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Research Article

Antibacterial activity of snake venoms against bacterial clinical isolates

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Abstract

Recently, many antibacterial agents have been found in the venoms of animals from different sources. However, multidrug-resistant strains of bacteria are an important health problem in need for new antibacterial sources and agents. This study aimed to evaluate the antibacterial activity of several snake crude venoms in Elapidae family against several strains of gram-positive and gram-negative bacteria as new sources of potential antibacterial agents. Current studies revealed that king cobra (*Ophiophagus hannah*) crude venom showed selective antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) more efficient than tested antibiotics currently on the market. King cobra crude venom showed the minimum inhibitory concentration (MIC) = 8 µg/ml against MRSA, whereas standard antibiotics (ampicillin, penicillin, chloramphenicol and tetracycline) showed MIC in the range of 8-64 µg/ml. The result of scanning electron microscope revealed that king cobra crude venom exerted antibacterial activity against gram-positive bacteria via its membrane-damaging activity and it is a feasible source for exploring antimicrobial prototypes for future design new antibiotics against drug-resistant clinical bacteria.

1. INTRODUCTION

Bacterial infections have become increasingly difficult to treat as microorganisms have been developing resistance to a variety of antimicrobial agents, for example, *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Acinetobacter*, *Mycobacterium*, *Salmonella*, *Staphylococcus*, *Streptococcus* and *Enterococcus* spp.[1]. Antibiotic-resistant organism is a serious problem and it is a major challenge in medicine since not many new antibiotics are being produced. Moreover, bacteria resistant to currently available drugs are increasing. To overcome the multidrug resistant bacteria (MDR), new antimicrobial agent that are broadly effective is the strategic option[2]. It has been reported that natural products are an important source of medicinal compounds and they have been shown to be able to kill bacteria[3,4]. Currently, it is known that venoms can be useful and valuable as pharmacological substances in drug research [5].

Snake venoms contain many proteinaceous and non-proteinaceous components. Their venoms are a mixture of proteins

and peptides (90-95%), including nucleotide, amino acids, lipids, carbohydrates and metallic elements bound to proteins (5%)[6, 7], for example; neurotoxin, cytotoxins, myotoxins, proteases, nuclease, and peptides. Most venomous snakes belong to two families, the Elapidae (cobras, mambas, kraits, coral snake and sea snake) and Viperidae (rattle snakes, copperheads, cottonmouths, European viper etc.). Snake venoms are varying in proportions and characteristics of the specific biochemical activities among different species[8, 9]. They contain numerous components of bioactive compounds. The broad spectrum of snake venom activities results from the action of their constituents. One in particular has become more significant in their antimicrobial activities. It has been reported that snake venoms and other animal's venoms are a rich source of protein and non protein of pharmacological interest. Antibacterial components in snake venoms may protect the host after eating prey contaminated with pathogens[10]. Several antimicrobial of snake venoms have been described in the literature, ex. the inhibitory effects of *Naja naja sputatrix*, *Vipera russelli*, and *Crotalus adamanteus* in *E. coli*[11]. In 1991, the antibacterial activities of 30 different snake venoms had been studied by Sitles et al and found that the Asian and African snakes (*Naja* sp.), Australian elapids (*Notechis scutatus* and *Pseudechis australis*) and North American snakes (*Crotalus* sp.) presents the highest antibacterial activities[12]. Antibacterial effects of viperid venoms had been described by Skarnes et al[13]. Bactericidal effects of rattle snake venoms were observed on gram-positive *Sarcina* species, but there was little effect against *Bacillus subtilis*, *E. coli*, or *S. aureus*. More recently report, snake venoms has shown promising activities against common infectious bacteria, such as *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *B. subtilis*, *Proteus mirabilis*, *Proteus vulgaris*, and *Enterobacter aerogenes*[11, 12, 14, 15]. Gomes et al reported that crude venom and isolated peptide from the *Bothrops jararaca* showed the activity against *S. aureus* and different fungi[16].

In spite of several works reported on this field, the present study was conducted to evaluate the antibacterial activity of snake venoms against different strains of gram-positive and gram-negative bacteria.

