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THE ANTIBACTERIAL PEPTIDES (AMPs) ORIGINATED FROM TRYPTIC HYDROLYSIS OF *Naja sumatrana* VENOM FRACTIONATED USING CATION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Bioactive peptides bring revolutions in the medical field and become an attractive target for new antibiotics. The bioactive peptides can be derived from the hydrolysis of protein. This study aims to explore the antibacterial peptide from venom hydrolysate of the Spitting cobra (*Naja sumatrana*). The venom protein was isolated from venom using a gel filtration technique. The digestion of venom protein was performed using trypsin with and without alkylation and reduction. After that, fractionation was carried out using a strong cation exchange solid-phase extraction, followed by antibacterial activity testing. The active peptides present in the active fractions were identified using HRMS. The protein hydrolysis with pretreatment using alkylation and reduction gave almost two-fold higher than that of untreated hydrolysis. Both the venom protein and the hydrolysate showed antibacterial activity against *Escherichia coli* but there was no activity against *Staphylococcus aureus*. Antibacterial activity has been detected in numerous cation exchange fractions against *Escherichia coli* and *Staphylococcus aureus*. The four best bioactive peptides were identified with amino sequences of VYGGDSR, YTPTNK, TQFSDR, and TFQDSR. In addition, molecular docking analysis revealed that the peptides showed high-affinity scores, and the model best matched the DNA gyrase subunit B by salt bridge formation, whereas hydrogen bond interactions proved inhibition of bacterial replication as an antibacterial mechanism.

Keywords: Anti-bacterial Activity, Bioactive Peptides, Hydrolysis, *Naja sumatrana*, Venom, Molecular Docking.

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INTRODUCTION

The use of antibiotics to treat infectious diseases has become increasingly ineffective against certain types of pathogenic bacteria due to the antibacterial resistance of pathogens.[1,2] These pathogenic bacteria are becoming a severe clinical problem worldwide.[3,4] This can be proved by the rapidly increased resistance to conventional antibiotics; immediate action, however, has not been carried out so far.[5] Thus, there is an urgent need to produce natural agents that are less toxic and more efficient against various microbial diseases with new modes of action. AMPs (Antimicrobial peptides) could become a viable alternative to current therapeutic strategies. Generally, AMPs contain 15–45 amino acid residues, each of which exhibits a completely different spectrum of activity. Several natural AMPs have been reported from various sources. In animals, AMPs can be found in neutrophil granules and secretions from epithelial cells coating the skin and mucosal surfaces.[6] Snake venom contains various cytotoxic components and has a strong antibacterial and antiviral impact. L-amino acid oxidase, a protein found in snake venom, possesses several important biological features, including bactericidal and virucidal activity. Snake venom's antibacterial action is linked to enzymes such as snake venom L-amino acid oxidases, metalloproteinases, and phospholipases A2. [7] The first peptide found in snake venom was melittin; snake venom also comprises an antibacterial ribosome-targeting peptide.[8] Antimicrobial peptides such as cathelicidin, vgf1 (nerve growth factor1), and omwaprin, are obtained from various poisonous snake species.[9]

The sequence of the amino acids governs the various actions of bioactive peptides as they interact with other proteins in the body and control natural processes. The target sequence is subjected to enzymatic treatment at a certain pH and temperature. Peptides produced by the hydrolysis of proteins with proteolytic enzymes have higher activity. It is easy to significantly increase and frequently responds faster than bacterial fermentation.[10] Proteolytic cleavage of polypeptides can provide the desired peptides with a unique amino acid sequence. Protein cleavage by particular peptidase enzymes, such as trypsin, can produce R or K-ending peptides.[11] Brucin is a peptide with an amino acid sequence of HTLCMAGGATY containing 16 times more antibacterial activity than that of penicillin against *Streptococcus pyogenes*. This peptide is produced by hydrolysis of *Brucea javanica* fruit with pepsin.[12] Trypsin is a serine proteolytic enzyme that catalyzes arginine or lysine at the C-terminus of a protein to produce tryptic peptides. The presence of an important residue (R or K) at the end of a tryptic peptide boosts ionization and, consequently, also boosts the signal intensity of mass spectral peaks.[13] Antibacterial proteins and peptides are almost all cationic, and they kill bacteria by permeabilizing or weakening their membranes.[14] Molecular docking is a well-studied approach for identifying potent peptides without excessive effort and investment in research to investigate the activity as a result of binding affinity (kcal/mol).[15] Molecular docking of peptides with microbial inhibitors predicts the most suitable location of the peptides that bind to a certain target protein molecule to evaluate the activity and affinity of bioactive peptides.[16] Mode of action of bioactive peptides can be confirmed by molecular docking after confirmation of interactions of bioactive peptides with the inhibitor of bacteria. Although many bioactive components of snake venom have been extracted and characterized, the systematic search for antibacterial peptides of *Naja sumatrana* venom has never been studied before.[17] In previous research, the primary focus was *N. sumatrana* Venom protein and its activity against pathogenic bacteria concentrates, not on the antibacterial bioactive peptides produced from hydrolysis.[18] In this study, the AMP resulting from the hydrolysate of venom protein was analyzed. The hydrolysate of venom protein derived from *N. sumatrana* (Spitting Cobra) was further tested against multidrug-resistant bacteria including *Staphylococcus aureus* and *Escherichia coli*. In addition, antibacterial activity was evaluated by determining (MIC) and (IC_{50}). Finally, the mechanism of action of bioactive peptides was examined through the docking process by determining the binding affinities, RMSD, and the interaction of peptides with bacterial proteins.

