Snake venom as an antibacterial agent

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1. Abstract

Snake venom is composed of different proteins and enzymes which have been shown to exert beneficial effects in the treatment of certain diseases due to its various biological activities. The present study highlighted the antibacterial effect of *Najanigricollis* and *Cerastes cerastes* venom**.** Only 2 snake venoms ware collected (*Najanigricollis* and *Cerastescerastes)* and examined against 3 typed of bacteria (*Staphylococcus aureus, Salmonella* Typhimurium and *E.coli)* to show the effect of each venom against them. SDS-PAGE profile ware proceded to realize the difference between both venoms. *Cerastes cerates* venom showed variable zones of inhibition against *Staphylococcus aureus* ranged from 16 to 33mm with concentration ranged from 40 µg to 0.3µg and from 9 to 23mm among *Salmonella* Typhimuriumand11-17mm among *E.coli* with concentration ranged from 40µg to 0.3µg. *Najanigricollis* venom showed the best effect on *Staphylococcus aureus* while no effect on Gram-negative bacteria with the same concentrations. The SDS-PAGE profile analysis of the venoms showed great differences in protein contents.

Keywords:Venom, *Najanigricollis, Cerastescerastes, S. aureus, Salmonella* Typhimurium, *E. coli*, SDS-PAGE.

2. Introduction

The discovery, commercialization and routine administration of antimicrobial compounds to treat infections revolutionized modern medicine and changed the therapeutic paradigm. Indeed, antibiotics have become one of the most important medical interventions needed for the development of complex medical approaches such as cutting edge surgical procedures, solid organ transplantation, and management of patients with cancer, among others. Unfortunately, the marked increase in antimicrobial resistance among common bacterial pathogens is now threatening this therapeutic accomplishment, jeopardizing the successful outcomes of critically ill patients. In fact, the World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century [1]**.**

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 of new antibacterial sources and agents [2]**.**

High resistance of *Staphylococcus aureus* against ciprofloxacin, amoxicillin, and chloramphenicol has been documented by **Jamil & Parveen** [3]. The problem has led to a wider search for antimicrobial agents from other sources against the antibiotics resistance bacterial strains. Among these new sources are plant extracts, which have shown promising activities against methicillin-resistant *S.aureus* (MRSA), vancomycin-resistant *S.aureus* (VRSA) and vancomycin-intermediate resistant *S.aureus* (VISA) [4] [5]**.**

With the threat of a post-antibiotic era looming, the search for new and effective antibiotics from novel sources is imperative. Not only has crude snake venom been shown to be effective, but also specific components within the venoms, such as Phospholipase A2s and L-amino acid oxidases have been isolated and demonstrated to be effective as well. Despite numerous studies being completed on snake venoms, there is a heavy bias towards utilizing the venoms from the highly toxic Elapidae and Viperidae species [6]**.**

Considering the latest research; the antibacterial activity of snake venoms may be due to enzymatic components such as L-amino acid oxidase (LAAO). Effectively, the resistance of the Gram-negative bacteria could possibly be attributed to the lipopolysaccharides (LPS) on the outer membrane of the bacteria which affect the uptake of an antimicrobial peptide. The antimicrobial mechanism of the snake venom is complex and could be affected by many factors as outer membrane composition, the net charge of the protein, salinity of the environment. These factors could explain the differences in susceptibility among the different bacterial strains [7].

There are several reports in the literature on the antibacterial activity of snake venom against Gram-positive and Gram-negative bacteria [8] [9] [10] including *Bacillus subtilis*, *Sarcina* spp., *Escherichia coli*, and *S.aureus*.

Snake venom exerts biological and pharmacological effects which attributed to its various contents of hundreds of peptides, proteins, enzymes, and chemicals. These proteins have potential antiviral, antiparasitic and antibacterial activities [11] [12]**.** The present study aimed to investigate the antibacterial potentials of *Naja nigricollis* and *Cerastes cerastes* venoms.

3. MATERIALS AND METHODS

VENOM

Naja nigricollis snake venom and *Cerastes cerastes* viper venom were kindly supplied by Natural Venoms Department, Center of Laboratory Animal Facility and Crude AntiSera Production *(CLAVCAP)*, Holding Company for Biological Products and Vaccines production (*VACSERA*), Giza, Egypt. The venom was collected in Eppendorf tubes and immediately frozen at -4°C. The venom was then lyophilized and stored at -20°C.

