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ORIGINAL RESEARCH ARTICLE

Antibacterial Activity and Silico Molecular Prediction of Snake Venoms (*Bitis arietans* and *Naja nigricollis*) Against Some Clinical Bacterial Isolates

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ABSTRACT

Over the years, the venoms of various animals have been found to include a variety of antibacterial compounds. One of the greatest challenges of public health is multidrug-resistant bacteria strains which always call for new and potent antibacterial agent to help curb these strains of bacteria. In the quest to source antibacterial agents active against multidrug-resistant bacteria, this research work was designed to investigate the antibacterial activity of crude venoms of *B. arietans* and *N. nigricollis* against gram-positive and gram-negative bacterial strains. Current studies revealed that Puff adder (*Bitis arietans*) of the Viperidae family crude venom showed distinct antibacterial activity against the clinical isolates and more efficient than (*Naja nigricollis*) of *Elapidae* family as well as tested antibiotics available today. The methods include antibacterial activity screening assay, followed by scanning electron microscope and molecular docking techniques. The minimum inhibitory concentration (MIC) for puff adder crude venom was 8 g/ml against *Staphylococcus aureus* ATCC. However, the MIC for common antibiotics (ampicillin, penicillin, chloramphenicol, and tetracycline) was in the range of 8-64 g/ml. The venom of the puff adder (*Bitis arietans*) exhibited antibacterial action against gram-positive bacteria through the cell wall and membrane damage, according to the results of scanning electron microscopy. The molecular docking established a mechanism of action between venom protein and the ligands in the cell wall of gram-positive bacteria. The result identified high docking energy scores and interacting amino acid residue. Puff adder (*Bitis arietans*) of the *Viperidae* family crude venom demonstrates a workable source for investigating antimicrobial prototypes for upcoming novel antibiotics against clinical microorganisms with medication resistance.

ARTICLE HISTORY

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KEYWORDS

B. arietans, minimum inhibitory concentration, Molecular Docking, *N. nigricollis*, and scanning electron microscopy

INTRODUCTION

According to the Centre for Disease Control (2019) and Blair et al., (2015), death in Nigeria, the United States of America and Europe as a result of multidrug-resistant bacteria was estimated to be Fifty Thousand (50,000), Twenty-Three Thousand (23,000) and Twenty-Five Thousand (25,000) respectively. Multiple drug-resistant bacterial strains that are already present and are still emerging pose a severe threat to human life, yet we still lack effective antibacterial drugs to fight them. Numerous antibiotics are now in various stages of development and clinical testing phases. However, it is still evident that developing new, powerful antibacterial agents that can combat drug resistance and develop antibacterial with a novel mechanism of action remain of the utmost importance (Perumal *et al.,* 2017).

Existing Age, sex, and the type of food consumed are additional variables that affect how snake venom is made. However, it should be noted that some systematic groupings, like those containing boas and pythons, do not include poisonous snakes. But in some other categories, all the species listed there are deadly. According to Warrell *et al*., (2019), venomous snakes are divided into four families: *Colubridae* (sea snakes), Hydrviperids, including vipers and rattlesnakes), and *Elapidae* (elapids, including cobras, mambas, and taipans) (colubrids, although only some of them are venomous). Snake venoms are intricate blends of different families of protein-origin substances that fall into four categories. The four dominants are snake venom metalloproteases (SVMP), phospholipases A2 (PLA2), three-finger toxins

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(3FTx), and snake venom serine proteases (SVSP). The second group contains proteins that are often found in venom but in considerably lesser amounts: Cysteine-Rich Secretory Proteins, or Kunitz Peptides (KUN) (CRiSP),), Disintegrins (DIS), l-amino acid oxidases (LAAO), C-type lectins (CTL), and natriuretic peptides (NP). Venom nerve growth factor (VNGF), vascular endothelial growth factor (VEGF), acetylcholinesterase (AChE), hyaluronidases, 5'-nucleotidases, phosphodiesterase (PDE), snake venom metalloprotease inhibitors, and others are among the proteins found in the third group of venoms that are less frequently found in venoms. Cobra venom factors (CVF), galactose-binding proteins, aminopeptidases, and waprins are a few examples of the unusual proteins found in the final category. Naturally, not all poisonous snake species include all protein groups. Phospholipases A2 and 3FTx, for instance, are the two most prevalent proteins among elapids; however, this is not the case, for example, in mambas. Most of their venom is made up of Kunitz peptides. However, PLA2s and proteases with various subtypes are the most prevalent categories among viperids overall. (Tasoulis and Isbister, 2017).

The quantities and specific biochemical properties of snake venom vary depending on the species. They come in a variety of bioactive chemical components. The interaction of their components causes aide range of snake venom activities. Notably, one has increased the importance of their antibacterial activities. It has been claimed that the venoms of snakes and other animals are a rich source of pharmacologically valuable proteins and non-proteins. After consuming pathogen-contaminated prey, the host may be protected by antibacterial components in snake venom. The literature has described the antibacterial properties of many snake venoms, such as the inhibitory effects of Naja naja sputatrix, Vipera russelli, and Crotalus adamanteus in *E. coli.* Titles *et al*., (1991) examined the antibacterial properties of 30 distinct snake venoms and discovered that Asian and African snakes had the most potent antibacterial effects (Naja sp.).

MATERIALS AND METHOD

Reagent and Equipment

All the equipment and reagent used in the research were purchased from media hi-tech, and all the reagents used is of analytical grade. The equipment includes; an autoclave, incubator, magnetic stirrer, weighing balance, centrifuge, and scanning electron microscope. The other materials and reagents include; Petri dishes, conical flasks, sample bottles,

Sample Collection

Venom Collection

The National Veterinary Research Institute (NVRI) in Vom was where the venom of *B. arietans* and *N. nigricollis* was purchased. The venoms from captive animals were carefully extracted by milking. After being freeze-dried, each sample was kept at -20^0 C.

