B, mytilins A and B, and myticins A and B have also been isolated from arthropods (Zhong et al. 2013, Mitta et al. 1999, Díaz, 2010.

Antimicrobial peptides in invertebrates represent the major humoral defense system against infection, most of them being involved in plasma membrane disturbance and lethal alteration of microbial integrity (Otero-González et al. 2010, Tincu et al. 2004). Several peptides from the hemolymph of molluscs and arthropods exhibit a broad-spectrum of antimicrobial activity against Gram-positive (Gram+) as well as Gram-negative (Gram-) bacteria, fungi and yeasts (Ramesh kumar et al. 2009, Conti et al. 2013, Chae et al., 2012, Keitel et al. 2013, Defer et al. 2013). So far, six different groups concerning their mechanism of attack were observed: chemotactic peptides, mastoparans, tachykinins, kinins, antibiotic peptides, and a group of long-chain peptides with one or two disulfide bonds and yet undefined biological activities (Saidemberg et al. 2011).

Our group also identified four novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *Rapana venosa* snails which showed strong antimicrobial activities against *Staphylococcus aureus* (Gram+) and *Klebsiella pneumonia* (Gram-) (Dolashka et al. 2011). Especially, the *Staphylococcus* species are becoming increasingly resistant to many commonly used antibiotics including penicillins, tetracyclines etc., which make studies on new natural antimicrobial peptides of enormous importance.

We now report on the primary structure and masses of peptides isolated from the hemolymph of the molluscan garden snail *Helix lucorum***,** and compare their antimicrobial activities with those from other physiologically similar peptides isolated from the hemolymph of the marine snail *R. venosa* and from the shrimp *Penaeus vannamei*.

Materials and Methods

Collection of hemolymph and purification of peptides from the hemolymph of H. lucorum snails

The hemolymph was collected from the leg of the snails, solubilized in 50 mM-sodium acetate buffer, pH 5.8, and centrifuged at 10000 rpm for 15 min to remove hemocytes in the supernatant. The hemocyanin was sedimented in a Beckman L-80 ultracentrifuge, equipped with an UZ rotor Ti 45, at a speed of 40 000 rpm, for 4 hours, at 5°C. After removal of the blue native

hemocyanin pellet, the supernatant was separated using Milipore filters (3, 10 and 100 kDa). Three fractions were obtained: Fraction A (masses between 0-3 kDa), Fraction B (masses between 3-10 kDa), and Fraction C (masses above 10 kDa). Fraction B was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.10% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and B (80% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.5 ml/min, over 60 min. Ultraviolet absorption was monitored at 214 nm. The eluted fractions were collected and lyophilized. The fractions were reconstituted in Milli Q water containing 0.10% TFA (v/v). Additional purification of the isolated peptides was performed using the same equipment and conditions.

N-terminal sequence determination and mass spectrometric analysis

Isolated HPLC fractions were dried and**,** after dissolving them in 40% methanol/1% formic acid**,** their N-terminal amino acid sequences were determined by automated Edman degradation using a 473 a gas-phase Protein Sequencer; (Applied Biosystems GmbH, Weiterstadt, Germany).

The molecular masses of the isolated fractions were analysed by MALDI-TOF-TOF mass spectrometry on a 4700 Proteomics Analyser 263 (Applied Biosystems, Framingham, MA) which uses a 200 Hz frequency-tripled Nd–YAG laser operating at a wavelength of 355 nm. Some 50 pmol of the HPLC fractions were dissolved in 0.1% (v/v) TFA and applied to the target, covered with α-cyano-4-hydroxycinnamic acid as a matrix. A total of 3500 shots were acquired in the MS mode and a collision energy of 4200 was applied. A solution of protein standards was used to calibrate the mass scale. The mass values assigned to the amino acid residues are average masses.

Antibacterial assays of the peptides

The Gram-positive bacterial strains (*Staphylococcus aureus*, *Enterococcus faecium* and *Staphylococcus epidermidis*) as well as the Gram- strain *Escherichia coli* were used in the antibacterial assays. They were chosen because they are human pathogenic species and commonly used in antimicrobial tests.

