

## Identification and functional characterization of antimicrobial peptide from the marine crab *Dromia dehaani*

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### Abstract:

Many naturally occurring biologically active compounds are derived from marine organisms. Brachyuran crab *Dromia dehaani* is also known as sponge crab. Hence, this study was undertaken to isolate and characterize the antimicrobial peptide from the marine crab hemolymph. Therefore the objectives of this research work was to high light the functions of biologically active peptide Dromidin. Dromidin was identified and confirmed as a new antimicrobial peptide in the hemolymph of the crab *D. dehaani*. Antimicrobial peptide was characterized and confirmed by using various analytical techniques. ESI-MS analysis of the active antimicrobial hemolymph fraction resulted in the value 513.0 Da. In addition, current findings strongly suggest that this peptide is functionally important against the pathogens.

**Keywords:** *Dromia dehaani*, Antimicrobial, Peptide, Hemolymph, Biological activity

## 1. Introduction

Antimicrobial peptides or proteins (AMP's) are one of the major components of the innate immune defense and are ubiquitously found in all kingdoms from bacteria to mammals, including fungi and plants. AMP's are primarily known as “natural antibiotics” because of their rapid and efficient antimicrobial effects against a broad range of microorganisms, including Gram positive and Gram negative bacteria, yeast, filamentous fungi and, to a lesser extent, protozoans and enveloped viruses. The antimicrobial factors might therefore have an important function as a first line of defense against pathogenic micro organisms. After immune challenge, these antimicrobial peptides are released into the hemolymph [1–3]. Antimicrobial peptides (AMP's) are generally small cationic molecules, have broad-spectrum of non-specific activity that plays an important role in innate immune defense against a wide range of microorganisms including viruses, bacteria, protozoa, yeasts and fungi, and may also be hemolytic and cytotoxic to cancer cells [4].

Many AMP's have been found and characterized in crustaceans [5–10]. Marine crabs depend upon AMP's as a major component of innate immunity, as they are rapidly synthesized and diffuse upon pathogen invasion. We present here the best source of antimicrobial peptide isolated from the hemolymph of marine crab. In crabs, few molecules displaying antimicrobial activity have been fully characterized. Initial investigations revealed that the antimicrobial activity of *D.dehaani* was extensive among the tested crabs. Hence, this study was carried out for the first time on isolation and characterization of an AMP from the hemolymph of sponge crab *D. dehaani*. In this paper, we propose the functionally active novel antimicrobial peptide from the marine crab.

## 2. Experimental procedures

### 2.1. Animals and sample collection

Healthy premoult crabs from both sexes were used in this study. Hemolymph was collected by dissecting walking legs of the live animal with a sterile scissor. To prevent the hemocyte degranulation and coagulation, the hemolymph was collected with sodium citrate buffer, pH 4.6 (2:1, v/v) to which equal volume of physiological saline (0.85%, NaCl, w/v) was added. This was followed by centrifugation at 2000 rpm for 15 min at 4 °C to remove hemocytes from the hemolymph. The cell-free hemolymph was immediately used or stored as aliquots at –20 °C for further studies.

### 2.2. Antimicrobial assay

The antimicrobial activity was determined against 10 different strains of bacteria and fungi. *D. dehaani* showed notable activity. Antimicrobial activity was performed using paper disc diffusion

method [11]. Each sample was tested in triplicate and the zone of inhibition was measured as millimeter (mm). The MIC, MBC and MFC were carried out. The highest dilution that yielded no bacterial growth on solid medium was taken as MBC [12]. The highest dilution that yielded no fungal growth on solid medium was taken as MBC [13]. All the assays were carried out in triplicate.

### 2.3. Thin-Layer Chromatography (TLC)

A diluted extract of methanol were applied to the Thin Layer Chromatographic plate (MERCK) with a capillary tube and placed in a chamber containing BAW (butanol 5: acetic acid 1: water 4) as mobile phase. After development compounds were visualized as pink spots on spraying with ninhydrin as detecting agent followed by heating at 100 °C till the spots were visible.

### 2.4. Peptide purification

Lyophilized crab hemolymph was purified through GFC reconstituted in 10 mL of 0.1 M phosphate buffer solution (PBS), containing 5 mM EDTA, pH 6.0. The sample was applied to a Sephadex G-25 (Superfine, Amersham Biosciences, 2.6 cm × 90 cm) gel filtration column was previously equilibrated with 0.1 M PBS. The column was eluted with the same buffer, and 3 ml fractions were collected. Fractions having major antimicrobial activity was pooled, lyophilized, re-suspended and further purified by RP-HPLC. The analysis was carried out on a HPLC system (Shimadzu-LC 20 A T) equipped with UV detector, pressure controlled by prominence pump. C18 column (250 mm × 4.6 mm particle size 5 µm) was used for separation.