Snake Milking Process

The snake's body was placed between the handler's arm and trunk, and its head was held between his index finger and thumb, slightly behind the angle of its mouth, to collect the venom. The snake's fangs were forced to the surface as its jaws were gently forced open. The venom was expelled after the fangs were forced through a plastic or parafilm membrane that had been looped over the lip of a glass cup. Any venom sample that contained blood was discarded. Following venom removal, the fangs were gently removed from the collection vessel to avoid harming the snake's mouth and dentition and impaling itself with its own fangs.

All equipment used for milking snakes were sterilized with flame after each venom milking and then cooled with a draft of air before the next snake was milked.

Bacterial Strains Collection

To create a pure culture, the bacterium strains were taken from the Abubakar Tafawa Balewa Teaching Hospital. Gram-positive bacteria used in antibacterial screening include Staphylococcus aureus (ATCC 25923), Enterococcus faecalis (ATCC 259212), and Streptococcus pneumonia (ATCC 6303). In contrast, Gram-negative bacteria including *Escherichia coli (E. coli)* ATCC 25922, *Pseudomonas aeroginosa* (ATCC 27853), and *Klebsiella pneumonia* (ATCC 43816). (*K. pneumonia*).

Antibacterial Activity Assay

According to the Clinical Laboratory Standards Institute (CLSI 2014), an antibacterial activity screening assay by disk diffusion test and MIC determination were carried out (Ferreira *et al.,* 2011)

Disk Diffusion Method

Preparation of Trypticase Soy Agar

Using a measuring scale, 30 grams (30g) of tryptic soy broth (TSB) powder were measured, and the powder was then dissolved in 1000 milliliters (1000ml) of distilled water in a conical flask. Ten (10ml) aliquots of the media in the conical flask were transferred to each of the 13*100 mm glass spiral tubes after the magnetic stirrer was used to mix the medium in the flask. All tubes are put into an autoclave at 121°C for 15 minutes after adding the aliquot. The tubes were taken out, and the tube caps were tightened. Before placing the stock in the 4°C refrigerators and avoiding light, the tubes were removed and allowed to cool at room temperature (CLSI, 2014).

Preparation of Mueller- Hinton Agar

This was done according to the method described by the Clinical and Laboratory Standard Institute (2014). 38g of Muller-Hinton Agar was weighed and dissolved in 1L of distilled water and was rocked until a homogenous mixture was formed. This medium was sterilized in an

autoclave for 15 minutes at 121^{O}C, after which it was cooled to room temperature. The medium was poured into clean Petri dishes and was allowed to solidify.

Preparation of McFarland Standard

Sulfuric acid and anhydrous barium solution were combined in a beaker to make the standard. Prepared and mixed are 1% solutions of anhydrous barium chloride ($BaCl_2$) and 1% solutions of sulfuric acid (H_2SO_4). To create a turbid suspension, add $BaSO_4$ in a precise ratio for each McFarland turbidity standard; the mixture was well blended. A spectrophotometer with a 1-cm light path and a matching cuvette was used to measure absorbance to confirm that the density of the turbidity standard was accurate. For the 0.5 McFarland standard, the absorbance at 625 nm should be between 0.08 and 0.13. The finished mixture was put into a screw-cap tube wrapped in foil.

Procedure for the Disk Diffusion Method

The method of Ferreira *et al.,* (2011) was adopted, solutions of Snake venoms were prepared by suspending 25mg/ml in sterile deionized water and 1 µL of this solution was applied on a Whatman paper disk of 6mm diameter in size. Thereafter, Trypticase Soy Agar was used to grow the bacterial strains overnight at 37^{O}C, normal saline was used to adjust the turbidity to McFarland standards (1.5 x 108 cfu/ml). The surplus inoculums were removed by pushing a sterile swab firmly against the tube's side above the liquid level after being dipped into the inoculum. Three times, the swab was used to streak the Mueller-Hinton agar plates' surface. By measuring the inhibitory zones surrounding the disk, the results were confirmed. As positive and negative controls, chloramphenicol, ampicillin, penicillin, tetracycline disks, and sterile deionized disks were employed. The minimum value for positive antibacterial activity was deemed to be an inhibition zone greater than 15 mm. As a positive bacterial control, S. *aureus* ATCC 25923 was employed (Ferreira *et al., 2*011).

Minimum Inhibitory Concentration

The broth microdilution method was used to carry out the Minimum Inhibitory Concentration (MIC) test following the guidelines set out by the Clinical Laboratory Standards Institute (CLSI, 2014).

Broth Micro dilution Method

The test organism was prepared as a suspension in saline or Mueller-Hinton broth at 1 108 CFU per milliliter 0.5 McFarland standard turbidity. A disposable plastic inoculator was used to transfer 1 to 5 l of the suspension, which had been diluted 1:20 in saline and contained doubling dilutions of the antimicrobial agents to be tested (often 8 to 12 antimicrobial agents per tray) (the remaining well is a broth sterility control). The final inoculum size is either 5 105 CFU/ml or 5 104 CFU/well.

Preparation of Mueller- Hinton Broth

The thirty-eight gram (38 g) media powder was weighed and dissolved in one litre (1000 ml) of distilled water. To fully dissolve the medium, it was heated with frequent agitation and brought to a boil for one minute. After autoclaving the medium at 121°C for 15 minutes, it was cooled to room temperature. The Mueller Hinton broth was placed into bottles when it had cooled. The final pH reading was 7.3 0.1 at 25^{o}C after being allowed to drop to room temperature. At 2 to 8oC, the plates were kept (CLSI, 2014).

Serial Dilution of the Antimicrobial Agents

Each well in column 1 received 50 l of Muller Hinton medium that was 2-folded, 50 µl of original Muller Hinton medium that was 1-folded, each well in columns 2 through 11, and each well in column 12 received 100 l of Muller Hinton medium. Pipette 50µl of a suitable 4-fold concentrated peptide solution in two or four separate passes into the wells in column 1 (antibiotic concentration was diluted in a ratio of 1:1). Using a 100-l manual multichannel pipette, adjust the peptides' two-fold dilution in the following columns from 1 to 10. 50µl ratio of the crude venom was taken out, mixed, and transported into column 2 using a Manual Multichannel Pipette, where they were dissolved 1:1. With MMP, the dilution process was repeated up to column 10 an additional three times, and 50µl of the solutions from this final column were discarded. Using column 12 as a negative control for medium sterility and column 11 as a positive growth control (no antibiotic) (no cells). Place microtiter plates in the container (with a lid) that can be closed until the time for the inoculation (to avoid evaporation, which most often occurs on the plate edges when no care is taken).