Antimicrobial assays of isolated Fraction A (spots 5), Fraction B (spots 6), Fraction C (spots 4), unfractionated hemolymph (spots 2) and control (spot 1) were obtained on agar plates containing the likewise Gram+ bacterium *S. aureus* and *S. epidermis* and the Gram- *E. coli* results. Each fraction was spread on agar medium with two different amounts (10 and 30 µl of the peptide solutions). The incubation was for 24-36 h at 37°C**.**

The eluted peptides were qualitatively proven according to the liquid growth inhibition assay, using two different amounts (10 and 30 µl of the peptide solutions) used for testing their antimicrobial activity, and were afterwards also quantitatively checked. Briefly, 10 µl of the samples was mixed with 6 ml of a mid-logarithmic bacterial culture (measured by OD_{600}) in poor broth nutrient medium (1% dextrose). Microbial growth was assessed by an increase in the McF value after incubation (24 h, 35°C). A bacterial culture without peptides and incubated under the same conditions was used as a control. 1 unit McF corresponds to 3×10^8 cells/ml. The turbidity was measured with a DENSIMAT (BioMerieux, France) instrument.

The antibacterial test was also performed using solutions of the peptides and standards that were serially diluted in sterile TSB in 96 well plates. A volume of 10 µl from each peptide solution was incubated for 24 h with the bacterial culture in Tryptic Soy Broth (TSB) medium, in a total volume of 120 µl.

The initial OD_{600} of the bacterial cultures was 0.4-0.6. The medium without bacteria served to verify the conditions of the test, and as a negative control the bacterial culture without any added peptide was used. Two different concentrations of each sample (10 µl and 30 µl) were tested.

Result and Discussion

The biochemically and pharmacologically active peptides in the hemolymph of *Helix lucorum* were subdivide into three fractions, obtained after separation over Millipore filters with a cut-off of 3, 10 and 100 kDa, respectively. Upon testing their antimicrobial activity on an agar medium after incubation for 24-36 h at 37°C, only Fraction 2 appeared to generate a zone of inhibition of *S. aureus* **(**Fig 1). The same result was obtained for *S. epidermis* as well as for the Gram- bacterium *E. coli* (not illustrated). Therefore, Fraction B was purified and the structure of the containing compounds analyzed. Its mass spectrum, containing peptides with masses between 2 and 10 kDa, is shown in Fig. 2.

Mainly 6 subfractions were eluted in by reversed-phase column chromatoghy of Fraction B (Fig. 3A). They were additionally purified on the same column; the resulting chromatogram for subfraction 6 is shown in Fig. 3B. The re-chromatography allowed to subsequently characterise four peptides (named Peptides 3, 4, 5 and 6). Their N-terminal amino acid sequences, determined by Edman degradation can be read in Fig. 4A. All of them appear to contain two consecutive Leucine residues, and peptides 3 and 6 contain a pair of Prolines which align without having to introduce a 'gap' between them and the pair of leucines.

Remarkably, also two Proline residues have previously been detected near the N-terminus of peptides from the hemolymphs of the marine snail *Rapana venosa* (Dolashka et al. 2011) and the shrimp *Penaeus vannamei* (Destoumieux et al. 2000). The molecular masses of the isolated Peptides, determined by MALDI/MS, revealed a main ion at m/z 5385.8 $(M+H)^+$ for Peptide 4 (Fig. 5A) and two ions at m/z 6580.0 and 6774.8 $(M+H)^+$ for Peptide 6 (Fig. 5B).

The peptides isolated from the hemolymph of garden snail *H. lucorum* were also tested against different species of Gram+ (*S. aureus*, *E. faecium* and *S. epidermidis*) and one Gram- bacterium (*E. coli*). The organisms were chosen, the first two Gram+ bacteria in particular, because they are human pathogenic bacteria and commonly used in antimicrobial tests. Although *S. epidermidis* is not usually pathogenic, patients with compromised immune systems are known to be at risk of developing infection, is generally hospital-acquired.

As Fig. 6 shows, the results on the antibacterial activity tested by the liquid growth inhibition assay pointed out that only Peptide 6 has a remarkable inhibitory effect on the growth of growth of three out of the four bacteria, with *E. faecium* not responding. Also none of the other 5 peptides appears to be able to interact in a growth-negative way with this bacterium. However, they do seem to inhibit *S. aureus*, although only at maximally 50%, whereas Peptide 6 is active at 90%.