### 2.5. UV-Vis spectroscopic analysis

The absorption spectrum of active hemolymph fraction was re-corded in the UV spectrum region (200–400 nm) by using a UV-Vis spectrophotometer (Perkin-Elmer Lambda 15).

### 2.6. FT-IR spectral analysis

FT-IR spectra were recorded on a Fourier Transform spectrometer Agilent resolutions pro resolution systems. All spectra were recorded at room temperature. The molar concentration of 1 varied from  $5 \times 10^{-5}$  to  $5 \times 10^{-3}$  M (in saturated solutions) and of models from  $5 \times 10^{-4}$  to  $5 \times 10^{-1}$  M. Depending on concentration of compound and the IR re-gion studied different cells were applied: quartz cells from 1 to 50 mm and KBr cells from 0.066 to 2.66 mm.

### 2.7. Nuclear magnetic resonance (NMR)

NMR spectroscopy can provide a principal of additional information about peptide. NMR spectroscopy was performed on purified hemo-lymph samples of (~5 mg) which is dissolved in D<sub>2</sub>O

(99.96 atom %) filtered through a 0.45 µm syringe filter, freeze dried twice to remove exchangeable protons and transferred to shigemi tubes. One dimensional  $^1\text{H}$  NMR was performed on at 400 MHz on a Bruker model 500 NMR type.

## 2.8. Hemolytic activity assay

The hemolytic activity of the AMP was tested using human erythrocytes [14]. A 2.5% (v/v) suspension of washed erythrocytes in PBS was incubated with the isolated peptide ranging from 10 µg to 160 µg in a 96-well plate for 2 h with intermittent shaking.

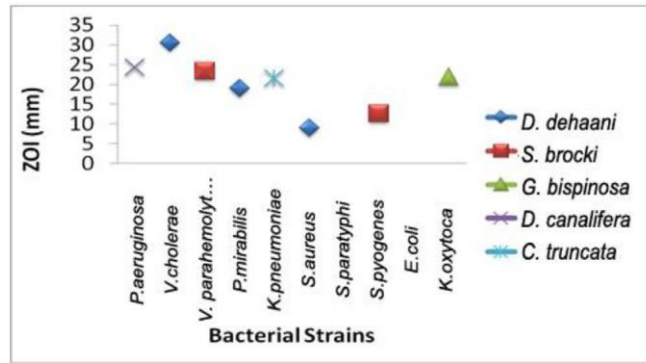
## 2.9. Molecular mass determination ESI- MS spectrometry

The molecular mass of the purified peptide was determined by Electron spray ionization mass spectrometer with an electrostatic ion spray source. A quadrupole is generally used as analyzer in ESI-MS. The quadrupole is essentially a mass filter, which allows ions of a given  $m/z$  to pass through to reach the detector. The electron multiplier is a combination of AC and DC voltage which facilitates the quadrupole to permit ions of a given ( $m/z$ ) value to pass through at a given time. Calibration of these ions using known ions  $m/z$  values enables assignment of peaks unambiguously. The fragment ion peaks are labelled according to the nomenclature proposed by Ref. [15], subsequently modified by Ref. [16].

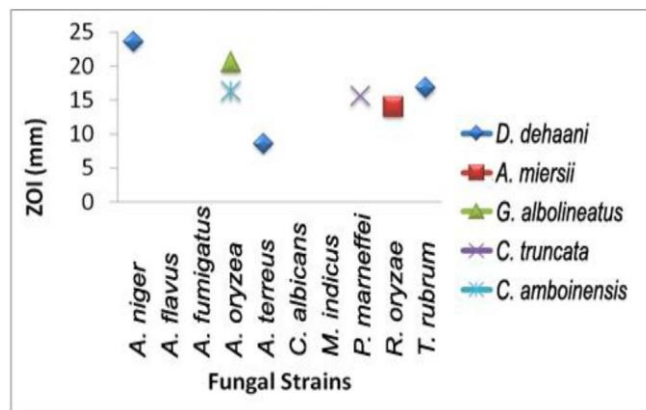
## 3. Results

### 3.1. Antimicrobial activity of marine crab *D. dehaani*

The current findings clearly demonstrated that the hemolymph of *D. dehaani* exhibit broad spectrum activity against pathogenic bacteria. The intensity of the antimicrobial action varied depending upon the microorganism, where the activity of *D. dehaani* displayed highest antimicrobial activity, the fractions collected through sephadex G25 was tested against the selected clinical isolates shown potent antimicrobial activity. Among the twelve fractions tested, fraction 5 was found to show maximum inhibition against the growth of bacterial pathogens and the most potent extracts were obtained from the crab *D. dehaani* which displayed activity against *Vibrio cholerae* at a concentration of 7.5 µg/mL and 15 µg/mL respectively. It was also shown to be highly active against fungal pathogens (Figs. 1 and 2).