Procedure of the Broth Micro Dilution

After the bacteria had grown for three hours, the culture was diluted to produce 106 CFU/ml. The bacterial culture was diluted twice with Mueller-Hinton broth before being added to the snake venom to achieve a final concentration that ranged from 256 g/ml to 0.125 g/ml. The MIC was ascertained by looking at the lowest concentration of venoms and antibiotics following an incubation period of about 17 hours. The samples with the least visible bacterial growth (i.e., turbidity) in relation to the positive growth were used to calculate the MIC (i.e. the control, which is medium plus bacterial without venom or antibiotics). MIC was determined as the lowest concentration of venom or antibiotic inhibiting visible bacterial growth in comparison to the positive growth control (medium with bacteria without venom or antibiotic), as well as in comparison to the negative growth controls, after 16–18 hours of incubation at 35°C (medium only).

Scanning Electron Microscopy (SEM)

Staphylococcus aureus ATCC 29213 was re-suspended in two tubes of 10 mM sodium phosphate buffer at a

concentration of around 108 CFU/ml (pH 7.4). The crude venom of *B. arietans* was applied to the other tube at a concentration of 80 g/ml (5X MIC) for 4 hours at 37 OC in the control tube. The bacteria were then fixed with an equal volume of 2.5% glutaraldehyde after being incubated. The fixed samples were kept in the fixative solution overnight at 4OC. The samples were then dehydrated three times for five minutes each by a sequence of alcohol concentrations of 30%, 50%, 70%, 95% and 100%. The samples were coated with gold for 3 minutes after being transferred in 100% ethanol to a critical point drier (Quorum K850) (Balzers SCD 040). In order to identify the bacterial cell structure, the bacteria were seen using the FESEM-EDS (7610F) (Field Emission Scanning Electron Microscope and Energy Dispersive X-ray Spectrometer scanning electron microscopy) (Watcharin *et al.,* 2019).

Protein Targets and Ligands

Protein Targets and Ligands Receptors: Crystal structure of Acidic phospholipase A2 of *Bititis aretians* is currently not available in PBD-RSCB databases hence the structure was predicted by structure homology modelling.

Ligands: PubChem, chem spider databases
Molecular Operating Environment, 2015 version.

Protein Structure Prediction and validation.

The 3-dimensional structures of Acidic Phospholipase A2 from Bititis aretians were automatically scored using the QMEAN scoring function using the SWISS-MODEL web server. This model predicts protein structure and function online. It was employed to estimate both the local and overall model quality. The shape, interactions, and solvent potential of the protein model serve as the foundation for its basic theory. In addition to these features, the SWISS-MODEL provides a z-score from 0 to 1 that compares to the expected value for any protein structure. Colovos and Yeasts (1993) description of the quality of the predicted protein was followed using PROCHECK and ERRAT. This model has the best quality was selected for further studies.

Protein (Receptor) Preparation

Ligand Selection and Preparation: Five ligands were used for this study to form a database. These ligands were biomolecules obtained from literature as components of the cell membrane. In the Molecular Operating Environment (MOE), the ligands were prepared for docking by protonation at 300 K and pH 7.0 and energy minimization using default settings. - The constraints were maintained at the rigid water molecule level, partial charges were also applied, and the Amber10-EHT force field was used without any periodicity (Ononamadu *et al.,* 2021).

Protein Target Preparation and Binding/docking Site Prediction: Using MOE's tools and processes, the chosen projected model (model 2) was prepared for docking. As mentioned above, the ligand preparation procedure involved the removal of water molecules and other heteroatoms, protonation, partial charges, and energy minimization. Moe format was used to save the completely prepared and optimized 3D structure for docking (Ononamadu *et al.,* 2021). Using the site finder methodology MOE, the expected model's active site was calculated or identified. (Ononamadu *et al.*, 2021).

RESULTS

The disk diffusion, MBC, and MIC assays were carried out in order to investigate the antibacterial effect of snake venoms, and the various conventional antibiotics were utilized as positive control. The findings showed that six (6) different bacterial strains—three (3) gram-positive and three (3) gram-negative—were evaluated for the antibacterial properties of crude venoms.

***In vitro* Antibacterial Activity of Snake Venoms against Gram-positive Bacteria**

Snake venom's antibacterial activity against gram-positive bacteria was evaluated using a variety of antibiotics. The results show different zone of inhibition prepared in a triplicate assay. Zone of inhibition is expressed as resistant, intermediate and susceptibility. The resistant zone of inhibition is given as <14 (less than 14 mm), the intermediate zone between 15-19mm and the susceptible zone is >20mm.

***In vitro* antibacterial activity of snake venoms against gram-negative bacteria**

Snake venom's antibacterial activity against gram-negative bacteria was evaluated using a variety of antibiotics. The results show different zone of inhibition prepared in triplicate assay. Zone of inhibition is expressed as resistant, intermediate and susceptibility. Resistant zone of inhibition is given as <14 (less than 14 mm), intermediate zone between 15-19mm and susceptible zone is >20mm (Table 1 and 2).

Table 1: Comparison of the *in vitro* antibacterial activity of snake venoms and the tested antibiotics against gram-Positive bacteria

Microorganism /crude venom antibiotics	*S. aureus* ATCC 259213	*E. faecalis* ATCC 259212	*S. pneumonia* ATCC 19659
B. aeritans	17.33±3.63	11±1.13	15±7.06
N. nigricollis	12.33±2.35	NI	14.67±1.72
Ampicillin	15.67±6.82	NI	18±6.48
Penicillin	14±6.30	12±3.39	NI
Tetracycline	17.33±4.28	NI	19.33±4.28

Values expressed as Mean+ SD of inhibition zone in mm. (P< 0.05, n=3). Interpretive Criterion: NI- no zone of inhibition, Susceptibility->20, intermediate-15-19 and Resistant-<14.