Figure.1 Antimicrobial assays against Gram+ *S. aureus* of isolated Fraction 1 (spots 5), Fraction 2 (spots 6) and Fraction 3 (spots 4). Spots 2 refer to the unfractionated hemolymph, and spot 1 is the control. Each fraction was applied on the agar medium in two different amounts (10 and 30 µl of the peptide solutions)

Figure.2 MALDI-MS, spectrum of the peptides in the hemolymph fractions of garden snail *Helix lucorum* with molecular masses between 2 and 10 kDa

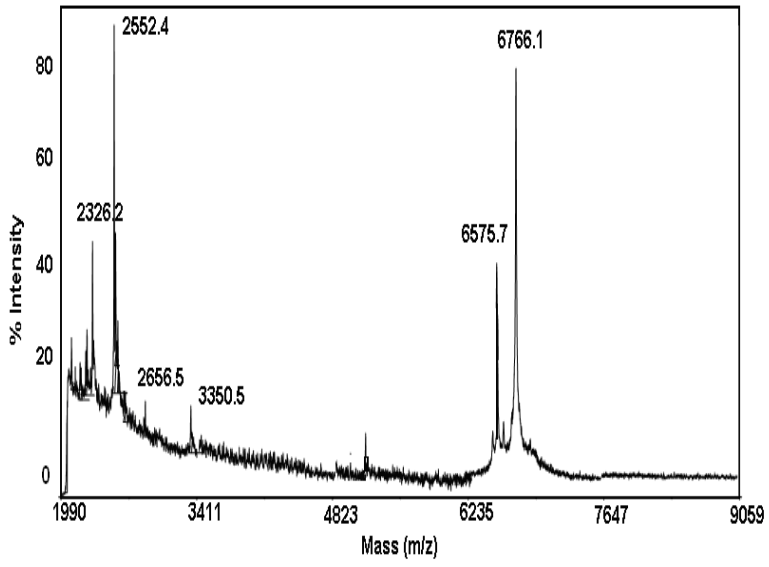
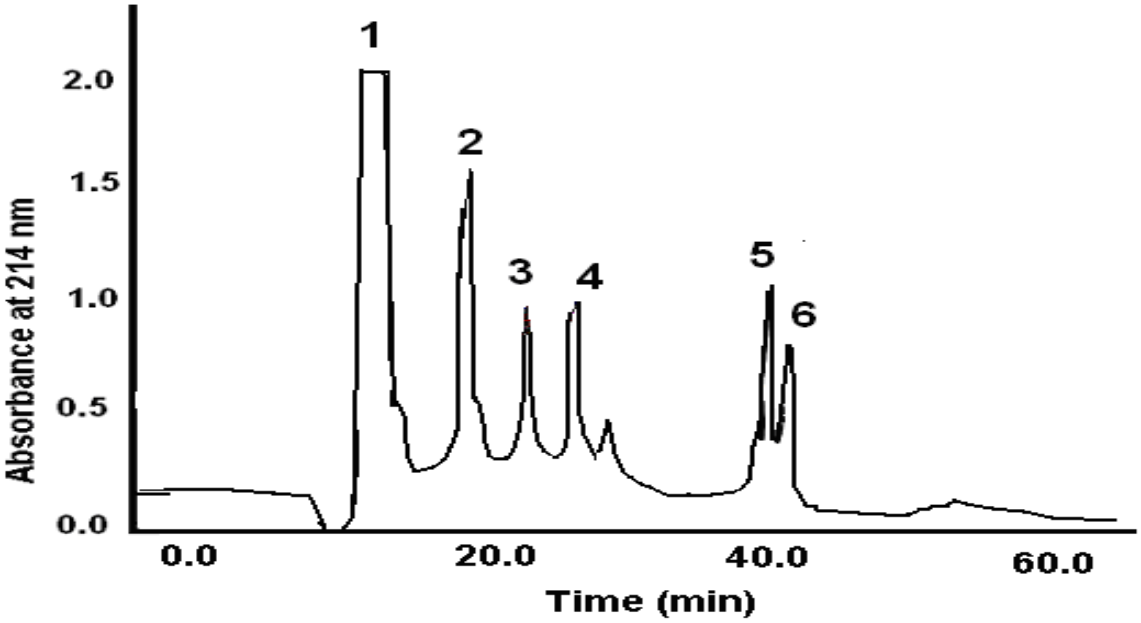