BACTERIAL STRAINS

Staphylococcus aureus ATTC No.25923, *Salmonella* Typhimurium ATTC No.14028, and *Escherichia coli* ATTC No.25922 were kindly provided from Animal Health Research Institute.

LD50 Determination, Cytotoxic activity of venom and SDS assay.

Determination of LD50 of the venom:-

Thirty-six Swiss albino mice of average body weight of (18-20) gm obtained from laboratory animal department *CLAVCAP-VACSERA* were used for determination of Median lethal dose (LD50) of tested snake venoms. It was measured according to the Spearman-Karber method[13]**.**

$$m = x100 \pm d/n \ [\Sigma r\text{-}n/2]$$

Determination of the Cytotoxicity of the venom using MTT assay:-

Vero cell line clone CCL-81 was supplied from the tissue culture department, *VACSERA*, were grown in MEM EARLES medium supplemented with 10 % fetal calf serum (FCS), 100 µg/ml penicillin and 10 µg/ml streptomycin. Tissue culture flasks showed fully grown monolayer were selected and were trypsinized using Trypsin 0.25% supplied fro Cell culture media department, VACSERA. The cell suspension was dispensed as 100 µl /well in sterile DNAse & RNAse free non-pyrogenic 96 well tissue culture plate (TPP-Swiss). Plates were incubated at 37°C, CO_2 5% incubator till confluent sheet was microscopically detected using an inverted microscope (HUND-Germany). MTT assay was performed for the in vitro cytotoxicity test according to **Alley** [14].

Twenty four hrs later post cell treatment within 2 fold serially diluted venoms, the treatment medium was decanted and detached cells were washed-out using phosphate buffer saline (PBS). MTT, as 0.05ml was dispensed to washed plates and plates, which were incubated at 37oC for 4 hrs. developed MTT formazan crystals were dissolved using 0.05 ml of DMSO (Sigma, St.Louis). plates were incubated for 30 minutes at 37ºC.

Optical density (OD) was measured using the ELISA reader (Dynatech, USA) at a wavelength of 550-570 nm. The mean optical densities of the test and control wells were recorded. Viability percentage was determined according to **Shin, Cho, Jung, Kim, & Im** [15] using Master-plex2010 software as follows:

The number of residual living cells = (OD of treated cells/ OD of untreated cells) X Number of negative control cells ($3\times10_3$ cells/0.1ml).

Percentage viability = (Number of residual living cells / Number of negative control cells) X 100.

SDS-PAGE(Polyacrylamide gel electrophoresis) of snake venom:-

O'Farrell [16] said that SDS is a technique has been developed for the separation of proteins by two-dimensional polyacrylamide gel electrophoresis. Due to its resolution and sensitivity, it is a powerful tool for the analysis and detection of proteins from complex biological sources.

- **Separating gel** was prepared by assembling the SE 600 vertical slab gel unit in the dual-gel casting stand using 1.5 mm spacers. In a 125 ml side-arm vacuum flask **resolving gel** was prepared by deaerating 13.3 ml acrylamide solution, 10 ml resolving gel buffer, 0.4 ml 10%SDS and 16.1 ml ddH_2O, then add 200µl 10%ammonium persulphate and 13.3 µl TEMED. Pipette the solution downed to a level about 4 cm from the top. Position the pipette filled with water-saturated n-butanol (resolving gel overlay) at about a 45° angle with the point at the top of the acrylamide next to a spacer. Gently applied 0.3 ml of n-butanol on both sides of the slab. Set the gel for full polymerization. Pour off the overlay and rinsed the surfaces of the gels twice with resolving gel overlay. Remove the n-butanol and add approximately 10 ml of resolving gel overlay solution to the top of each sandwich then add 1 ml of resolving gel overlay to each gel and allowed the gels to sit.
- In a 50 ml side-arm vacuum flask **stacking gel** was prepared by deaerating 2.66ml acrylamide solution, 5ml stacking gel buffer, 0.2ml 10%SDS, 12ml ddH_2O then add the 100µl ammonium persulphate and 10µl TEMED. Pour off resolving gel overlay from the gel and fill each sandwich with stacking gel solution and insert a comb into each sandwich then allowed gel to rest for complete polymerization.
- **The sample** prepared by combining equal volumes of protein sample and treatment buffer in a tube and place the tube in a boiling-water bath for 90 seconds. Apply 50 µg of protein per lane. Apply silver staining.
- Slowly remove the combs from the gels then rinse each well with tank buffer then load 5-10 µl of the sample using Hamilton™ syringe beneath the buffer.
- Finally, **Run the gel** by adjusting the current to 30 mA then disconnect the power when the tracking dye reaches the bottom of the gel.

