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Anti-pathogenic bacterial activities of fractionated venom of king cobra *(Ophiophagus Hannah*)

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Abstract

This study aimed at measuring the antibacterial activity of the semi-purified fractions isolated from King Cobra venom and investigated the mechanism of action. The protein fractions were isolated from King Cobra venom and designated F1-F7. The F5 showed the highest antibacterial activity. The F5 was further concentrated and separated by Sephadex G-75 column to obtain six fractions (F5.1-F5.6). The antibacterial assay showed that F5.1 could inhibit the growth of gram-positive *S. aureus* and gram-negative *E. coli, S. aeruginosa* and *S.* Typhimurium bacteria. The F5.1 showed antibacterial activity against *S. aureus* with the MIC of 1.8 μg/ml. Its molecular weight was 69 kDa, which was determined by SDS-PAGE. The mechanism of action may be damaging the cytoplasmic membrane, as shown from the scanning electron microscopy. The MIC concentration of F5.1 showed potent cytotoxicity on Human PBMCs cells. The results obtained from this study indicated that the F5.1 had antibacterial activity against the *S. aureus* through membrane damage, it was also cytotoxic to normal human cells. In conclusion, this study provides basic information on antibacterial effect of the semi-purified F5.1 of King cobra venom. The F5.1 should be further purified or modified to obtain the potential antibacterial compound which will be safe to the normal human cells.

Keywords: *Ophiophagus Hannah*, snake venom, fractionated, antibacterial, membrane damage

Introduction

Antibacterial proteins and peptides have been found and isolated from a variety of plants, animals and microorganism. Snake venoms contained a complex mixture of proteins and peptides (90-95%). Antibacterial proteins and peptides were found in the snake venoms, such as L-amino acid oxidase from *Bothrops alternates* (1), PLA_2 myotoxins from *Bothrops asper* snake venoms (2), metalloproteases from *Bothrops jararacussa* (3) and Small peptide from *Naja atra* venom (4). However, King Cobra venom (*Ophiophagus Hannah*) that are rich source of proteins and peptides have a variety of pharmacological activities such as antinociceptive activity, antiplatelet aggregation, anticonvulsant effects and cytotoxicity (5), however the antimicrobial activity has not been investigated.

The aim of this preliminary study was to find the antibacterial activity of fractionated venom of King Cobra against the pathogenic bacteria including both gram-negative and gram-positive bacteria in order to evaluate the potential use of its components as an antibacterial agent.

Materials and Methods

The crude venom of King Cobra was tested for antibacterial properties against the pathogenic bacteria including gram-positive *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC 19615) and gram-negative *E. coli* (ATCC 25922), *S.*Typhimurium (ATCC 25922) and *P. aeruginosa* (ATCC 27853) bacteria by using disc diffusion assay for in vitro susceptibility testing. The F1-F7 obtained from the Snake Venom Research Division, Queen Saovabha Memorial Institute were tested for the antibacterial activity. MIC were determined using microbroth dilution method. The highest active fraction was collected and then purified by Sephadex G-75 column equilibrated and eluted with 20 mM TBS buffer. Protein elution was monitored at uv absorption of 280 nm. Each fraction was collected, dried and resuspended in 10 mM sodium phosphate buffer and tested for antibacterial activity by microbroth dilution method. The molecular weight of the highest potent fraction was determined by 12.5% SDS-PAGE. Tested bacteria was treated with F5.1, the highest potent fraction, at the MIC for 3 and 6 hours. The bacteria was visualized using scanning electron microscopy to determine the bacterial cell structure. Cytotoxicity of the MIC of F5.1 was assessed by using rezasurin assay on the human PBMCs cells. Viability of the cells was measured by microplate reader spectrophotometer.

Statistical analysis

Data was presented as mean ± standard error (mean ± S.E.). Statistical comparisons were made by one-way ANOVA. Any p-value < 0.05 was considered statistically significant.