Figure.3A RP-HPLC purification of the peptides of 3-10 kDa residing in Fraction 2; **B)** RP-HPLC rechromatography of Peptide 6

3A



3B

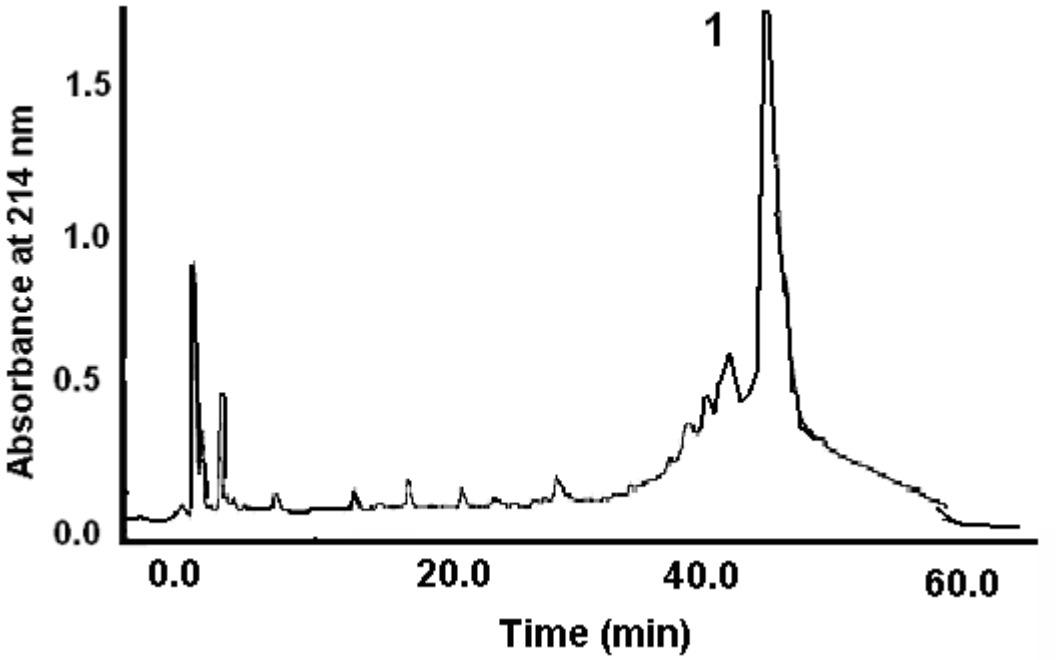


Figure.4 N-terminal sequence comparisons of peptides from Fraction 2 of the hemolymph of *H. lucorum* determined by Edman degradation with peptides from the hemolymph of the marine snail *Rapana venosa* and the shrimp *Penaeus vannamei.* Proline and glycine residues are in boldface, and identical amino acids at given positions are on a gray background