2. MATERIAL AND METHODS

2.1. Venoms

Lyophilized venoms- *Naja siamensis* (Thailand), *Naja sputatrix* (Indonesia), *Naja naja* (Pakistan), *Naja naja* (India), *Naja naja* (Sri Lanka), *Naja atra* (China), *Naja kaouthia* (Thailand), *Naja oxiana* (Pakistan), *Naja karachiensis* (Pakistan), *Bungarus multicinctus* (China), *Bungarus sindanus* (Pakistan), *Bungarus candidus* (Indonesia), *Bungarus fasciatus* (Thailand), *Bungarus caeruleus* (Pakistan), *Ophiophagus hannah* (Thailand). Venoms from captive specimens were collected manually by milking. Each sample were freeze-dried and stored at -20°C.

2.2. Bacterial strains

The bacterial strains used in the antibacterial screening were *Staphylococcus aureus* (*S. aureus)* ATCC 25923, *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA, clinical isolate), *Staphylococcus epidermidis (S. epidermidis)* (clinical isolate), *Staphylococcus saprophyticus* (clinical isolate), *Staphylococcus lugdunensis*, (clinical isolate), *Enterococcus faecalis* (clinical isolate), and *Escherichia coli* (*E. coli*) ATCC 25922.

2.3. Antibacterial activity assay

Antibacterial activity screening assay by disk diffusion test and MIC determination was performed according to the Clinical Laboratory Standards Institute (CLSI 2014) and Ferreira BL et.al.[17]. Briefly, the bacterial strains were grown at 37°C in trypticase soy agar for 16-18 hours and turbidity was adjusted to the 0.5 McFarland standards ($1.5x10^8$ colony forming unit/ml) with sterile normal saline. A sterile swab was dipped into the inoculum and the excess inoculums were removed by pressing the swab firmly against the side of the tube above the level of the liquid. The swab was streaked all over the surface of Mueller-Hinton agar plates three times. Each snake venom solution was prepared at 25 mg/ml by dissolved in sterile deionized water. Then, 1 µL of the solution was applied on Whatman paper disks (6 mm diameter) and further put on bacterial culture in triplicate assay, and then incubated for 16-24 hours at 35±2°C. The results were verified by measuring the inhibition zones surrounding the

disk. Chloramphenicol, ampicillin, penicillin, tetracycline disks and sterile deionized water disk were used as positive and negative controls. The inhibition zone >15 mm was considered the minimum value for positive antimicrobial activity. *S. aureus* ATCC 25923 was used as positive bacterial control.

The snake venoms which showed the highest diameter of inhibition zones were chosen for minimum inhibitory concentration (MIC) determination. MIC test was performed according to the Clinical Laboratory Standards Institute (CLSI 2014), the broth microdilution method. After 3 hours of the bacterial growth, the culture was diluted to obtain 10^6 CFU/ml. The snake venom was diluted by two-folded dilution with Mueller-Hinton broth and then the diluted bacterial culture was added in order to reach a final concentration from 256 µg/ml to 0.125 µg/ml. After 16-18 hours of incubation at 35±2°C, MIC was defined as the lowest concentration of venom or antibiotic preventing visible bacterial growth compared to the positive growth control (medium plus bacteria without venom or antibiotic) that presented high turbidity, and to the negative growth controls (medium only).

2.4. Scanning electron microscopy (SEM)

Approximately 10^8 CFU/ml of *S. aureus* ATCC 29213 was re-suspended in 2 tubes of 10 mM sodium phosphate buffer (pH 7.4). One tube was set as control and the other tube was treated with crude venom of *O. hannah* at a concentration of 80 µg/ml (5X MIC) at 37°C for 4 hours. After incubation, the bacteria were washed and fixed with an equal volume of 2.5% glutaraldehyde. The fixed samples were stored overnight at 4°C in the fixative solution. Then the samples were dehydrated by a series of alcohol (30, 50, 70, 95%) once and 100% totally 3 times and 5 minutes each. The samples were transferred in 100% ethanol to a critical point dryer (Quorum K850, UK) and coated with gold for 3 minutes (Balzers SCD 040 Germany). The bacteria were visualized using FESEM-EDS (7610F) (Field Emission Scanning Electron Microscope and Energy Dispersive X-ray Spectrometer scanning electron microscopy) with an accelerating voltage of 10 kV to determine the bacterial cell structure.