Table 2: Comparison of the *in vitro* antibacterial activity of snake venoms and the tested antibiotics against gram-negative bacteria

Microorganism /Crude Venom Antibiotics	*E. coli* ATCC 25922	*K. pneumonia* ATCC 43816	*P. aeruginosa* ATCC 27853
B. aeritans	16±5.19	152.26	12.67±6.43
N. nigricollis	13±1.96	NI	15.±4.52
Ampicillin	NI	18.33±6.91	21.67±7.53
Penicillin	NI	9.33±1.72	NI
Tetracycline	12.33±3.74	NI	24±5.18

Values expressed as Mean+ SD of inhibition zone in mm. (P< 0.05, n=3). Interpretive Criterion: NI- no zone of inhibition, Susceptible ->20, intermediate-15-19 and Resistant-<14.

Comparison of MIC of Crude Venom and tested antibiotics on Gram-positive Bacteria

Antibacterial susceptibility pattern of snake venom against gram-positive bacteria tested against several antimicrobials. The results were analysed based on minimum inhibitory concentration. Minimum inhibitory concentration was expressed as resistant, intermediate, and susceptibility MIC. Resistant MIC was at the concentration greater than 32 µg/ml, intermediate at 8-16 µg/ml and susceptibility at less than 4µg/ml (Table 3).

Table 3: Comparison of MIC (µg/ml) of Crude Venom and tested antibiotics on Gram-positive Bacteria

Microorganism /Crude Venom Antibiotics	*S. aureus* ATCC 259213	*E. faecalis* ATCC 259212	*S. pneumonia* ATCC 19659
B. aeritans	8	16	8
N. nigricollis	2	4	1
Ampicillin	16	8	2
Penicillin	8	16	0.25
Tetracycline	8	4	8

Values are expressed as a minimum inhibitory concentration in **µg/ml**. Interpretive Criterion: Resistant MIC is given as >32 (greater than 32**µg/ml**), intermediate zone between 8-16**µg/**ml and susceptible zone is <4 (less than 4**µg/**ml).

Comparison of MIC of Crude Venom and tested antibiotics on Gram-Negative Bacteria

Antibacterial susceptibility pattern of snake venom against gram-negative bacteria tested against several antimicrobials. The results were analysed based on minimum inhibitory concentration. Minimum inhibitory concentration was expressed as resistant, intermediate and susceptibility MIC. Resistant MIC is given as >32 µg/ml intermediate at between 8-16µg/ml and susceptibility was at less than 4µg/ml (Table 4).

Table 4: Comparison of MIC (µg/ml) of Crude Venom and tested antibiotics on Gram-Negative Bacteria

Microorganism /crude venom antibiotics	*E. coli* ATCC 25922	*K. Pneumonia* ATCC 43816	*P. aeruginosa* ATCC 27853
B. aeritans	8	*4*	*16*
N. nigricollis	4	0.5	*4*
Ampicillin	16	8	8
Penicillin	8	0.5	4
Tetracycline	8	4	64

Values are expressed as a minimum inhibitory concentration in µg/ml. Interpretive Criterion: Resistant MIC is given as >32 (greater than 32 µg/ml), intermediate zone between 8-16 µg/ml and susceptibility zone is <4 (less than 4 µg/ml).

Comparison of MBC of Crude Venom and tested antibiotics on Gram-positive Bacteria

Antibacterial susceptibility pattern of snake venom against gram-positive bacteria tested against several antimicrobials. Based on the minimum bactericidal concentration, the findings are examined. To calculate the concentration below which bacterial growth is impossible on a single scale, the minimum inhibitory concentration is utilized (Table 5).

Table 5: Antibacterial Activity Assay of Crude Venom by Broth Microdilution Method against Gram-positive Bacteria compared to some tested Antibiotics (Minimum Bactericidal Concentration MBC) (MBC (µg/ml), Bacterial Strains)

Microorganism /Crude Venom Antibiotics	*S. aureus* ATCC 259213	*E. faecalis* ATCC 259212	*S. pneumonia* ATCC 19659
B. aeritans	32	128	64
N. nigricollis	16	32	8
Ampicillin	32	32	16
Penicillin	64	128	4
Tetracycline	256	32	64

Values expressed as a minimum bacteriocidal concentration in **µg/ml**

Comparison of MBC of Crude Venom and tested antibiotics on Gram-Negative Bacteria

Antibacterial susceptibility pattern of snake venom against gram-negative bacteria tested against several antimicrobials. The results are analysed based on minimum bactericidal concentration. Minimum inhibitory concentration is the concentration at which no single growth of bacteria is possible (Table 6).

Relationship of Crude venom/ Antibiotics for the minimum inhibitory concentration and minimum bactericidal concentration in Gram-positive bacteria strains

The relationship is shown in Figure 1. The concentration between the MIC and MBC are compared to each other to evaluate the potency of the venom against the bacteria

strains measuring the concentration it starts to inhibit the bacteria to the concentration of no single growth recorded.

Relationship of Crude venom/ Antibiotics for the minimum inhibitory concentration and minimum bactericidal concentration in Gram-negative bacteria strains

The relationship is shown in Figure 2. The concentration between the MIC and MBC are compared to each other to evaluate the potency of the venom against the bacteria strains measuring the concentration it starts to inhibit the bacteria to the concentration of no single growth recorded.