A Peptides from hemolymph of *Helix lucorum* determined by N-terminal sequences

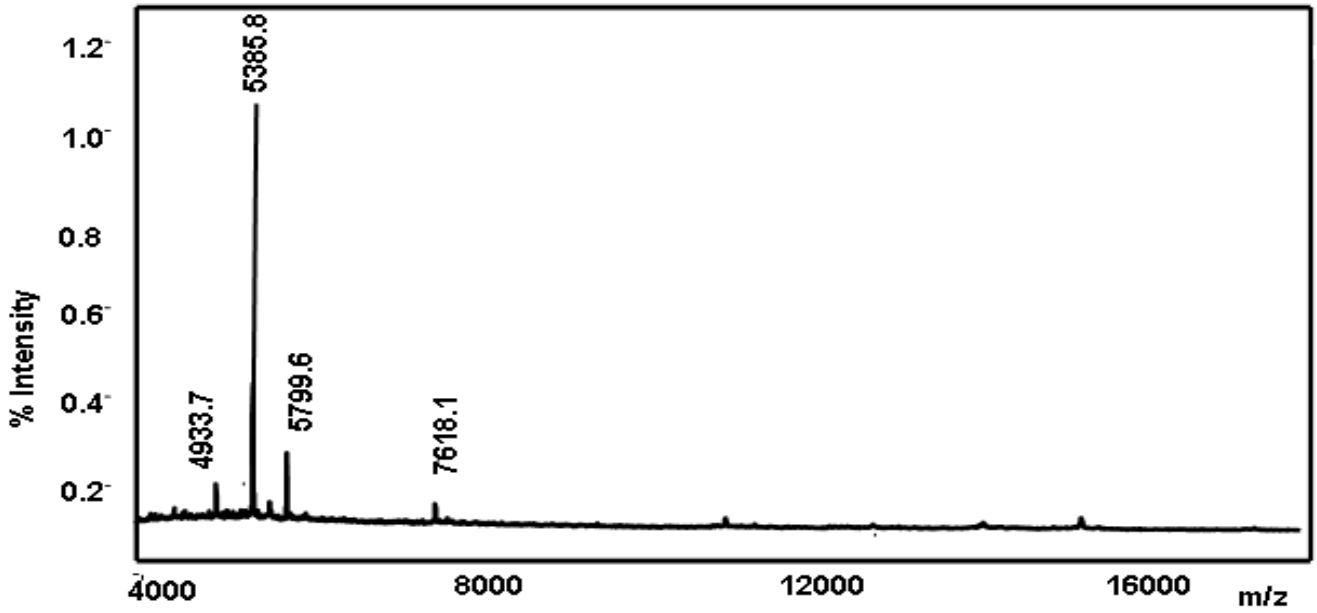
	1	5	10	15	20	25
Peptide 3		X **P** **P** D V	**L** **L**			
Peptide 4		A	**L** **L** F E N Y D D K V X D H			
Peptide 5	D Q I	T K V E R	**L** **L** R			
Peptide 6		E **P** **P** Y Q	**L** **L** A K F I K A **G** N **G** R			

B) Peptides from hemolymph of *R. venosa* determined by N-terminal sequences

Peptide 3	E	L	V	R	K	N	V	D	H	**L**	S	T	P	D	V	L	E	L	V								
Peptide 2			S		**P**	**P**	N	Q	P	S	I	M	T	F	D	Y	A	K	T	N	K						
Peptide 4			S	L	**P**	**P**	T			**L**	E	E	E	F	N	M	K	K	M	**G**							
Peptide 5			S		**P**	**P**	S	E	Q	L	**G**	K	S	F	N	F											
Peptide 6			S		**P**	**P**	**P**		**G**	E	S	K	V	D	M		S	F	N	Y	A	L	S	N	P	A	Q
Peptide 7				A	**P**	**P**	**P**		**G**	**L**	S	A	G	V													
Peptide 8				A	**P**	**P**	**P**		**G**	Y	A	M	E	S	D		S	F	S								
Peptide 9				F	**P**	**P**	**P**		**G**	E	S	A	V	D	M		S	F	F	Y	A	L	S	N	P		
Pen-1				R	**P**	**P**	**P**	I	**G**	R	**P**	**P**	L	R	L		V	V									
Pen-2				R	**P**	**P**	**P**	I	**G**	R	**P**	**P**	F	R	P		V										

Figure.5 MALDI-MS spectra of the Peptides 4 and 6 isolated from Fraction 2 shown in Figure 2