**Fig. 1. Antibacterial activity of selected crabs**



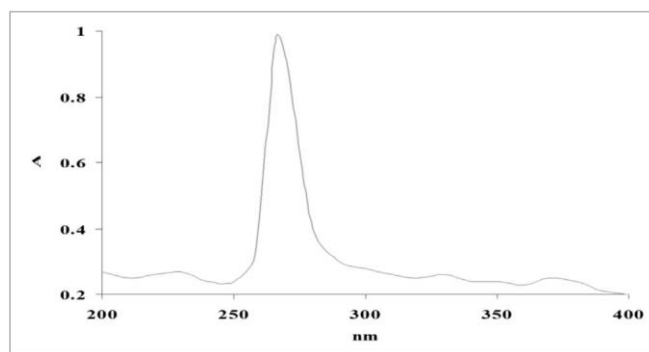
**Fig. 2. Antifungal activity of selected crabs.**

### 3.2. Thin-Layer Chromatography

Thin-Layer Chromatography profiling was done for the purified hemolymph samples in two different solvent systems (A & B). Solvent A consisted of methanol: chloroform (1:9) and B was a combination of butanol, acetic acid and water (B: A: W) in proportions of 5:1:4. The plate with fractions developed in BAW as the solvent system and sprayed with ninhydrin, showed pink spots indicating the presence of amino acids and peptides.

### 3.3. UV VIS spectroscopy

UV Spectral Data of active hemolymph fraction exhibited strong absorption maxima (Y max) at 277 nm and there was no appreciable absorption after 300 nm (Fig. 3).