EXPERIMENTAL

Materials

Materials and chemicals utilized in this research included *N. sumatrana* venom, cation exchange SPE column (Discovery DSC-SCX; Supelco, PA, USA), and trypsin (*Bioworld, OH, USA*). The antibacterial activity test was carried out using *E.coli* (ATCC) and *S. aureus* (ATCC). Other chemicals utilized for performing the fractionation process and determining the antibacterial activity were resazurin, chloramphenicol, ammonium bicarbonate, HCl, NaOH, citric acid monobasic, trisodium citrate, and resin from *Sigma-Aldrich*. Acetonitrile (*hyper grade for LC-MS LiChrosolv, Merck, and Germany*), distilled water (*M.S. grade, Merck, Germany*), centrifuge (*Biofuge Primo R*), and Amicon® *ultra-15 with 3000 Da MWCO* Centrifugal Filter were used for peptide identification.

Milking of Snake Venom

N. sumatrana venom was collected from one of the private zoos (Bhumi Merapi, Yogyakarta). An expert at the Faculty of Veterinary Medicine, Universitas Gadjah Mada Yogyakarta confirmed the *N.sumatrana* and collected the venom. The collecting procedure was performed by following the protocol approved by the Faculty of Veterinary Medicine, Universitas Gadjah Mada, under the letter number (0052/EC-FKH/Eks./2020). The venom was then lyophilized in a sterile flacon and stored in a refrigerator at −20 °C until further use.

Digestion of Venom Protein

The lyophilized venom was dissolved in PBS buffer solvent. The venom protein and small-molecule fraction were separated using Amicon® ultra-15 with 3000 Da MWCO. The protein was diluted with 20 mM ammonium bicarbonate pH 8, mixed with trypsin (20:1 ratio), and incubated overnight at 37 °C. The hydrolysis was stopped by putting the sample in the oven at 80 °C for 15 minutes. The second type of hydrolysis of venom protein employed reduction and alkylation using dithiothreitol and iodoacetamide

respectively before trypsin digestion.[19] The digested venom was centrifuged using an Amicon® ultra-15 with 3 kDa MWCO to get the hydrolysate. The concentrate was undigested venom, while the filtrate was considered hydrolysate. The amount of undigested protein was measured using a UV-Vis spectrophotometer to determine the degree of hydrolysis.

Fractionation of Hydrolysates

Hydrolysates were fractionated by cation-exchange chromatography as well as by using cation-exchange SPE. Both columns were conditioned with a pH three buffer after being washed with distilled water. Protein hydrolysate at pH three was gently poured over the column. The elution procedure was conducted in series using buffers at pH values of 4, 5, 6, 7, and 8 at a flow rate of 1–2 mL/min to elute roughly twice the column capacity. The concentration of the peptides in each fraction was measured by UV spectrometer at λ 280 nm.[20]

Antibacterial Testing of the Peptides

The hydrolysate and venom proteins were tested for determining the antibacterial activity using the disc diffusion method. A bacterial culture of *S. aureus* and *E. coli* was uniformly layered on Muller Hinton agar plates using a sterile swab. The hydrolysate and venom protein-treated discs were placed on the bacterial plates. Subsequently, the plate was incubated for 18 to 24 hours at 37 °C before measuring the zones of inhibition. Meanwhile, the antibacterial evaluation of the peptide fractions was performed by employing the microdilution method. *S. aureus* and *E. coli* were grown in the MHB medium by incubating them overnight at 37 °C until they reached 1×10^7 CFU/mL. Fifty µl of bacterial culture was then added in 96-well plates, added with 10 µL of 0.1 percent resazurin, and followed by various peptides fractions ranging from 0,5,10,15,20,25 µL. The plates were then incubated at 37 °C for 20 hours and measured by an Elisa reader at λ 590 nm.