A



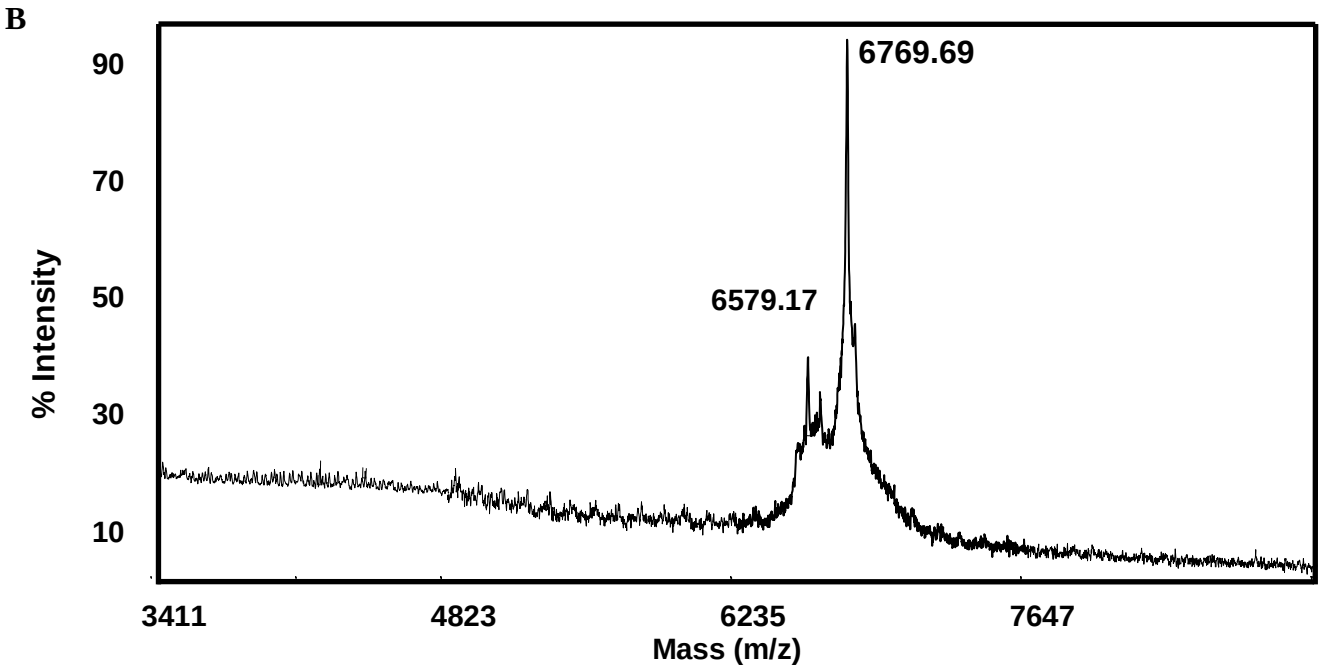
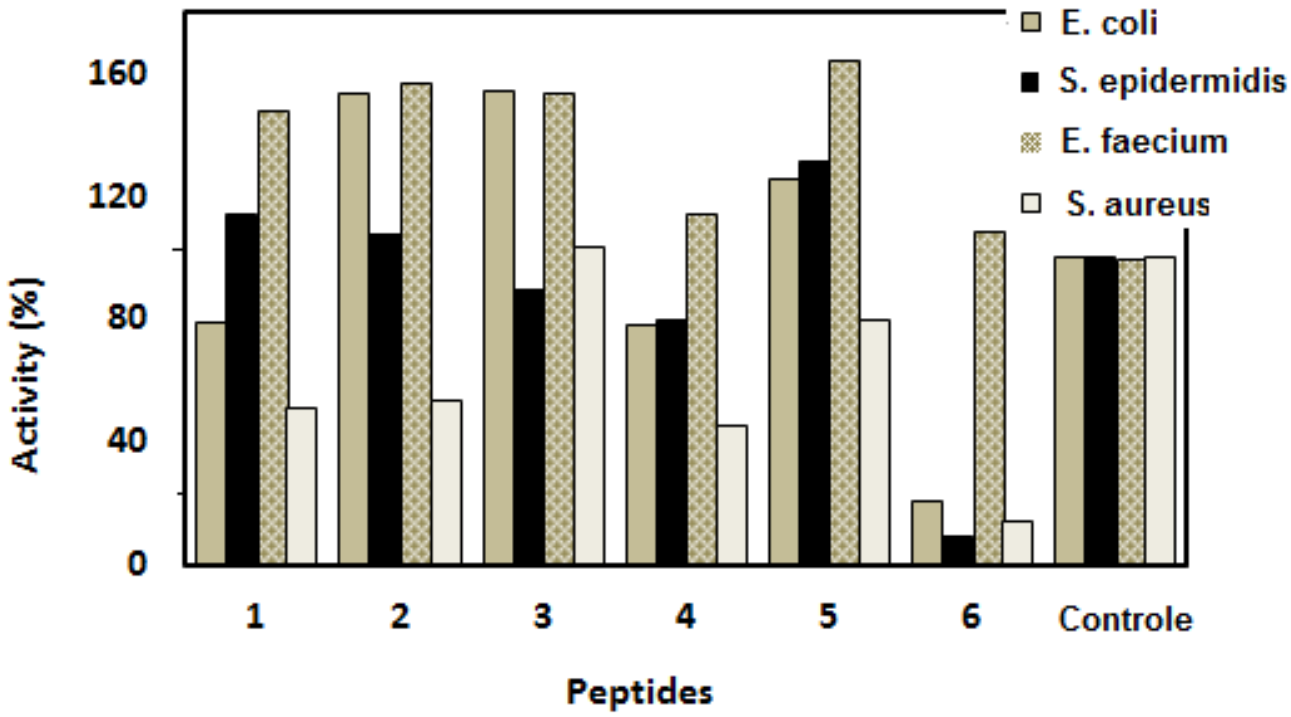