Table 6: Antibacterial activity assay of crude venom by broth microdilution method against gram-negative bacteria compared to some tested antibiotics (Minimum Bactericidal Concentration MBC) (MBC (µg/ml), Bacterial Strains)

Microorganism /Crude Venom Antibiotics	*E. coli* ATCC 25922	*K. Pneumonia* ATCC 43816	*P. aeruginosa* ATCC 27853
B. aeritans	16	8	32
N. nigricollis	8	4	8
Ampicillin	32	16	16
Penicillin	4	8	8
Tetracycline	8	16	64

Values expressed as a minimum bacteriocidal concentration in µg/ml

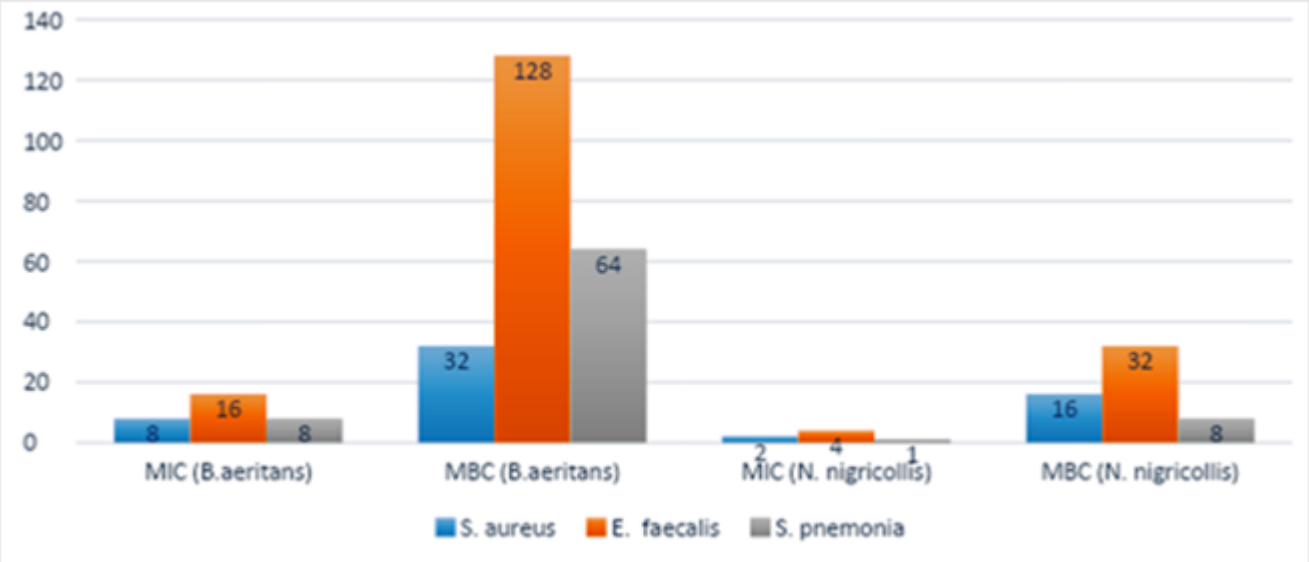

Figure 1: Relationship between Crude venom and Antibiotics for the MIC and MBC in Gram-positive bacteria strains

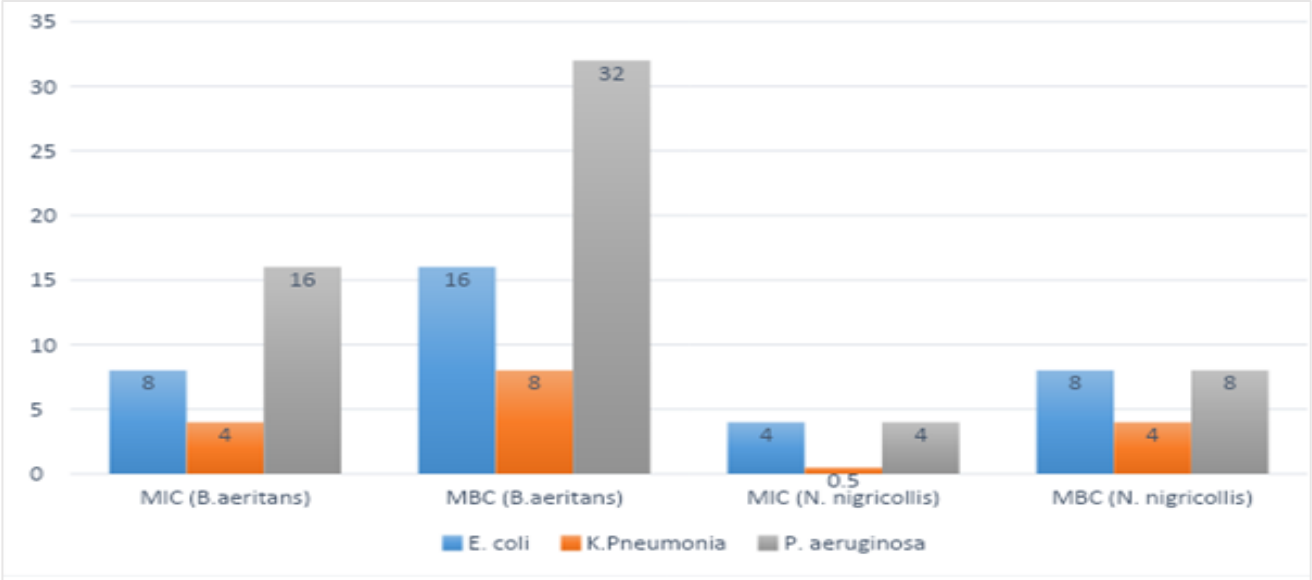

Figure 2: Relationship between Crude venom and Antibiotics for the MIC and MBC in Gram-negative bacteria strains