2.5. Statistical analysis

Descriptive statistics was performed with SPSS and Data were expressed as means ± *standard deviation* (SD)

3. RESULTS

In order to evaluate the antibacterial effect of snake venoms, the disk diffusion and MIC assay were performed and the different standard antibiotics were used as a positive control. However, only venoms that showed the best activity of anti-bacterial activity was chosen for MIC assay.

Table 1 showed the antibacterial effects of 15 crude venoms tested against 8 different strains of bacteria; 7 different strains of gram-positive and 1 strain of gram-negative bacteria. The results showed that gram-negative bacteria were resistant to all the crude venoms as no inhibition zone was observed in the antibacterial assay. In three independent experiments, all venoms from Elapidae family had shown no antibacterial activities against *E. coli* and *Enterococcus faecalis*. However, interestingly, all crude venoms demonstrated significant inhibition zones against *S. aureus* ATCC 25923 between 7 - 14 mm and *O. hannah* showed the largest inhibition zone (14 ±1 mm). Only some crude venoms (*N. siamensis* (Thailand), *N. naja* (Pakistan)*, N. oxiana* (Pakistan), *N. karachiensis* (Pakistan), *N. naja* (India), *N. kaouthia* (Thailand), *B. sindanus* (Pakistan), *B. fasciatus* (Thailand), and *O. hannah* (Thailand) which showed top nine of largest inhibition zone (≥ 10 mm) against *S. aureus* ATCC 25923 were chosen to test against other strain of gram-positive bacteria (*S. aureus* ATCC 29213, MRSA, *S. epidermidis, S. saprophyticus, S. lugdunensis. E. faecalis*). The results showed that the tested venoms showed the inhibition zone against those six gram-positive bacteria (*S. aureus* ATCC 29213, MRSA, *S. epidermidis, S. saprophyticus, S. lugdunensis*, *E. faecalis*) between 8-16 mm, 7-13 mm, 9-18 mm, no inhibition zone (NI)-8 mm, 7-14 mm, NI respectively. Among these nine venoms, O. *hannah* showed the largest inhibition zone against all six different gram-positive bacteria (Figure 1).

Table 1. In vitro antibacterial activity of snake venoms against gram-positive and gram-negative bacteria compare to some tested antibiotics. Each number was presented as mean ± SD of inhibition zone in mm.

Microorganism Crude Venoms/ Antibiotic	*S. aureus* ATCC 25923	*S. aureus* ATCC 29213	*E. coli* ATCC 25922	MRSA	*S. epider-midis*	*S. sapro-phyticus*	*S. lugdu-nensis*	*E. faecalis*
1. *N siamensis* (Thailand)	10 ± 0	8 ± 1	NI	8± 1	10± 1	7±1	8 ±1	NI
2. *N. sputatrix*	9 ± 0	ND	NI	ND	ND	ND	ND	NI
3. *N. naja* (Pakistan)	11±1	10 ± 0	NI	8± 1	9± 1	8±1	8 ± 1	NI
4. *N. atra* (China)	7±0	ND	NI	ND	ND	ND	ND	NI
5. *N. oxiana* (Pakistan)	11± 1	10± 1	NI	10±1	12 ± 1	7±1	8 ±1	NI
6. *N. karachiensis* (Pakistan)	11±1	11±1	NI	10± 1	12 ± 1	7± 1	8±1	NI
7. *N. naja* (Sri Lanka)	6±1	ND	NI	ND	ND	ND	ND	NI
8. *N. naja* (India)	10±1	10±1	NI	7±1	10±1	7±1	7±1	NI
9. *N. kaouthia* (Thailand)	10±1	9±1	NI	9± 1	11±1	7±1	7± 1	NI
10. *B. multicinctus* (China)	9±0	ND	NI	ND	ND	ND	ND	NI
11. *B. sindanus* (Pakistan)	11±1	10±1	NI	9± 1	10 ± 0	NI	8 ± 1	NI
12. *B. candidus* (Indonesia)	9±1	ND	NI	ND	ND	ND	ND	NI
13. *B. fasciatus* (Thailand)	10±1	10±1	NI	10±1	11±1	7±1	8±1	NI
14. *B. caeruleus* (Pakistan)	8± 1	ND	NI	ND	ND	ND	ND	NI
15. *O. hannah* (Thailand)	14±1	16±1	8± 0	13±1	18±0	8± 1	14±1	NI
Ampicillin	35±1	29±1	18±0	NI	24±0	40±1	40±1	25±0
Penicillin	36±1	30±1	NI	NI	21±0	34±1	48±1	16±1
Chloramphenicol	26 ± 1	26 ± 1	27± 1	26± 1	27±1	30±1	32± 1	21±1
Tetracycline	25±0	25±1	21±0	NI	26±0	26±0	28±0	20±0