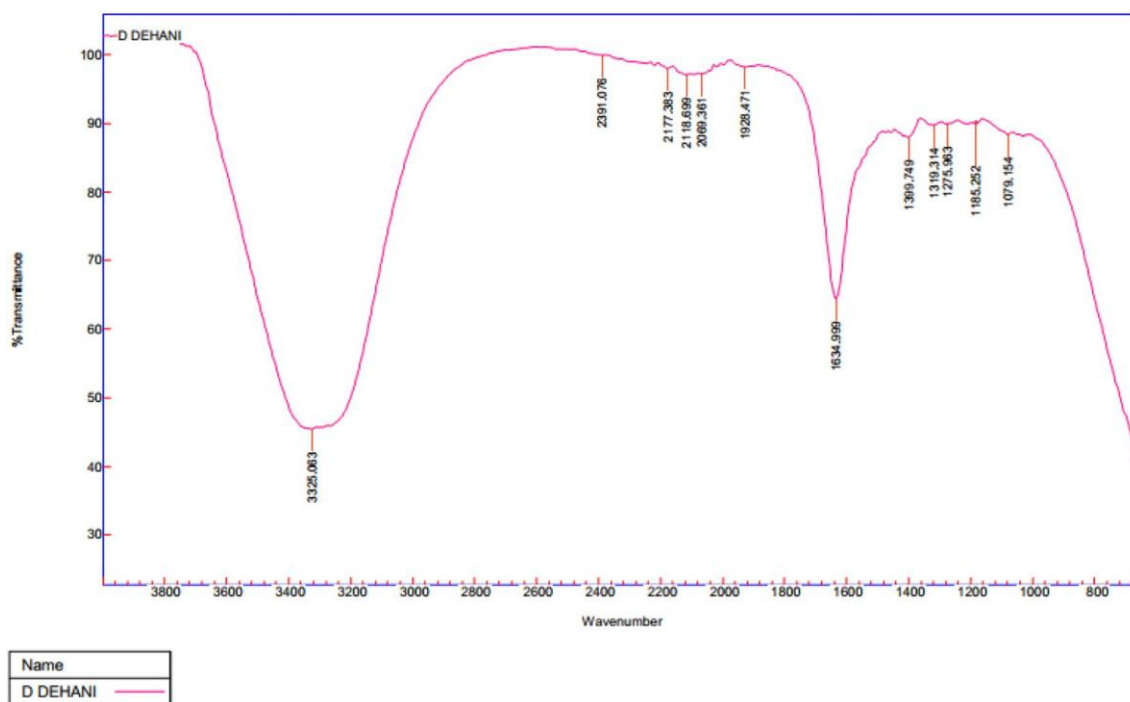


**Fig. 3. UV spectrum of the chloroform extract of crab *D. dehaani*.**

### 3.4. FT-IR

The FT-IR spectrum of the peptide isolated from *D. dehaani* was recorded and shown in (Fig. 4). The intense bands which are strong occurred at 3325, 2391, 2177, 2118, 2069, 1928, 1634, 1399, 1319, 1275  $\text{cm}^{-1}$ , 1185  $\text{cm}^{-1}$  and 1079  $\text{cm}^{-1}$ . The peak area at 3325  $\text{cm}^{-1}$  which falls in the range of 3300–3260 is correspond to N-H stretching secondary sulphonamide. The spectrum show peak at 1634  $\text{cm}^{-1}$  indicates the presence of vinyl halides. The peak areas 1399  $\text{cm}^{-1}$  and 1319  $\text{cm}^{-1}$  indicates the presence of C-H stretching of aromatics com-pound. The weak band of 1220  $\text{cm}^{-1}$  are showed thiophenes. The band at 1275  $\text{cm}^{-1}$ , 1185  $\text{cm}^{-1}$  and 1079  $\text{cm}^{-1}$  indicated presence of alde-hyde group. The spectrum showed a peak at 978  $\text{cm}^{-1}$  indicating the presence of methylene compound.

#### Agilent Resolutions Pro



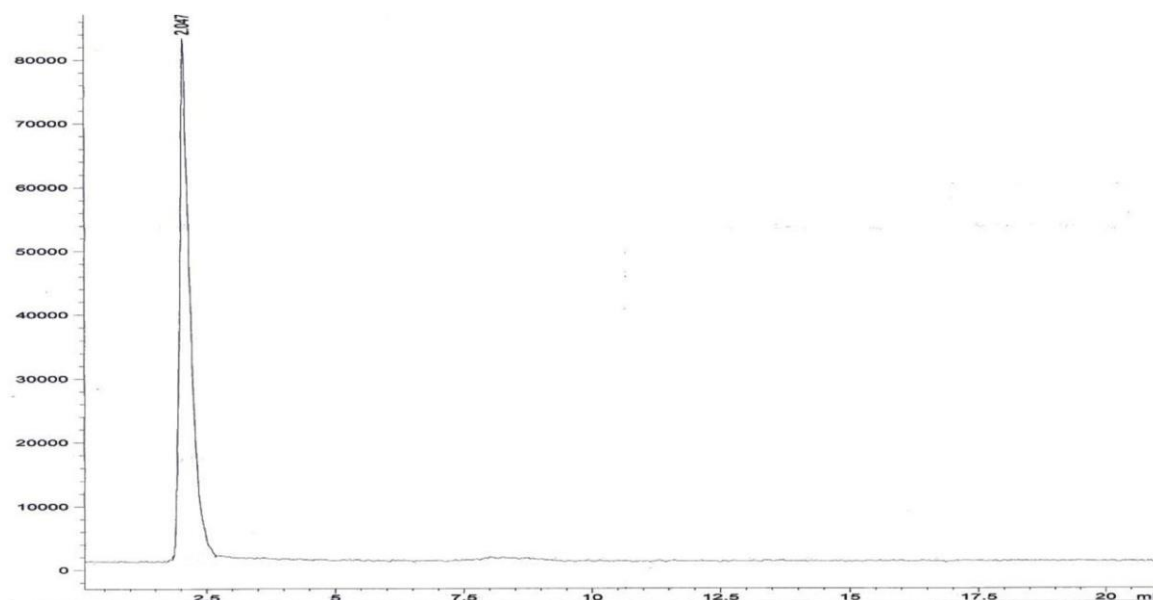
**Fig. 4. Infrared spectrum of the isolated peptide from crab *D. dehaani*.**