Identification of Peptides

The peptides of the most antibacterial active fraction were analyzed using LC-HRMS equipped with the Acclaim® PepMap RSLC column (C18, 75 µm x 150 cm). The mobile phase consisted of mobile phase A (0.05% of TFA in water) and mobile phase B (water/acetonitrile 20/80 and 0.1% TFA). The mobile phases A and B were then used with a gradient at a flow rate of 0.3 µL/minute. A positive ion mode was employed for MS detection, operated with a high resolution and accurate mass mode. MS/MS peptide analysis was carried out with a scan range of 350-1,800 m/z with full MS/ddMS2 mode. The full M.S. parameter used was the resolving power set to 140,000 (FWHM), while the ddMS2 parameter used was set at a resolution of 17,500 (FWHM). The raw chromatogram MS data were processed using the Proteome Discoverer program version 2.5. The data was processed using the Fusion Basic Sequest HT mode and a simple consensus algorithm was utilized to identify the peptides. The algorithm employed in the peptide analysis was Sequest HT using the UniProt protein database of *N. sumatrana*.

Molecular Docking

Molecular docking analysis was carried out using Auto Dock 4.2 Vina to investigate the binding mechanism of the identified peptides from *N. Sumatrana* venom to the bacterial DNA gyrase. Bioactive peptides were docked against the DNA Gyrase of *E. coli* to assess binding affinity, hydrophobicity, and possible interactions with the enzyme. DNA Gyrase subunit B (PDB ID: 1KZN) was downloaded from RCSB PDB Protein Data Bank for *E. coli*. The peptides were also docked against *S. aureus* DNA Gyrase (PDB ID: 4URM). Peptides' structures were drawn using the Gaussian view, and all the peptides' structures were optimized by utilizing Gaussian with the semi-empirical method. The optimized peptides were computed by Autodock vina, while data were visualized by employing BIOVIA Discovery studio.

RESULTS AND DISCUSSION

Degree of Hydrolysis of Venom Protein

Protein digestion with the alkylation and reduction process can modify proteins in solution and gel slices before proteolytic digestion and mass spectral analysis. Cystine bonds in proteins are frequently opened with reducing agents during the alkylation process; sometimes it is done to accelerate digestion and more often than not, it is performed to eliminate disulfide linkages which make the digestive process difficult to

analyze.[21] Reducing disulfide bridges and alkylating sulfhydryl groups are common steps in a bottom-up proteomics approach. Without reduction and alkylation, the peptides involved in disulfide bridges can be difficult to identify during database searches. The results indicate that the degree of hydrolysis of venom protein in the alkylated and reduced sample was higher (49.71%) than in samples without reduction and alkylation (29.27%). However, the degree of hydrolysis was concluded to be relatively low compared to other samples using the same digestion protocol. Digestion of the *Ricinus communis* seed protein using the same protocol without alkylation and reduction steps could digest up to 82.15%.[15] The presence of protease as one of the toxic components in the venom [22] also digests external proteins, including trypsin, which is used to digest the venom protein. The fact that the hydrolysate mainly contains native protein hinders the possibility of assaying the fractionation of the resulting peptides. Therefore, the undigested protein must be separated using a filtration column such as Amicon ultra. The molecular weight cut-off (MWCO) of 3000 Da is sufficient to separate the peptides (MW < 3000 Da) from the proteins.