Note: NI ; No inhibition zone, ND; Not Done

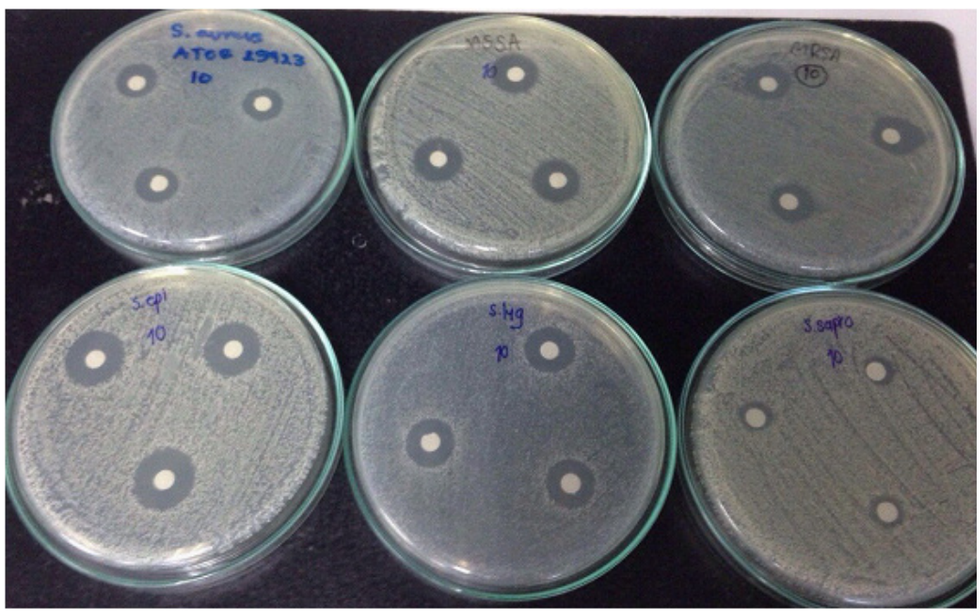

Figure 1. Antibacterial activity of king cobra (*O. hannah*) crude venom against gram-positive bacteria. *S. aureus* ATCC25923 (A); *S. aureus* ATCC29213 (B); MRSA (C); *S. epidermidis* (D); *S. lugdunensis* (E); *S. saprophyticus* (F). The disks were performed in triplicate assay each plate.

Since the crude venom from *O. hannah* demonstrated the most significant inhibition zones against six strains of *Staphylococcus* spp., including the MRSA strain, therefore, *O. hannah* venom was chosen to test its antibacterial activity by broth microdilution method. Table 2 showed the MIC values of *O. hannah* venom on gram-positive bacteria; *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, MRSA, *S. epidermidis*, *S. saprophyticus*, and *S. lugdunensis*. The MIC of *O. hannah* ranged from 4 to 16 µg/ml. When tested against *S. aureus, S. saprophyticus,* and *S. lugdunensis*, the MIC value of the venoms was found to be much higher than some antibiotics (ampicillin, penicillin, chloramphenicol, and tetracycline). However, interestingly, the MIC value of *O. hannah* venom was found to be much lower than ampicillin, penicillin and tetracycline when tested against MRSA and also lower than tetracycline when tested against *S. epidermidis*. The MIC of *O. hannah* venom when tested against *S. saprophyticus* was found to be similar to the MIC level of chloramphenicol but much higher than other antibiotics (ampicillin, penicillin, and tetracycline) (Table. 2)