### 3.5. Purification of antimicrobial peptides

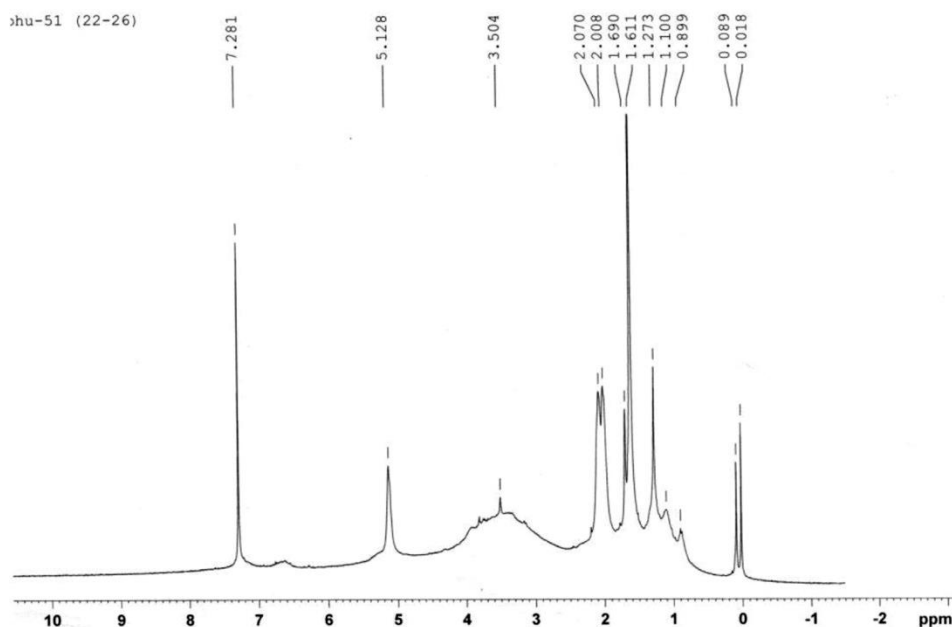
Fractions having major antimicrobial activity was pooled, lyophilized, and resuspended and further purified by reverse phase HPLC. Twelve major fractions were obtained when the lyophilized sample of *D. dehaani* hemolymph was resolved by gel filtration. Among these eluted fractions, fraction 5 was found to have antimicrobial activity. Thus it was further purified by RP-HPLC. The partially purified hemolymph was subjected to RP-HPLC using C-18 column. The chromatogram of the sample showed single peak at absorption of 280 nm with 40 min retention time (Fig. 5). The fractions was collected, lyophilized and stored at 4 °C. Therefore, we undertook the characterization of the molecule responsible for this antimicrobial activity.

### 3.6. $^1\text{H}$ NMR

The active RP-HPLC fraction of *D. dehaani* showed the presence of peptides and confirmed by  $^1\text{H}$  NMR spectra shown in (Fig. 6). Hence, it was selected for further studies. Since the concentration of the peptide is low  $^1\text{H}$ -NMR of the peptide rich fraction has been magnified manifold. The  $^1\text{H}$  NMR results show that the purified hemolymph contains a modified dipeptide which was characterized by a peak for the presence of NH proton (Fig. 6).



**Fig. 5. RP-HPLC of the active fraction 5 of the crab *D. dehaani*.**



**Fig. 6.**  $^1\text{H}$  NMR spectrum of the chloroform extract of the crab *D. dehaani*.

### 3.7. Hemolytic activity assay

The absorbance in the supernatant was measured at 415 nm. Hemolysis caused by PBS and 1% (v/v) Triton X-100 were used as 0 and 100% controls respectively shown in (Table 1). The hemolysis percentage was calculated using the following equation: % hemolysis =  $[(\text{Abs}_{414 \text{ nm}}$  in the peptide solution -  $\text{Abs}_{414 \text{ nm}}$  in saline) / ( $\text{Abs}_{414 \text{ nm}}$  in 0.1% Triton X-100 -  $\text{Abs}_{414 \text{ nm}}$  in saline)] X 100.

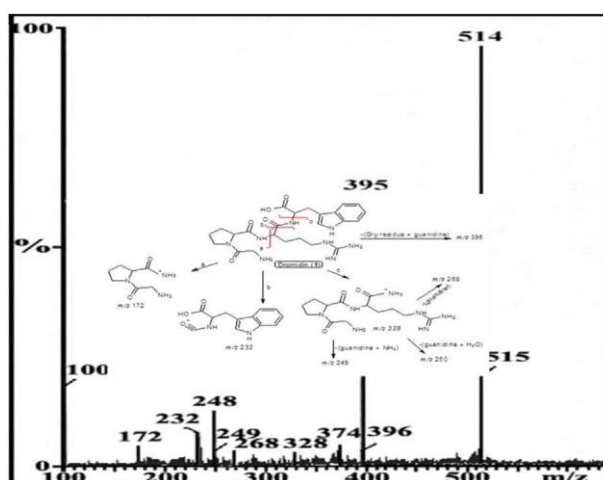
**Table 1:** Hemolytic activity of antimicrobial peptide.