Antibacterial Activity of Venom Protein and the Hydrolysate

The disc diffusion method was employed to carry out the antibacterial evaluation. As can be seen in Fig.-1, both the venom protein and the hydrolysate had significant antibacterial activity against the gram-negative bacterium *E. coli*, as evidenced by the clear zone of inhibition in the petri dish of *E.coli*. However, there was no activity observed in both samples against a Gram-positive bacterium *S. aureus*. The resulting data were analyzed using Davis and Stout's (1971) formula. According to the formula, a clear zone with a 10–20 mm diameter will be considered as strong inhibition. The measurement result of the inhibition zone showed that venom protein had an inhibition diameter of 8.3 mm for *E. coli* (Table-1). In contrast, in *S. aureus*, there was no inhibition diameter. Referring to the previous report formula, the diameter of 8.3 mm was classified in the intermediate category, indicating that venom protein had a moderate to strong response against *E. coli* growth.[23] Meanwhile, the protein hydrolysate showed a 9.1 mm diameter inhibition zone for *E. coli,* which means that the protein hydrolysate had an intermediate to a strong reaction against *E. coli*.
The absence of the inhibition zone in the *S. aureus* instance might be due to several factors. One of the factors was that the diffusion rate of antibacterial peptides might influence the diameter of the zones in a Petri dish. Thus, it can be concluded that it cannot precisely represent the potency of the venom protein and protein hydrolysate's antimicrobial activity. In some studies, related to extracts, the disc preparation technique could be considered a particular problem. It was possible that venom protein and the hydrolysate were not evenly and adequately infused into the paper discs. The standardization of inoculum size to 0.5 McFarland turbidity might be a critical component influencing the inhibitory zone produced by the sample. This inoculum size is important to ensure confluent or nearly confluent lawn growth, whereas a lesser inoculum size might result in false wide inhibition zones. A larger inoculum size (thick bacterium lawn) may result in falsely smaller zones.[24] The negative results in *S.aureus*'s case do not indicate that there were no bioactive compounds or molecules, nor does it indicate that venom protein was inactive. Bioactive peptides may have fewer quantities both in the hydrolysate and venom protein to show activity against bacteria.[25] Gram-positive bacteria have a thicker cell wall than that Gram-negative bacteria; the cell wall thickness of the Gram-positive bacteria was around (20–80 nm), and served as the outer shell of the cell. Gram-negative bacteria, on the other hand, have a thinner cell wall (10 nm) but a thicker outer membrane with many pores and appendices.[26] Thus, large doses must be used to demonstrate a lack of action.[27] Alternatively, if the bioactive peptides are present in large quantities, other elements may use the bioactive compounds' resistant effects.[28] Another possibility is, despite the absence of antibacterial activity, the venom protein and hydrolysate might be effective against uninvestigated microorganisms. Some extracts failed to show antibacterial activity when evaluated using the disc diffusion assay. When examined using the micro-dilution method, they clearly showed antibacterial activity. It might be owing to the effect of agar on bioactive peptide diffusion.[29]

Peptide Fraction Antibacterial Activities

Since the mechanism of the AMP is related to the peptide charge, the fractionation was performed using a cationic exchange system. The fraction elution was carried out by varying the pH of the elution system and using the pH value as the name of the resulting fraction (pH fraction 3 to pH fraction 10). The MIC value

was measured using the microdilution method with a 96-well plate, which was the most effective quantitative assay method for antibacterial activity.[30] Again, the antibacterial activity test on peptide fractions was performed against *E. coli* and *S. aureus*. The potential peptide fraction indicated by the MIC values is shown in Table-2.

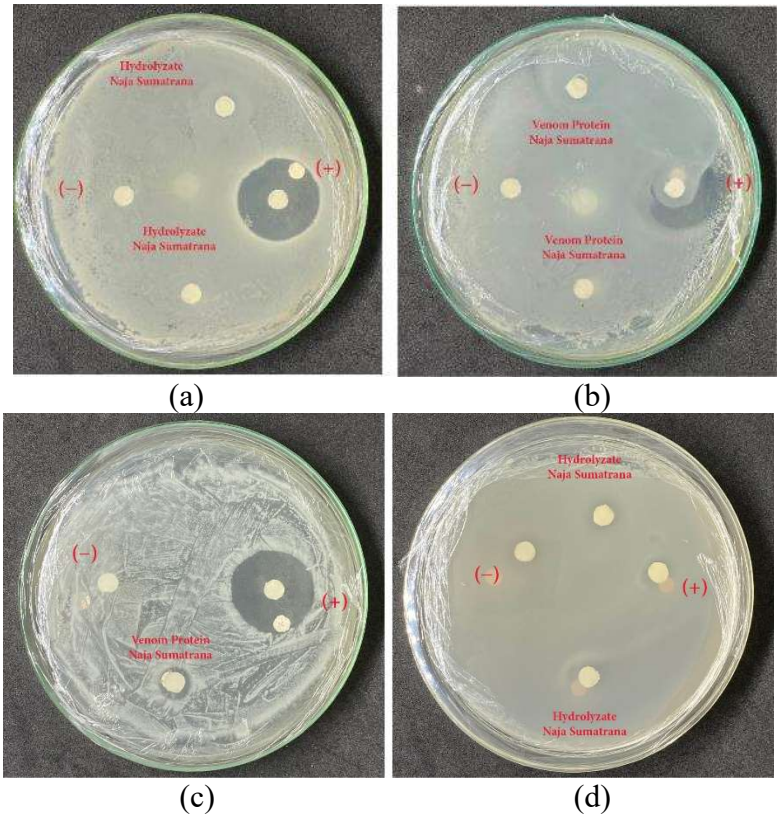


Fig.-1: (a) *Naja sumatrana* Hydrolysate Antibacterial Activity Against *Staphylococcus aureus*, (b) Venom Protein Antibacterial Activity Against *Staphylococcus Aureus*, (c) Venom Protein Antibacterial Activity Against *Escherichia Coli*, (d) Venom Hydrolysate Antibacterial Activity Against *Escherichia coli*