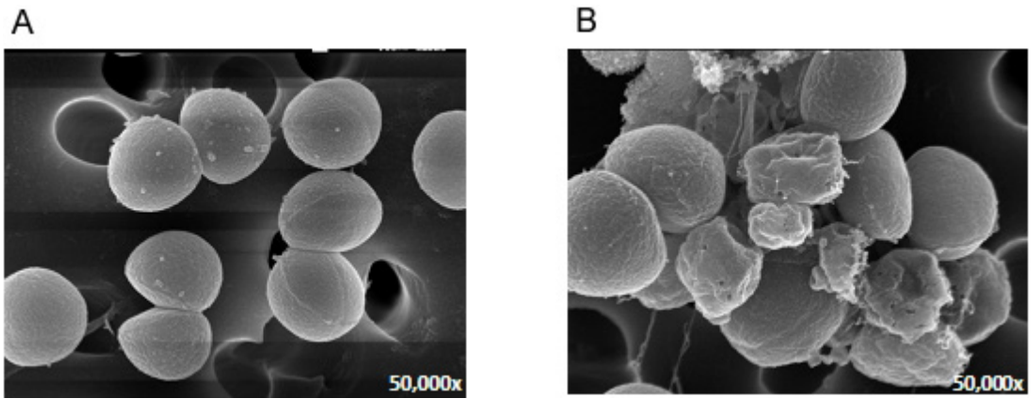

Figure 2. Scanning electron micrographs of *S. aureus* ATCC 29213 incubated with 10 mM sodium phosphate buffer (pH 7.4) (A) and treated with *O. hannah* crude venom at a concentration of 80 µg/ml (B), at 37 °C for 4 hours.

Table 2. Antibacterial activity assay of *O. hannah* crude venom by broth microdilution method (each concentration was performed a triplicate).

Antibiotic/ Crude Venoms	MIC (µg/ml), Bacterial Strains					
	S. aureus ATCC 29213	*S. aureus* ATCC 25923	MRSA	*S. epidermidis*	*S. saprophyticus*	*S. lugdunensis*
Ampicillin	2	0.125	64	0.5	1	0.5
Penicillin	2	0.06	16	8	0.25	0.125
Chloramphenicol	4	2	8	2	4	1
Tetracycline	0.25	0.5	32	32	0.25	0.125
O. hannah (Thailand)	8 -16	8	8-16	4-8	4-8	8

Furthermore, the antibacterial activity was examined by ultrastructural studies. Representative micrographs were shown in Fig. 2. The control cells displayed normal and smooth surface morphology, while *S. aureus* treated with *O. hannah* crude venom showed morphological changes. *O. hannah* crude venom treated *S. aureus* displayed significant roughening, wrinkling, blebbing with several holes on bacterial cell membrane subsequently led to leakage of cytoplasmic content.

It appeared that *O. hannah* crude venom treated cells lost their shape and membrane integrity after treatment.

4. DISCUSSION

Development of new drugs represents one of the most promising in pharmaceutical industry, because recently, a number of microbial drugs resistances have been increasing. New drugs have been extracted and isolated from plants, animals, and microorganism toxins, including snake venoms. In spite of snake venoms toxicological effects, their proteins and peptides have been found practical application as pharmaceutical agents[18]. Concerning the treatment of other critical diseases, there is a great interest in drug design, in which venom toxins could provide new antimicrobial and anticancer agents. The biological significance of antibacterial proteins in the snake venoms is still unclear. These antibacterial venom proteins may help to inhibit bacterial cell growth in the swallowed prey or to protect the snake from bacterial pathogens in the ingested prey [10].

Fifteen snake venoms in Elapidae family have been examined their antibacterial activity using disk diffusion, broth microdilution, and scanning electron microscope. The results of disk diffusion showed that king cobra crude venom was the highest potent antibacterial against gram-positive (*S. aureus* ATCC 25923, MRSA, *S. epidermidis*, *S. saprophyticus, S. lugdunensis*) whereas it does not show activity against gram-positive (*E. faecalis*) and gram-negative bacteria (*E. coli*). It is clear that king cobra venom does not have a wide spectrum antibacterial effect. This selective antibacterial activity may be due to several factors, including structure of lipopolysaccharide and charge density in the case of gram-negative bacteria, or lipid composition of the cytoplasmic membrane and electrostatic potential across this membrane in gram-positive bacteria. These results are consistent to the report of Perumal Samy et al.[4]. Because snake venoms contain many different substance like proteins and enzymes, which are responsible for its biological activities, therefore, these compounds may interact with specific molecules of some bacteria while may not affecting other strains of bacteria. In addition, the resistance to gram-negative bacteria could possibly be due to the outer membrane of the bacteria, which the charges on lipopolysaccharides (LPS) can affect the uptake of some bioactive compound. Although, all venoms are from the same family (Elapidae), only some of them contain antibacterial activities against gram-positive bacteria. These results suggested that the different of proteins, peptides and enzymes from different species of snakes can have varied on antimicrobial activities, resulting in the differences of susceptibility among the different bacterial strains.