Antibacterial activity:-

Antibacterial activity of the venom was carried out well diffusion assay and compared with antibiotics challenge using amoxicillin with clavulanic acid (AMC) 30 µg, ciprofloxacin (CIP) 5µg and chloramphenicol (C) 30µg.

Wells were cut out of the agar then 40 µl of different concentrations of the venom poured into each well. Plates were incubated at 37°C for 24 hours and the inhibitory zone was determined [17].

In other Petri dishes, bacterial culture ware prepared and antibiotic discs ware added to make the challenge against venom concentration.

4. RESULT

LD50 of the venoms for mice:-

Evaluated LD50 of test venoms was determined using mice inoculation assay and it was calculated recording 8 µg/ ml and 12 µg/ ml for *Naja nigricollis* (NN) and *Cerastes cerastes* (CC) respectively. Data recorded that there was an insignificant difference in the LD50 (P>0.052). Also, it was clear that *N.nigricollis* venom was more toxic than *C.cerastes* venom (**Figure 1**)

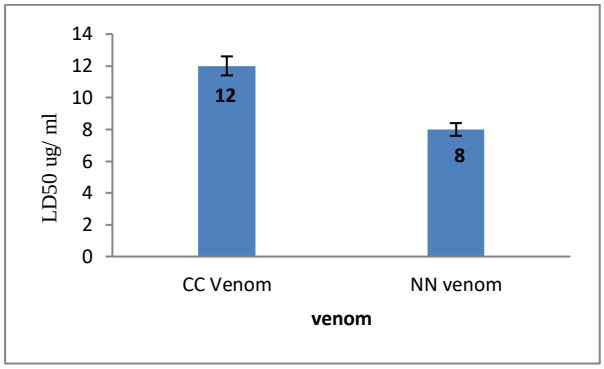

Figure 1: Show different LD50 of venoms used.

Cytotoxicity of the venoms to Vero cells:-

The present study showed that the effect of both *Naja nigricollis* and *Cerastes cerastes* on Vero cells. Data recorded revealed that IC50 of *Naja nigricollis* venom showed lower value compared with that of *Cerastes cerastes* venom (**Figure 2&3**). *N. nigricollis* showed a lower IC50 value than *C.cerastes* venom and the safe concentration was 16µg/ml, while *C.cerastus* showed safe concentration starting from 0.25 µg/ml.

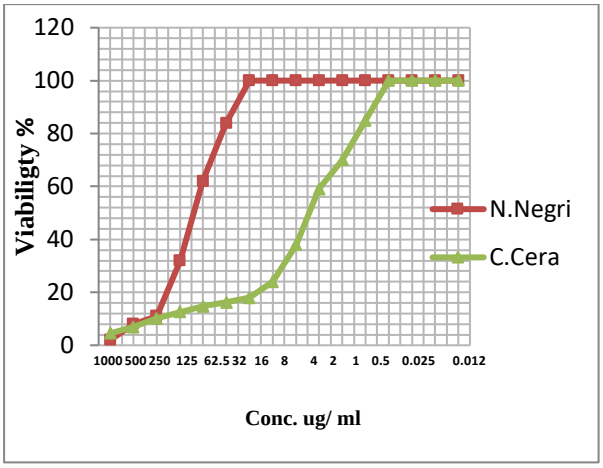

Figure 2: Evaluation of viability % post Vero cell treatment with snake venoms relative to concentration using MTT Assay

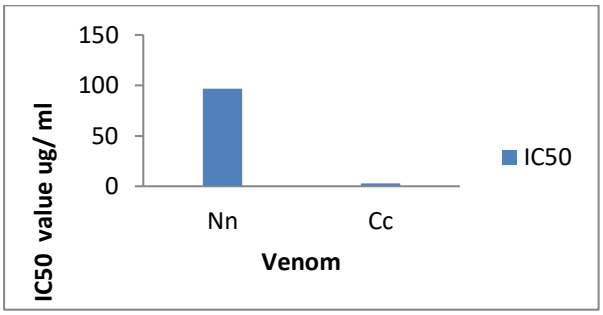

Figure 3: Determination of the IC50 of *N. nigricollis* and *C.cerastus* Vero m using MTT assay

SDS-polyacrylamide gel electrophoresis of snake venom

Figure 2 shows SDS-PAGE, among the venoms, it is clear that many protein bands ware observed for crude *Naja nigricollis* (column 1 and 2) and *Cerastes cerastes* venom(column 4,6 and 8) in compare with molecular weights protein standards (Precision Plus Protein) ranged from 15-205kDa Crude *Naja nigricollis* venom shows various clear protein bands which was distinctly ranged between 14-180 kDa while the crude *Cerastes cerastes* venom shows more many clear protein bands than *Naja nigricollis* venom distinctly ranged between 13-200 kDa (**Figures 3,4&5**).