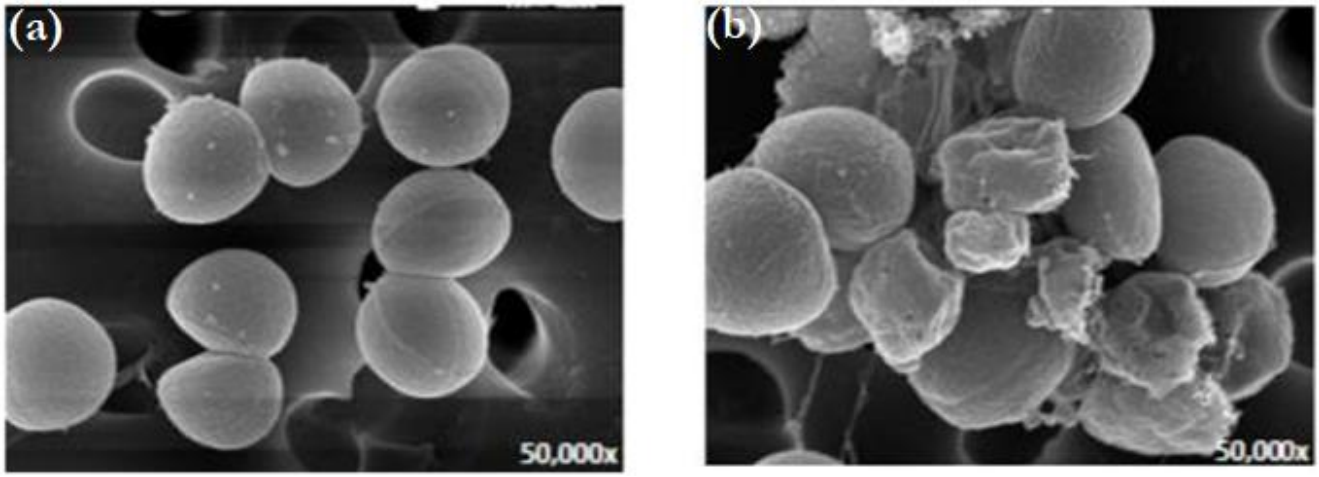

Figure 3: Scanning electron micrographs of *S. aureus* ATCC 29213 incubated with (a) 10 mM sodium phosphate (pH 7.4) and (b) buffer 10 mM sodium phosphate buffer (pH 7.4) and treated with *B. arietans cru*de venom at a concentration of 80 µg/ml (B), at 37 °C for 4 hours.

Table 7: Molecular docking of selected chemicals on acidic phospholipase A2 models from *Bitis arietans* venom.

SN	Compound	Binding Energy	Interacting Amino Acid Residue(s)
	Model 1 (1OZ6.1. A)		
1	Aristolochic acid	-6.5668	[a]LUE 18
2	N-acetylglucosamine	-7.4417	[b]gly 45, [b]met 34
3	Glycolipid component of teichoic acid	-5.1537	[c]Asp 64
4	Sugar amine component of Lipoteichoic acid	-8.6670	[d]Cys 44, [b]Met 34
5	Phosphodiacylglycerol	-8.5750	[c]Asp 64
	Model 2 (1VPI.1. A)		
1	Aristochic acid	-6.5084	[d']Try 37
2	N-acetylglucosamine	-8.0237	[c]Asp 64
3	Glycolipid moety of teichoic acid	-5.5887	[d]cys 60, [a']Tyr 43, [b]cys 44
4	Sugar-amine moety of Lipoteichoic acid	-7.2371	[c]Met 34, [b]Ser 128
5	Phosphodiacylglycerol	-8.0406	[c]Met 34, [d]Cys 44 [d']Trp 48

Table 8: Interpretation and verification of models

S/N	Models	(Errata)	Ramachanda Parameters (Procheck)			
		Overall Quality Factor	Residues in most favoured regions	Residues in additional allowed regions	Residues in generously allowed regions	Residues in disallowed regions
1	Model 1	88.69657	85 / 80.2%	20 / 18.9%	1 / 0.9%	0 / 0.0%
2	Model 2	96.522	80 / 75.5%	25 / 23.6%	1 / 0.9%	0 / 0.0%

Model 1
Remainders in the most advantageous regions [A, B, L] 85 80.2%
Additional permitted regions with residues [a, b, l, p] 20 18.9%
Residues in places with generous permission [a, b, l, p] 1 0.9%
Residues in prohibited areas 0 0.0%

Model 2
Remainders in the most advantageous regions [A, B, L] 80 75.5%
Additional permitted regions with residues [a, b, l, p] 25 23.6%
Residues in places with generous permission [a, b, l, p] 1 0.9%
Residues in prohibited areas 0 0.0% ---- ------

DISCUSSION

In order to determine the antibacterial activities of the Crude Snake Venoms, MIC assay and disc diffusion method were carried out. Tests were performed on three (3) Gram-positive bacteria and three (3) Gram-negative bacteria using the venoms of two different snake species (*B. aeritans and N. nigricollis*). Ampicillin, Penicillin, and Tetracycline were the three (3) antibiotics utilized as controls. The study shows that *B. aeritans* had a zone of inhibition of 17.33±3.63, *N. nigricollis* had a zone of inhibition of 12.33±2.35, ampicillin had a zone of inhibition of 15.67±6.82, and Penicillin had the zone of inhibition of 14.6.30 while Tetracycline had the zone of inhibition of 17.33 ±4.28 on *S. aureus*. On *E. feacalis,* it was seen that there were only 2 samples that had antibacterial activity on this samples which were recorded in *B. Americans* with the zone of inhibition of 11±1.13 and Penicillin at 12±3.39.

The third clinical isolate, *S. pneumonia* which is a virulent bacterial was inhibited by the four samples except for Penicillin which had no antibacterial activity on this organism but *B. aeritans, N. nigricollis,* Ampicillin and Tetracycline had the zone of inhibition of 15±7.06, 14.67±1.72, 18±6.48 and 19.33 ± 4.28 respectively. Comparing the antibacterial activities of these samples on the three clinical isolates, it can be seen that on *S. aureus*, *B. aeritans* and Tetracycline had the same zone of inhibition with the value of 17.33±3.63 and 17.33±4.28 respectively which were the highest inhibition zones on *S. aureus* while the minimum zone of inhibition was seen in Penicillin. Though there was no inhibition of the growth of *E. faecalis* by *N. nigricollis*, Ampicillin and Tetracycline, *B. aeritans* still have some zones of inhibition on the organism but *Penicillin* contained more inhibitory activity than the latter. *S. pneumonia* was inhibited the most by Tetracycline, followed by ampicillin. The venom of *B. aeritans* was proven to contain some antibacterial agents more than that of *N. nigricollis* and Penicillin. This result correlates with that of Rangsipanuratni *et al.,* (2019). They tested 15 snake venoms against nine clinical isolates; they found out that snake venoms from Viperidae family were more active than snake venoms from the family of Elapidae. *S. pneumonia* and *S. aureus* have been reported to be well inhibited by Viperidae family snakes by San et al., (2010) and Phua *et al.,* (2012).