		% Hemolysis					
AMP( $\mu\text{g/mL}$ )	5	10	20	40	80	160	
	0	0	0	0	2	2	



### 3.8. ESI-MS

The ESI-MS of the ninhydrin positive chloroform fraction is shown in Fig. 7. The result shows the presence of molecular mass of  $m/z$  513. To retrieve a sequence from the mass spectrometric data of the peptide was applied the criteria that mass difference of peaks showed precisely fit the mass of an amino acid residue. Based on the arrangement of amino acid in the hemolymph of *D. dehaani* was categorized peptide in  $m/z$  433. The ESI-MS showed presence of several molecular species in the range of  $m/z$  100- 515. To retrieve a sequence from the mass spectrometric data of the new peptide was applied the criteria that the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue. Depending upon the fragmentation pattern of the sequence for new peptide was assigned. Based on the arrangement of amino acid in the *D. dehaani*, isolated compound was categorized peptide (Fig. 7). ESI-MS analysis of the active antimicrobial hemolymph fraction resulted in the value 513.0 Da.



**Fig. 7.** ESI-MS spectrum of the chloroform extract of the crab *D. dehaani*

### 4. Discussion

In this present investigation, we report purification and character-ization of the AMP from hemolymph of sponge crab *D. dehaani*. Due to the harsh conditions of the habitat, crabs are endowed with an excellent chemical defense system composed of pharmacological biomolecules including AMP's. In recent times a considerable variety of AMP's have been characterized from crabs. Because of the special habitat, *D. dehaani* carries some sponges most of its life. Such living environment is extremely different from other crabs living in the marine. Thus, it is reasonable to infer that the AMP's from hemolymph of *D. dehaani* might be different from other characterized AMP's in structures and functions. In the current study, an AMP was purified and characterized from the

hemolymph of sponge crab *D. dehaani*. This peptide was purified and characterized using a combination of reverse-phase chromatography, mass spectrometry and  $^1\text{H}$  NMR.

The results of the present study showed that *in vitro* antimicrobial activity is present in hemolymph tested. Antimicrobial peptides are paramount substances of the immune defense reactions, developed by living organisms to fight infection by microorganisms. Antimicrobial activity has previously been described in a wide range of crab species. In most of the species studied, the hemolymph and the hemocyte's have been tested for activity. The present study demonstrated the presence of antimicrobial factors in hemolymph. The antimicrobial factors might therefore have an important function as a first line of defense against pathogenic microorganisms. After immune challenge, these anti-microbial peptides are released into the hemolymph [17,18] and fused with phagocytic vesicles following infection [19]. In crustacean, hemocyte's have been shown to play the main role in innate immunity. As such, they are involved in the prophenoloxidase-activating system, the clotting reaction of the antimicrobial peptide synthesis and releasing upon pathogen infection. Moreover, in decapods, hemocyte's participate in the production and the storage of antimicrobial peptides [20].

The UV spectral data for the purified peptide had shown maximum absorbance peaks at 270 nm and the characteristics of absorption peaks indicate a highly peptide bond. The spectral data were consistent with those obtained by Ref. [21]. It is reported that most of peptide anti-biotics exhibit absorption maxima at 210–230 and 270–280 nm [21–23]. A peptide antibiotic, cerein, obtained from *Bacillus cereus*, shows UV absorption maxima at 198, 255 and 273 nm and weak absorbance peak at 250 and 273 nm [24]. FTIR spectra of antimicrobial peptide had a broad band centering around  $1644.87\text{ cm}^{-1}$  indicating an amino and hydroxyl group of amino acids. Analysis of the spectrum also shows typical absorption bands ( $1670.5\text{ cm}^{-1}$  and  $1539.8\text{ cm}^{-1}$ ) corresponding to N-H stretching of proteins and peptide bonds [25]. The band at  $1275\text{ cm}^{-1}$ ,  $1185\text{ cm}^{-1}$  and  $1079\text{ cm}^{-1}$  showing presence of aldehyde group. Additional absorption values  $1634\text{ cm}^{-1}$  and  $1399\text{ cm}^{-1}$  indicating (C-H) aliphatic side chain may be related with predominance of hydrophobic amino acids such as Val, Leu and Ileu [24].

Based on the investigation of TLC ninhydrin positive spots showed the presence of peptide was further confirmed using NMR. On the basis of solubility, chloroform soluble fraction was selected for further studies by ESI-MS analysis. The ESI-MS of the ninhydrin was positive chloroform fraction. The result showed presence of several molecular ion peak at  $m/z$  514  $[\text{M}+\text{H}]^+$ . This molecular ions  $[\text{M}+\text{H}]^+$  at  $m/z$  514 was selected for identification and it has been tentatively assigned the structure as peptide as dromidin (1) for molecular mass of  $m/z$  513.