Results

The crude venom showed concentration-dependent antibacterial activity against the pathogenic gram-positive strain, *S. aureus*, and gram-negative strains such as *E. coli*, *S.*Typhimurium and *P. aeruginosa* (Figure 1). The F5 had the strongest inhibitory activity against *S. aureus* and *P. aeruginosa* with the MIC of 3.90 µg/ml and 62.5 µg/ml respectively (Table 1). The F5 was further separated by Sephadex G-75 column and obtained six fractions (F5.1, F5.2, F5.3, F5.4, F5.5, F5.6). The F5.1 was found to possess the strongest inhibitory activity against all the tested bacteria with the MIC ranging from 1.95 to 7.81 µg/ml. The F5.2 possessed very low antibacterial activity with the MIC of 125-500 µg/ml. The F5.3, F5.4 and F5.6 were inactive against all of the tested bacteria. The F5.5 was active against only *P. aeruginosa* (Table 2). The MIC for *S. aureus* was further determined by measuring the turbidity at 600 nm, the MIC was found to be 1.8 µg/ml (Figure 2). From the SDS-PAGE, the molecular weight of F5.1 was 69 kDa (Figure 3). The mechanism of action of F5.1 was explored, the morphological changes induced by F5.1 on *S. aureus* were examined using SEM. Figure 4 showed the SEM micrographs of the buffer-treated and F5.1-treated cells of *S. aureus* at 3-6 hours of exposure. The buffer-treated *S. aureus* had smooth and normal surface morphology (Fig. 4A, 4C). The F5.1-treated *S. aureus* showed large globular surface protrusions on the bacterial cell surface (Fig. 4B, 4D). The F5.1 had cytotoxic activity on Human PBMCs cells at MIC concentration.

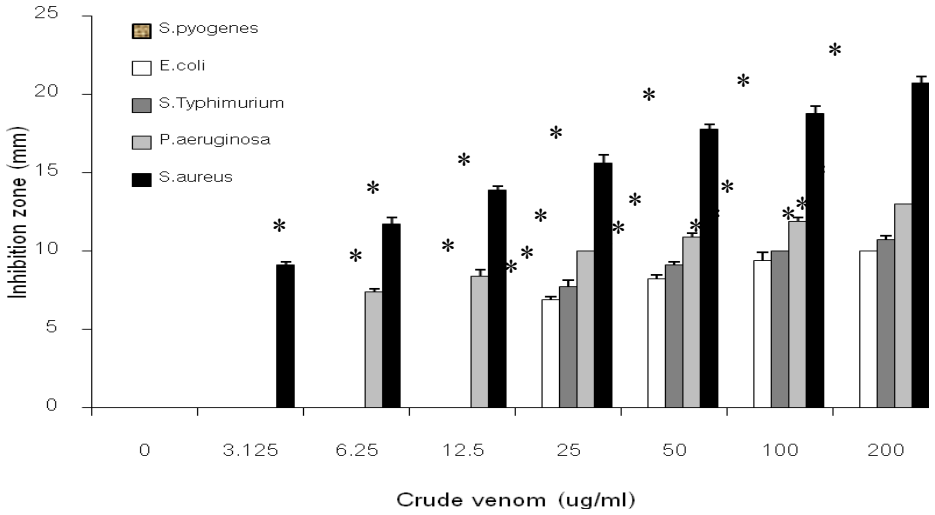


Figure 1 Antibacterial activity of King Cobra crude venom Disc diffusion assay. Antibacterial activity of crude venom of King Cobra. The inhibition zone was shown as mean ± S.E. of five replicate discs. Data was analyzed by one way ANOVA, $p < 0.05$ was considered sigfinicantly different when compared with the control.

Table 1 Antibacterial activity of F1-F7 peak fractions determined by microbroth dilution method

Microorganism	MIC (µg/ml)		MIC (µg/ml) Q-Sepharose						
	Cipro-floxacin	Crude venom	F1	F2	F3	F4	F5	F6	F7
E. coli	0.015	-	-	-	-	500	250	125	250
P. aeruginosa	0.5	-	-	-	-	1,000	62.5	250	250
*S.*Typhimurium	2.0	-	-	-	-	-	1,000	62.5	250
S. aureus	0.5	15.62	-	1,000	-	62.5	3.90	250	500

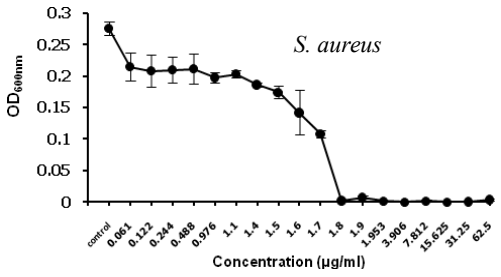
Each concentration was performed a triplication, MIC: Minimum inhibitory concentration, -: no antibacterial activity detected at concentrations up to 1,000 µg/ml

Table 2 Antibacterial activity of Sephadex G-75 peak fractions determined by microbroth dilution method (each concentration was performed a triplication)

Microorganism	Sephadex G-75 fractions of F5 (µg/ml)											
	F5.1		F5.2		F5.3		F5.4		F5.5		F5.6	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	7.81	7.81	500	500	-	-	-	-	-	-	-	-
P. aeruginosa	7.81	7.81	125	250	-	-	-	-	500	500	-	-
*S.*Typhimurium	7.81	62.5	500	500	-	-	-	-	-	-	-	-
S. aureus	1.95	1.95	125	125	-	-	-	-	-	-	-	-