Figure.6 Antimicrobial assays of the isolated Peptides 1-6 against Gram+ (*S. aureus*, *E. faecium* and *S. epidermidis*) and Gram- (*E. coli*) bacteria. The methodology is described in Materials and Methods.



To determine the minimal antimicrobial concentration of the most active peptides**,** bacterial suspensions of *S. aureus*, *S. epidermidis,* and *E. coli* were incubated with peptide concentrations ranging from 10 to 30 □ M. It was found that Peptides 4 and 6 cause agglutination of the Gram+ *S. aureus* and *S. epidermidi*s as well as of Gram- *E. coli*, at a concentration of 10 □ M, at 50 % affinity.

Several antibacterial and antifungal peptides of moderate molecular masses and rich in Cys, Pro, Ser or Gly residues have already been isolated and characterized in the past, also from molluscs other than *Helix lucorum*. As mentioned before, eleven proline-rich peptides with molecular weights ranging from 3000 and 9500 Da were isolated from the hemolymph of *R. venosa* snails, some of them exhibiting high antimicrobial activity against *S. aureus* and low activity against *Klebsiella pneumoniae* (Dolashka et al., 2011). The N-terminal sequence of the presently characterized Peptide 6 from Fraction 2 adds to this category of peptides. The cysteine-rich peptide mytimacin-AF from the *Achatina fulica* snail, composed of 80 amino acid residues, and the mytilins A and B from the hemocytes and plasma of the bivalve mollusc *Mytilus galloprovincialis*, composed of 40 residues, exert, as does Peptide 6, the strongest antimicrobial activity against *S. aureus* (Zhong, et al. 2013). To put the glycine content in the peptides studied in the present paper in context, it should be noted that a new 14-kDa AMP (tenecin 4), from the larval hemolymph of the beetle Tenebrio contains 14% glycine residues and shows a bactericidal activity against *E. coli* (Chae, et al. 2012). It is possible that the Gly- and Pro-content in *H. lucorum* Peptide 6 also plays a structural role in the activity against this Gram- bacterium. Finally, it may be reminded that, although the mechanism of the inhibition effect of most peptides is still unknown, studies of the 21 residue peptide buforin II, with a single proline moiety in a mid-chain position, was found to penetrate the bacterial cell membrane and to accumulate in the cytoplasm (Brogden, 2005). In contrast**,** proline-free magainin remains associated with the inner leaflet of the lipid bilayer after translocation of an artificial membrane (Matsuzaki, 1998). Understanding the function and mechanism of action of the antibacterial Peptide 6 from the hemolymph of *H. lucorum* may contribute to the potential of this compound in anti-infection therapeutics.

Acknowledgement

This work was supported by Deutsche Forschungsgemeinstchaft (DFG-STE 1819/5-1/2012), Germany; Bulgarian Ministry of Education, (projects TK01-496/2009; ДНТС/01/6; "Young researchers" DMU 03/26); grant № BG051PO001-3.3.06-0025), by the European Social Fund and Operational Programme Human Resources Development (2007-2013)**,** and was co-financed by the Bulgarian Ministry of Education, Youth and Science, and by the Exchange Programme VS.O25.12N of the Flemish Fund for Scientific Research (FWO) and the Bulgarian Academy of Sciences (BAS). We thank to Dr. Maren Paulmann and Prof. Birgit Schittek from Eberhard-Karls-Universität Tübingen Hautklinik Sektion Dermatologische Onkologie, Tübingen, for their support in the antimicrobial analyses.

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