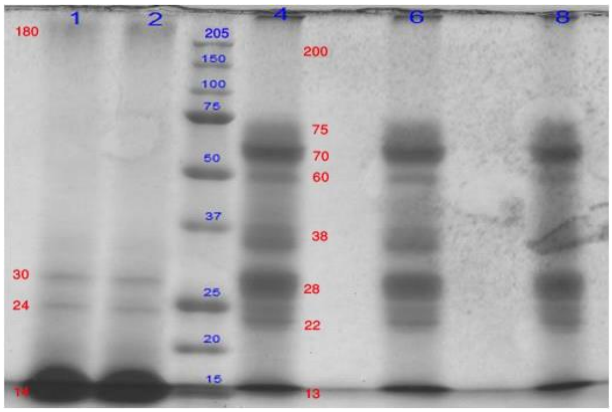

Figure 3 SDS-PAGE showing venoms protein bands.

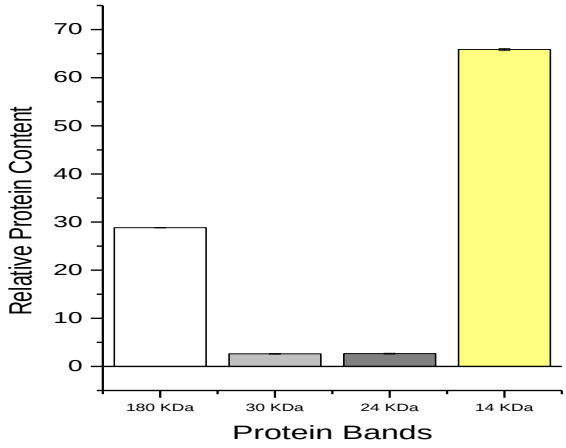

Figure 4: *N.nigricollis* relative protein content and protein bands.

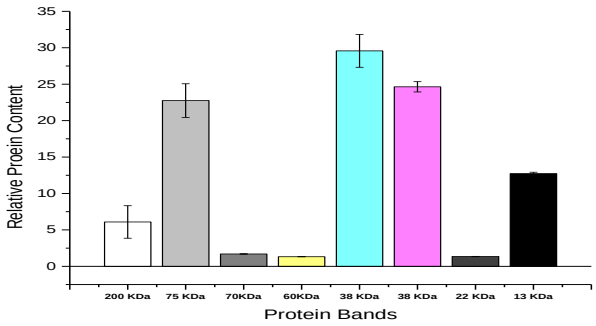

Figure 5: *C.cerastes* relative protei content and protein bands.

Well diffusion assay and challenge against antibiotics:-

The well diffusion assay was carried out for examining the antibacterial activity of both *Naja Nigricollis* crude venoms and *Cerastes cerastes* crude venom.

It was observed that 2 fold serially diluted venom starting with 40 µg of crude venom to 0.3µg with 2 fold serial dilution.

Cerastes cerastes venom showed the best antibacterial effect against *Staphylococcus aureus* with a clear zone of inhibition, it's diameter ranged from 16-33mm (**Figure 6**) respectively.

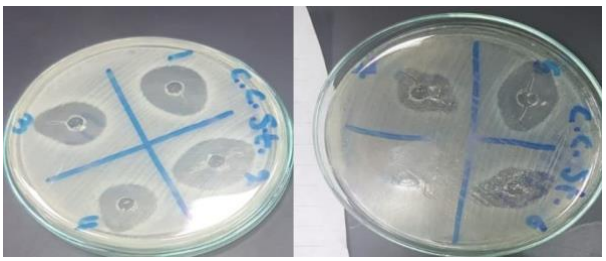

Figure 6: *Staphylococcus aureus* showing a clear zone of inhibition in all concentrations.