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, -: no antibacterial activity detected at concentrations up to 500 µg/ml

Figure 2 Minimum inhibitory concentration (MIC) of F5.1 peak fractions against *S. aureus* determined by microbroth dilution method (each concentration was performed a triplication)



Microorganism	F5.1 MIC (µg/ml)
E. coli	7.0
P. aeruginosa	6.0
*S.*Typhimurium	6.0
S. aureus	1.8

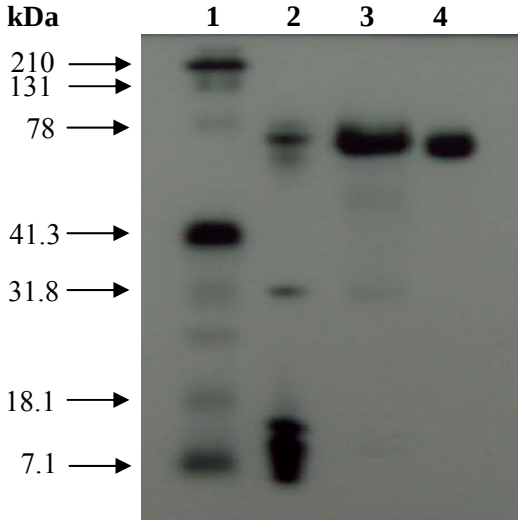


Figure 3 Molecular weight of F5.1 analyzed by 12.5% SDS-PAGE: Lane 1: protein markers including myosin (210 kDa), *β*-galactosidase (131 kDa), bovine serum albumin (78 kDa), carbonic anhydrase (41.3 kDa), soybean trypsininhibitor (31.8 kDa), lysozyme (18.1 kDa) and aprotinin (7.1 kDa); Lane 2: *O.hannah* crude venom; Lane 3: F5; Lane 4: F5.1. The molecular masses (kDa) of the markers are indicated on the left column.

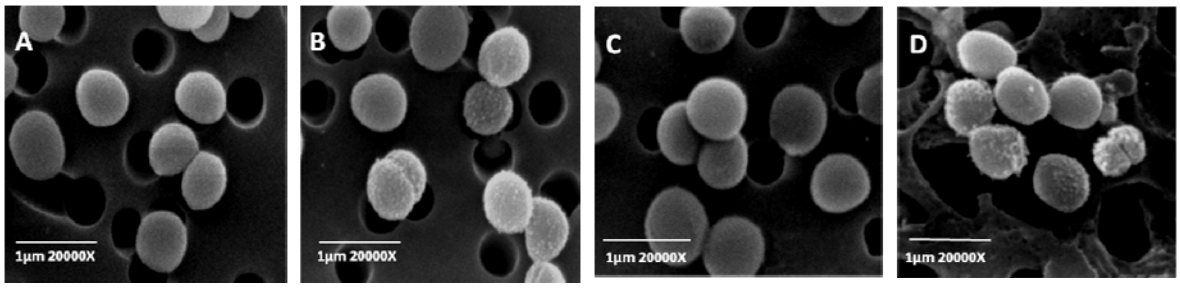


Figure 4 Scanning electron micrographs of *S. aureus* treated with the F5.1. Incubated *S. aureus* at 37 ^{o}C for 3 h, with 10 mM sodium phosphate buffer (pH 7.4) (control, A) and with 1.8 µg/ml F5.1(B) and for 6 h, with10 mM sodium phosphate buffer (pH 7.4) (control, C) and with 1.8 µg/ml F5.1(D).

Discussion and Conclusion

The results obtained from this study indicated that F5.1 with molecular weight of 69 kDa showed inhibitory activity against pathogenic bacteria. *S. aureus* was more susceptible to the F5.1 than *P. aeruginosa, E. coli* and *S.* Typhimurium. The mechanism of antibacterial activity may be through the membrane damage. The MIC of F5.1 was cytotoxic to the human normal cells. From the previous report, the pEM-2, a modified synthetic peptide derived from a snake venom Lys49 phospholipase A_2 showed reduced toxicity towards muscle cells, while retaining high bactericidal potency (3). Therefore, F5.1 should be further studied in terms of

cytotoxic components or modified the amino acid sequence of this protein to decrease toxicity and retain antibacterial activity. It will be useful in the search and developing for new potential antibacterial agents against pathogenic bacteria.

Acknowledgements

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