The study also observed the antibacterial activities of the tested samples on Gram-negative clinical isolates, the analysis shows the five samples, *B. aeritans, N. nigricollis,* and Tetracycline had the antibacterial activity of 16±5.186, 13 ± 1.96 and 12.33 ± 3.74 respectively on the *E. coli* ATCC 2592 which is a gram-negative bacteria. Most *E. coli* intestinal bacteria are not harmful, but a small number can contaminate food, causing diarrhea, while others can cause pneumonia, lung ailments, urinary tract infections, and other illnesses. These gram-negative bacteria have been reported to be a marker in water contamination (CDC, 2021). The result shows that *B. aeritans,* Ampicillin and Penicillin have the zone of inhibitions of 15± 2.26, 18.33 ± 6.91 and 9.33 ± 1.72 on *K. pneumonia* ATCC 43816 while *N. nigricollis* and Tetracycline have no any antibacterial activity on this clinical isolate. The third isolate, *P. aeuriginosa* ATCC 7853 resist the antibacterial effects of Penicillin while *B. aeritans, N.nigricollis,* Ampicillin and Tetracycline had the zone of inhibition of 12.67 ± 6.43, 15 ± 4.52, 21.67 ± 7.53 and 24 ± 5.18 respectively. The result shows that apart from Penicillin with no zone of inhibition, *B. aeritans* have the least zone of inhibition, followed by *N. nigricolllis*. The results show that the antibiotics have more inhibition activities than the 2 snake venoms.

According to Rangsipanuratn1 *et al.,* (2019), the mixture of proteins and peptides found in snake venoms included nucleotides, amino acids, lipids, carbohydrates, and metallic elements bound to proteins (5%), such as neurotoxins, cytotoxins, myotoxins, proteases, nucleases, and peptides. The structure of the lipopolysaccharide and the charge density in the case of gram-negative bacteria, or the lipid composition of the cytoplasmic membrane and the electrostatic potential across this membrane in the case of gram-positive bacteria, may be the causes of this selective antibacterial activity. These findings are in line with the work of Rangsipanuratni *et al.,* (2019) and Perumal Samy *et al.,* in 2006. (2019).

Antibacterial activity of crude venom of broth Microdilution method against Gram-positive bacteria. The table shows that the 2 tested samples (*B. aeritans* and *N. nigricollis*) and the three (3) tested antibiotics (Ampicillin, Penicillin and Tetracycline) had the Inhibitory concentration of 8 mm, 2 mm, 16 mm, 8 mm and 8 mm respectively on *S. aureus*. Compared to *B. aeritans, penicillin*, and tetracycline, the results suggest that *S. aureus* was least vulnerable to the venom of *N. nigricollis* (2 mm) (8 mm). When comparing the MIC findings of the tested samples and the antibiotics, it is evident that Ampicillin, which has the highest Minimum inhibitory concentration of 16 mm, was able to stop the growth of *S. aureus* ATCC 259213. *E. faecalis* ATCC 259212 was less susceptible to *N. nigricollis* (4 mm) compared to the venom of *B. aeritans* and *Penicillin*, whose MIC were (16 mm) which on *E. faecalis*. This is supported by the results of the antibacterial activity of the snake venoms and the antibiotics. The result shows that the antibacterial activities of Tetracycline (4 mm) is the same as that of *N. nigricollis* (4 mm). Finally, the result shows that ampicillin (16 mm) also has the same antibacterial activities as that *B. aeritans,* this implies that *B. aeritans* and ampicillin have the highest MIC on the tested *E. faecalis* ATCC 259212. The results of the MC on *S. pneumoniae* show that *B. aeritans, N. nigricolli,* Ampicillin, Penicillin and Tetracycline had the MIC of 8mm, 1mm, 2mm, 0.25mm and 8mm. The result shows that the highest MIC was recorded at 8 mm with *B. aeritans* and Tetracycline while the lowest was recorded at 1 mm which was found in *N. nigricollis*. This

result shows the big *Naja nigricollis* venom did not have much antibacterial activity on *Streptococcus pneumoniae.*

Antibacterial activity of crude venom of broth Microdilution method against Gram-negative bacteria. The table shows that the 2 tested samples (*B. aeritans* and *N. nigricollis*) and the three (3) tested antibiotics (Ampicillin, Penicillin and Tetracycline) had the Inhibitory concentration of 8 mm, 4 mm, 16 mm, 8 mm, and 8 mm respectively on *E. coli* which is human pathogenic bacteria. The table shows that the highest MIC was recorded in ampicillin followed by *B. aeritans,* Penicillin and Tetracycline while the lowest MIC was recorded in the use of *N. nigricollis* (4 mm according to the findings, B. aeritans' MICs for E. coli ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853 were 8 mm, 4 mm, and 16 mm, respectively. For *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853 correspondingly, the MIC of *N. nigricollis* was 4 mm, 0.5 mm, and 4 mm for *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853 respectively, ampicillin showed MICs of 16 mm, 8 mm, and 8 mm. Penicillin, a different antibiotic, had MICs of 8 mm, 0.5 mm, and 4 mm for *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, respectively. Tetracycline, the most recent antibiotic, showed MICs of 8 mm, 4 mm, and 64 mm for *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, respectively. According to the findings, Tetracycline had the highest MIC (64 mm) against *P. aeruginosa* and *N. nigricollis* had the lowest MIC (0.5 mm) against *K. pneumoniae.*