While on Gram-negative bacteria represented by *Salmonella* Typhimurium and *E.coli* the zones of inhibition ranged from 9-23mm in *Salmonella* Typhimurium (**Figure 7**) and from 11-17mm in E. coli. (**Figure 8**).

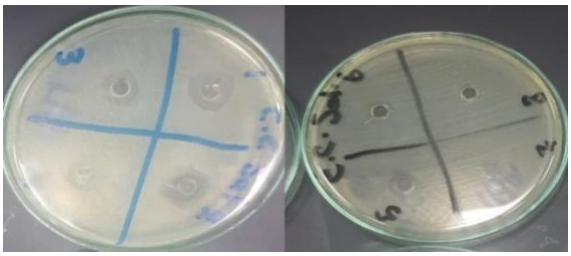

Figure 7: *Salmonella* Typhimurium showing a clear zone of inhibition in all concentrations except 7th and 8th well.

Figure 8: *Escherichia coli* showing a clear zone of inhibition in all concentrations except 7th and 8th well**.**

Otherwise, the *Naja Nigricollis* crude venom shows no bacterial growth on *Staphylococcus aureus* culture (**Figure 9**) and no significant activity against neither *Salmonella* Typhimurium nor *Escherichia coli* (**Figure 10&11**).

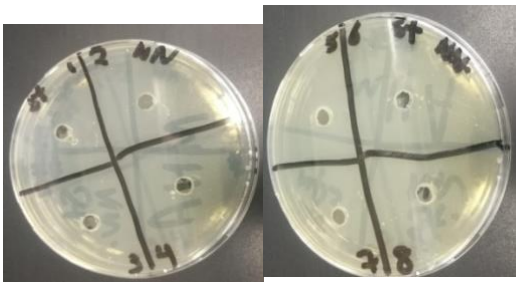

Figure 9: *Staphylococcus aureus* showing a clear zone of inhibition in all concentrations except in the last 4 wells.

Figure 10: *Escherichia coli* shows no zones of inhibition.

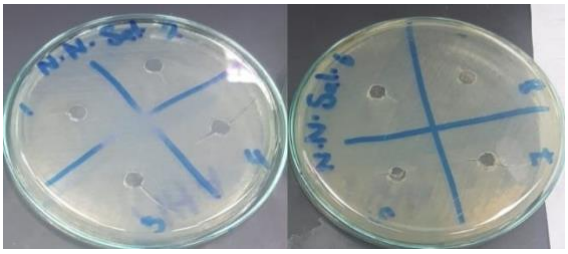

Figure 11*:* *Salmonella* Typhimurium shows no zones of inhibition.

Cerastes cerastes venom has a potential antibacterial effect against Gram-positive and Gram-negative bacteria with a more potent effect on Gram-positive bacteria. *Naja nigricollis* venom shows powerful antibacterial activity against Gram-positive bacteria in comparison with *C.cerastes* and shows no effect on Gram-negative bacteria (**Table 1).**

Table 1: Comparison of the difference between the concentration of venoms and related zone of inhibition on different types of bacteria.

Venom	Start conc. *µg*	*Staphylococcus aureus* inhibition zone	*Salmonella* Typhimurium inhibition zone	*E. coli* inhibition zone
Naja nigricollis conc.	40 µg	+ve whole culture	-ve	-ve
	20 µg	+ve whole culture	-ve	-ve
	10 µg	+ve whole culture	-ve	-ve

	5 µg	+ve whole culture	-ve	-ve
	2.5 µg	+ve whole culture	-ve	-ve
	1.25 µg	+ve whole culture	-ve	-ve
	0.625 µg	+ve whole culture	-ve	-ve
	0.3125 µg	+ve whole culture	-ve	-ve
Cerastes cerastes conc.	40 µg	+ve (33.85mm)	+ve (17.18mm)	+ve (22.16mm)
	20 µg	+ve (32.25mm)	+ve (15.85mm)	+ve (21.35mm)
	10 µg	+ve (29.15mm)	+ve (14.55mm)	+ve (16.84mm)
	5 µg	+ve (26.22mm)	+ve (12.9mm)	+ve (15mm)
	2.5 µg	+ve (24.1mm)	+ve (11.9mm)	+veb (11.74mm)
	1.25 µg	+ve (22.5mm)	+ve (10.8m m)	+ve (7.23mm)
	0.625 µg	+ve (19.35mm)	-ve	-ve
	0.3125 µg	+ve (16.3mm)	-ve	-ve