In the present report, using broth micro-dilution assay, we have demonstrated that king cobra (*O. hannah*) crude venom is a potent anti-bacterial agent when compared to 4 commons antibiotics. On a weight basis, the MICs of the king cobra venom against MRSA showed higher antibacterial activity than other 4 conventional antibiotics (ampicillin, penicillin, chloramphenicol and tetracycline). The MIC of the king cobra venom against *S. epidermidis* (4-8 µg/ml) was comparable and a bit more potential than penicillin (8µg/ml) however; ampicillin, chloramphenicol and tetra-cycline were more potential than king cobra venom. The MICs of the king cobra venom against *S. saprophyticus* was comparable to chloramphenicol, except for ampicillin, penicillin, and tetracycline which were more potential than the king cobra crude venom. However, MIC of the king cobra against *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, and *S. lugdunensis* were far less potential than all four antibiotics. Even though the MIC values obtained from king cobra crude venom against some gram-positive bacteria were higher than that of standard antibiotics, we believe that the purification of bioactive compound from king cobra venom would exhibit a significant higher inhibition than crude venoms[19].

Thus, in view of its highly potent against MRSA, king cobra crude venom may be a suitable bioactive agent to be developed into an antibacterial agent especially, against MRSA which recently resist to a variety of conventional antibiotics.

In order to study the mechanism of action of antibacterial activity of king cobra venom, morphological changes induced by crude venom on bacteria were studied by SEM. The micrographs of selected experiments are shown in figure 2. After *S. aureus* ATCC 29213 treated with king

cobra crude venom for 4 hours, the structure of bacterial cell membrane was examined. Figure 2 shows that the majority of the cells lost their shape and membrane integrity after treatment. Membrane damage including membrane wrinkling, blebbing, and subsequent leakage of cytoplasmic contents were observed whereas no morphological changes were observed in the control cells. The mode of action of king cobra crude venom presented in these studies is thought to be due to their interaction with the cytoplasmic membrane, leading to its ultimate disruption and leakage of cytoplasm and finally bacterial cell death.

O. hannah venom contains high enzyme activity[20]. Several enzymes from snake venoms have been shown antibacterial activities, for example, L-amino acid oxidase (LAO). The nonspecific cytolytic effects of LAO are due to production of H_2O_2. Recently, the antibacterial activity of phospholipase A_2 (PLA_2) against gram-positive bacteria was reported[21]. The positive charged residues are essential for their activity. The interactions between the PLA_2 molecules and bacterial membrane promote formation of negatively charged peptidoglycan pores in the bacterial cell membrane, allowing the penetration of PLA_2 into bacteria[21]. Samy et al reported that antimicrobial activity of snake venom is due to PLA_2[22]. Furthermore, some studies have demonstrated that pharmacological effects such as antimicrobial and antitumor activities can also be correlated with PLA_2 snake venom activities[23]. Therefore, based on the effect of king cobra venoms on bacterial cell membrane highly suggested that the antibacterial activity in the current study might be due to enzymatic components such as PLA_2.

Further studies on bioactive compound fractionation, structure and the nature of interaction of king cobra venom with the bacterial cell will provide new insight into its antibacterial activity.

5. CONCLUSION

This study reveals that king cobra (*O. hannah*) crude venom contains antibacterial activity against methicillin-resistant *S. aureus* (MRSA) more efficient than tested antibiotics on the market. This study reveals that king cobra snake venom is a feasible source for exploring antimicrobial prototypes for future new antibiotics against drug-resistant clinical bacteria.

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