The results revealed that B. aeritans has MBCs of 16mm, 8mm, and 32mm for *P. aeruginosa* ATCC 27853; *E. coli* ATCC 25922; and *K. pneumoniae* ATCC 43816. According to the findings, *P. aeruginosa* ATCC 27853 had the greatest MBC for *B. aeritans*, whereas *K. pneumonia* ATCC 25922 had the lowest. Compared to *B. aeritans, N. nigricollis* had significantly less MBC; the MBC measurements for *N. nigricollis* were 8mm, 4mm, and 8mm for the respective strains of *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853. *K. pneumoniae* was found to have the lowest. For *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853 correspondingly, ampicillin exhibits MBC of 32mm, 16mm, and 16mm. For *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853 respectively, penicillin exhibits MBC of 4 mm, 8 mm, and 8 mm. The outcome reveals that E. coli had the lowest level of (4mm). According to the Tetracycline MBC , *E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, and *P. aeruginosa* ATCC 27853 each had measurements of 8mm, 16mm, and 64mm. P. aeruginosa ATCC 27853 had the highest MBC (64mm), while *E. coli* ATCC 25922 had the lowest MBC (8mm).

Snake venoms have been described as complicated combinations of numerous families of protein-origin components that can be separated into four classes by Gold *et al.,* (20020), Warrell (2010), Burbrink and Crother (2011), and Warrell (2019). The most prevalent is snake venom metalloproteases (SVMP), phospholipases A2 (PLA2), three-finger toxins (3FTx), and snake venom serine proteases (SVSP). The second group consists of proteins that are frequently, albeit in much smaller proportions, found in the venom: Kunitz peptides (KUN), Cysteine-Rich Secretory Proteins (CRiSP), L-amino acid oxidases (LAAO), C-type lectins (CTL), disintegrins (DIS), and natriuretic peptides (NP). The third category of proteins includes those that are less frequently found in venoms, including venom nerve growth factor (VNGF), vascular endothelial growth factor (VEGF), acetylcholinesterases, hyaluronidases, 5'-nucleotidases, phosphodiesterases (PDE), and inhibitors of the metalloproteases found in snake venom. According to Helen (2015), these ingredients work well as bacterial growth inhibitors. Helen (2015) outlined the reasons some bacteria can be suppressed while others cannot. According to Helen (2015), the majority of Gram-positive and Gram-negative bacteria can grow more slowly when a chemical that produces proteins is present. According to her, all organisms require transition metal ions, such as iron, zinc, and manganese, to flourish. However, using proteins that chelate—or bind with—metal ions—will prevent the growth of germs. She also reported that if only iron is still present in Gram-Negative bacteria, they can still grow, but gram-positive bacteria need Manganese, Iron and Calcium for them to grow. Hence, there is the possibility to be of more inhibition in Gram-positive bacteria when compared with gram-negative bacteria.

Morphological changes induced by crude venom were studied using Scanning Electron Microscope to determine the lytic action of the antibacterial activity of *B. aeritans* venom. In figure 3, the micrographs from a few experiments are displayed. The structure of the bacterial cell membrane was investigated following a 4-hour treatment of S. aureus ATCC 29213 with crude venom from B. aeritans. Figure 3 demonstrates how most of the treated cells lost their shape and membrane integrity. While no morphological alterations were seen in the control cells, membrane damage, including membrane wrinkling and blebbing, and consequent leakage of cytoplasmic contents, were reported. According to these investigations, B. aeritans crude venom interacts with the cytoplasmic membrane, causing its eventual breakdown and cytoplasmic leaking, which ultimately results in bacterial cell death.

In order to further access the potential mechanism of action of the snake venom components, the study employed an in-silico approach. Using molecular docking, the main snake venom protein (PLA2) was shown to have an impact on the gram-positive Staphylococcus aureus cell wall components' structural makeup. Structural moieties of lipoteichoic acid, (glycolipid moiety, amino-sugar moiety), N-

acetylglucosamine, phosphodiacylglycerol (natural substrate) and aristochic acid (Inhibitor) were docked to the predicted model of PLA2. The docking result showed that the enzyme binds the cell wall components with high negative binding energy (affinity) which is comparable to that of the natural substrate phosphodiacyl glycerol (-7.2571 Kcal) and known inhibitor aristochic acid (-6.5084Kcal).

One of the main elements of the cell walls of gram-positive bacteria, such as S. aureus, is lipoteichoic acid. It is structurally made up of three parts: an amino acid moiety, a lipid, and a sugar. The large structure was spilt into amino-sugar and lipid-sugar moiety. The amino sugar moiety docked with a high binding score of -7.237kcal and was stabilized by hydrogen bonding with amino acid residue met 34 and serine 128. Whereas the glycolipid moiety produced a lower acute score with Hydrogen bond interaction with different sets of amino acid residue Cys 60, Tyr 43, Cys 44 and Asp 64.

N-acetylglucosamine is a sugar component of most bacteria (both gram-positive and negative). N-acetylglucosamine also docked to PLA2 presented a high docking score of -8.024kcal (stabilized by H-bond interaction with Asp 64). This strong binding interaction between the components of the cell wall bacteria and the PLA2 enzymes from the venom of *Bitis aretians* corroborates the antibacterial activity resulting in the observed cell wall disruption.

CONCLUSION

Generally, today, the world is facing serious challenges posed by many diseases with numerous names and other complications, researchers, scientists, and pharmacists are on a daily basis trying to produce drugs which will be effective and at the same time have no side effects. Though the venom of Snakes is very poisonous, this research work has opened a new way in which these venoms can be utilized. The development of cutting-edge antibacterial medicines is a significant challenge in the period of the serious threat posed by bacterium strains resistant to antibiotics. The hunt for substances with antibacterial potential among venom components is one of the trendiest developments. It has been consistently demonstrated that the required qualities of both complete snake venom and its individual members, or even their fragments, exist, making them a potential source of novel antibiotics. This strategy is even more promising because there are documented cases of the creation of efficient medications based on proteins and peptides extracted from snake venom.

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