The discovery of new bioactive peptides on specific cellular targets resulted in their emergence as promising lead drug candidates. To date, many AMP's, such as crustins, anti-lipopolysaccharide factors, penaei-dins, arasin and hyastatin had been identified from different crustacean species. Many of the peptides isolated from crabs show interesting biological properties. AMP's and proteins have previously been identified in *C. sapidus* [26], *C. maenas* [27,28], *S. serrata* [29]. In *P. bernhardus*, the active fractions were generally heat labile, whereas in *H. araneus* the activities were resistant to heat. Heat labile antibacterial factors have been detected in several crustaceans [30]. A marine-derived compound, abalone hemocyanin, from *Haliotis rubra* was shown to have a unique mechanism of antiviral activity against herpes simplex virus 1 (HSV-1) infections [31]. However, an antibacterial protein of 11.5 kDa isolated which was active even after heating for 10 min at 100 °C [28]. These results indicates that crustaceans have developed a variety of defense molecules against pathogenic micro-organisms and the kind dominating vary among the different species.

## **5. Conclusion**

Thus, on the basis of the results obtained from the analytical techniques HPLC, TLC, UV, FTIR, ESI-MS and NMR data, AMP from the crab had been characterized. A safe and more effective natural compound is a challenge to the pharmaceutical industries, especially with increase in antibiotic resistant pathogens. Current findings strongly suggested that the peptide plays an important role against broad range of bacteria and fungi. With this background, the biotechnological production potential of antimicrobial peptides from *D. dehaani* is noteworthy.

## **Conflicts of interest**

We have no conflicts of Interest.

## **Acknowledgement**

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## References

- [1] P. Bulet, R. Stocklin, L. Menin, Anti-microbial peptides: from invertebrates to vertebrates, *Immunol. Rev.* 198 (2004) 169–184.
- [2] N.Y. Yount, A.S. Bayer, Y.Q. Xiong, M.R. Yeaman, *Adv. Antimicrob. Pept. Immunobiol. Biopolym.* 84 (2006) 435–458.
- [3] E. Guaní-Guerra, T. Santos-Mendoza, S.O. Lugo-Reyes, L.M. Teran, Antimicrobial peptides: general overview and clinical implications in human health and disease, *Clin. Immunol.* 135 (2010) 1–11.
- [4] A. Doyen, L. Beaulieu, L. Saucier, Y. Pouliot, L. Bazinet, Demonstration of in vitro anticancer properties of peptide fractions from a snow crab by-products hydrolysate after separation by electro dialysis with ultra-filtration membranes, *Separ. Purif. Technol.* 78 (2011) 321–329.
- [5] S.P. Antony, I.B. Singh, N.S. Sudheer, S. Vrinda, P. Priyaja, R. Philip, Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile in response to various immunostimulants and challenge with WSSV, *Immunobiology* 216 (2011) 184–194.
- [6] S. Ravichandran, S. Wahidulla, L. D'Souza, G. Rameshkumar, Antimicrobial lipids from the hemolymph of brachyuran crabs, *Appl. Biochem. Biotechnol.* 162 (2010) 1039–1051.
- [7] S. Ravichandran, S. Wahidullah, L. D'Souza, R.M. Anbuchezhian, Antimicrobial activity of marine sponge *Clathria indica* (Dendy, 1889), *Russ. J. Bioorg. Chem.* 37 (4) (2011) 428–435.
- [8] J.L. Rolland, M. Abdelouahab, J. Dupont, F. Lefevre, E. Bachère, B. Romestand, Stylicins A new family of antimicrobial peptides from the Pacific blue shrimp *Litopenaeus stylirostris*, *Mol. Immunol.* 47 (2010) 1269–1277.
- [9] S.V. Sperstad, T. Haug, V. Paulsen, T.M. Rode, G. Strandskog, S.T. Solem, O.B. Styrvoid, K. Stensvåg, Characterization of crustins from the hemocytes of the spider crab, *Hyas araneus*, and the red king crab, *Paralithodes camtschaticus*, *Dev. Comp. Immunol.* 33 (2009) 583–591.
- [10] C. Sun, X.J. Du, W.T. Xu, H.W. Zhang, X.F. Zhao, J.X. Wang, Molecular cloning and characterization of three crustins from the Chinese white shrimp, *Fenneropenaeus chinensis*, *Fish Shellfish Immunol.* 28 (2010) 517–524.
- [11] A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, *Am. J. Clin. Pathol.* 45 (1966) 493–496.
- [12] I.B. Suffredini, H.S. Sader, A.G. Goncalves, A.O. Reis, A.C. Gales, A.D. Varella, R.N. Younes, Screening of antibacterial activity extracts obtained from plants native to Brazilian Amazon rain forest, *Braz J Med Ethnopharmacol* 62 (2004) 183–193.
- [13] J.R. Porter, A convenient microdilution method for screening natural products against bacteria and fungi, *Pharm. Biol.* 39 (2001) 221–225.
- [14] M.G. Ryadnov, G.V. Mukamolova, A.S. Hawrani, J. Spencer, R. Platt, RE coil: an antimicrobial peptide regulator, *Angew. Chem. Int. Ed.* 48 (2009) 9676–9679.