While using antibiotics such as Amoxicillin with Clavulanic acid, Ciprofloxacin, and Chloramphenicol on our targeted bacteria. The best zone of inhibition was obtained on *Staphylococcus aureus* was with AMC and CIP while the best zone of inhibition recorder with CIP in the case of *Salmonella* Typhimurium and *Escherichia coli* **(Figure 12)(Table 2).**

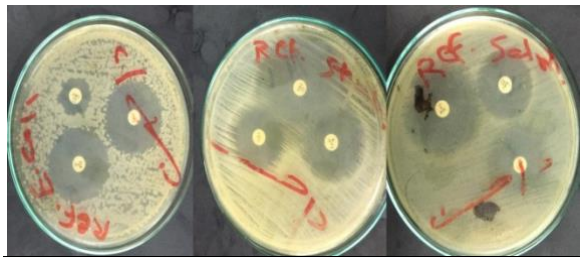

Figure 12: Effect of AMC, CIP, and Chloramphenicol on ref. *Staphylococcus aureus*, *Salmonella* Typhimurium and *Escherichia coli*.

Table 2: zone of inhibitions in relating to antibiotic used and type of bacteria.

	Staphylococcus aureus	*Salmonella* Typhimurium	*Escherichia coli*
AMC 30 µg	30mm	27mm	15mm
CIP 5µg	30mm	35mm	32mm
Chloramphenicol 30µg	25mm	30mm	30mm

5. DISCUSSION

Antimicrobial resistance (AMR) within a wide range of infectious agents is a growing public health threat of broad concern to countries and multiple sectors. Increasingly, the government's government's affairs are drawn attention to a problem so serious that it threatens the achievements of modern medicinal resources. A post-antibiotic era in which common infections and minor injuries can kill far from being an apocalyptic fantasy. In fact, the World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century[1]**.** The development of new drugs represents one of the most promising in the pharmaceutical industry because recently, the number of microbial drug 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]**.** There are several reports in the literature on the antibacterial activity of snake venom against Gram-positive and Gram-negative bacteria [8] [9] [10]**.**

Two major families have been examined for their antibacterial activity, family *Elapidae*, represented by *N.nigricollis* and family *Viperidae*, represented by *C.cerastes* using well diffusion assay and antibiotics challenge besides having different effects on bacterial culture, the biological activity of each venom were examined using LD50 determination, determination of the cytotoxic activity of venom and SDS assay.

The results of well diffusion assay showed that *Naja nigricollis* venom has a potential antibacterial effect against Gram-positive bacteria with no significant effect against Gram-negative bacteria while *Cerastes cerastes* venom has a significant effect on both Gram-negative and Gram-

positive bacteria and in comparison with antibiotic sensitivity, snake venom show powerful antibacterial effect against both bacterial categories.

But the actual difference between both venoms was referred the biological activity of each venom which appeared clearly by applying the SDS-PAGE which shows the complete difference between both venoms where *C.cerastes* composed of nine bands while the *N.nigricollis* venom is composed of only four bands.

A. Charvat [6] believed that the venom efficacy referred to some specific components within the venoms, such as Phospholipase A_2s and L-amino acid oxidases which have molecular weight ranged from 13-30kDa for Phospholipase A_2s and 50–70 kDa for L-amino acid oxidases, with comparison with our tested venom, *C.cerastes* venom shows prominent band in the range of both enzymes while *N.nigricollis* show support to the Phospholipase A_2s enzyme only and that may give the *C.cerastes* it's effect in compare with *N.nigricollis*.

6. 5.CONCLUSION

This study reveals that the two snake's crude venom has a potential antibacterial effect against bacteria. The first one has a potential effect against Gram-positive bacteria with low cytotoxic effect and the second one has an effect on both Gram-negative and Gram-positive bacteria with a higher cytotoxic effect. It was clear that the cytotoxic effect of the tested venoms and antibacterial potential were venom concentration and bacterial strain-dependent.

Recommendations

- It is recommended to arrange for fractionation and reevaluation of the antibacterial potential of each fraction.
- Combination of the dried fraction with an antibiotic that showed to be resisted by antimicrobial-resistant bacteria.
- Formulation of active fractions as a sustained released formula with minimal toxicity.
- Evaluation of dry formulae venom fraction containment stability.
- Evaluation of long-lasting or delayed toxicity of applied venom fraction as a preclinical monitoring protocol.

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