- [15] P. Roepstorff, J. Fohlman, Letter to the editors, *Biol. Mass Spectrom.* 11 (11) (1984) 601–601.
- [16] R.S. Johnson, S.A. Martin, K. Biemann, J.T. Stults, J.T. Watson, Novel fragmentation process of peptides by collision-induced decomposition in a tandem mass spectrometer: differentiation of leucine and isoleucine, *Anal. Chem.* 59 (21) (1987) 2621–2625.
- [17] M. Charlet, S. Chernysh, H. Philippe, C. Hetru, J.A. Hoffmann, P. Bulet, Innate immunity Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*, *J. Biol. Chem.* 271 (1996) 21808–21813.
- [18] D. Destoumieux, M. Munoz, P. Bulet, E. Bachere, Penaeidins, a family of anti-microbial peptides from penaeid shrimp (Crustacea, Decapoda), *Cell. Mol. Life Sci.* 57 (2000) 1260–1271.
- [19] G. Mitta, F. Vandenbulcke, T. Noel, B. Romestand, J.C. Beauvillain, M. Salzet, P. Roch, Differential distribution and defence involvement of antimicrobial peptides in mussel, *J. Cell Sci.* 113 (2000) 2759–2769.
- [20] D. Destoumieux, P. Bulet, J.M. Strub, A. van Dorsselaer, E. Bachère, Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp, *Eur. J. Biochem.* 266 (1999) 335–346.
- [21] K. Kurusu, K. Ohba, T. Arai, K. Fukushima, New peptide antibiotics LI-F03, F04, F05, F07, and F08, produced by *Bacillus polymyxa*. I. Isolation and characterization, *J. Antibiot.* 40 (1987) 1506–1514.
- [22] F. Besson, F. Peypoux, G. Michel, L. Delcambe, Identification of antibiotics of iturin group in various strains of *Bacillus subtilis*, *J. Antibiot.* 31 (1978) 284–288.
- [23] A.S. Motta, A. Brandelli, Characterization of an antibacterial peptide produced by *Brevibacterium linens*, *J. Appl. Microbiol.* 92 (2002) 63–70.
- [24] D. Bizani, A.P. Dominguez, A. Brandelli, Purification and partial chemical characterization of the antimicrobial peptide cerein 8A, *Lett. Appl. Microbiol.* 41 (2005) 269–273.
- [25] K. Maquelin, C. Kirschner, L.P. Choo-Smith, N. van den Braak, H.P. Endtz, D. Naumann, G.J. Puppels, Identification of medically relevant microorganisms by vibrational spectroscopy, *J. Microbiol. Meth.* 51 (2002) 255–271.
- [26] L. Khoo, D.W. Robinette, E.J. Noga, Callinectin, an antibacterial peptide from blue crab, *Callinectes sapidus*, hemocytes, *Mar. Biotechnol.* 1 (1999) 44–51.
- [27] D. Schnapp, G.D. Kemp, V.J. Smith, Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to Bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*, *Eur. J. Biochem.* 240 (1996) 532–539.
- [28] J.M. Relf, J.R. Chisholm, G.D. Kemp, V.J. Smith, Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, *Carcinus maenas*, *Eur. J. Biochem.* 264 (1999) 350–357.

- [29] V. Jayasankar, T. Subramoniam, Antibacterial activity of seminal plasma of the mud crab *Scylla serrate* (Forsk.) J Exp Mar Biol Ecol. 236 (1999) 253–259.
- [30] E.J. Noga, D.P. Engel, T.W. Arroll, S. McKenna, M. Davidian, Low serum anti-bacterial activity coincides with increased prevalence of shell disease in blue crabs *Callinectes sapidus*, Dis. Aquat. Org. 19 (1994) 121–128.
- [31] N.T. Zanjani, M. Miranda-Saksena, P. Valtchev, R.J. Diefenbach, L. Hueston, E. Diefenbach, F. Sairi, V.G. Gomes, A.L. Cunningham, F. Dehghani, Abalone he-mocyanin blocks the entry of Herpes Simplex Virus 1 into cells: a potential new antiviral strategy, Antimicrob. Agents Chemother. 60 (2) (2016) 1003–1012.