Table-1: Antibacterial Activity of Venom Hydrolysate And Venom Protein

Diameter of inhibition zone (mm)						
Sample	*Escherichia coli*			*Staphylococcus aureus*		
	Positive Control	Negative Control	Sample	Positive Control	Negative Control	Sample
Venom Protein	23.9	-	8.3	17.8	-	-
Protein Hydrolysate	8.5	-	9.1	22.9	-	-

The selected fraction presented in Table-2 exhibits the lowest MIC value against each *E. coli* and *S.aureus* from hydrolysate obtained from hydrolysis with and without reduction and alkylation. The lowest MIC values obtained from digestion with reduction and alkylation were 3.38 µg/ml from pH 6 fraction against *E. coli* and 2.450 µg/mL from pH 3 fraction against *S. aureus*. The lowest MIC values obtained from the hydrolysate without alkylation and reduction hydrolysis were 1.59 µg/mL from pH 6 against *E. coli* and 1.10 µg /mL from fraction pH 7 against *S. aureus.* Antibacterial peptides are usually cationic in composition and are hypothesized to exhibit bactericidal activity by permeabilizing bacterial membranes through a depletion process that includes membrane bilayer instability. The antibacterial mechanism of anionic antimicrobial proteins against Gram-positive bacteria such as *Staphylococcus aureus*, on the other hand,

includes interactions with the membrane lipid head group domain.[31] Antibacterial peptides and proteins kill bacteria by inhibiting the macromolecular synthesis and interacting with the basic bacterial components, as well as membrane permeabilization.[32]

Table-2: Antibacterial Activity of Free Peptides and the Hydrolysates

Bacteria	Digestion with Alkylation and Reduction (µg/ml)		Digestion without Alkylation and reduction (µg/ml)		Positive control chloramphenicol (µg/ml)
	Sample	MIC	Sample	MIC	MIC
Escherichia coli	pH 6	3.376	pH 6	1.586	7.078
Staphylococcus aureus	pH 3	2.450	pH 7	1.092	1.36

Peptides Sequence Characterized from Venom Protein Hydrolysate

The peptide sequences were obtained as selected peptides from identified proteins based on MS/MS analysis. Table-3 summarizes the peptides identified from the active fraction according to their MS spectra.

Table-3: Peptide Sequence Identified From the Antibacterial Active Fraction of *N. sumatrana* Venom Protein Hydrolysate

No.	Amino acid sequences	pI (predicted)	$[M-H]^+$ Da	The protein of king cobra *(Ophiophagus hannah)*	Amino acid position in the protein
1.	LMTSQK	8.75	723.37718	Olfactomedin-like protein 1 domain from gliomedin, putative	392 - 397
2.	TQFSDR	5.50	753.35179	Highly divergent homeobox, putative	445 - 450
3.	VYGGDSR	5.81	753.35179	Hydroxy acyl glutathione hydrolase, mitochondrial, putative	392 - 397
4.	YTPTNK	8.59	723.37433	Nebulin, putative	5113-5118
5	TIFKSK	10.00	723.43983	PR domain zinc finger protein 15, putative	569 -574
6	TFQDSR	5.50	753.35179	Nop9 protein, putative	282 - 287
7	TLMTNK	8.41	723.37511	Ankyrin repeat and BTB/POZ domain-containing protein 2, putative	564 - 569

One example of HRMS data in the identification of the peptide sequence, as can be seen in Fig.-2, exhibits MS/MS spectra of the parent ion with mass $(M-H)^+$ = 753.35179 Da which corresponds to the mass of TQFSDR. The peptide was identified as part of a highly divergent homeobox, a putative protein of *N. sumatrana*. The MS^2 detected the m/z = 377.17953 and z=+2, confirming spectra of y_3^+ and b_3^+ ions representing the sequence of TQF and SDR respectively. The b_1^+ ion was formed due to the termination of the C - N bond on the threonine (T) at the C - terminus with glutamine (Q). Threonine (T) had a theoretical monoisotopic mass of 101.0549 which then acquired one proton and appeared with m/z 102.07104. The b_3^+ was formed due to the cleavage of the C - N bond on the phenylalanine (F) at the C - terminus with serine (S). The theoretical mass of threonine (T), glutamine (Q), and phenylalanine (F) was 376.1819, which then m/z 377.18127 was obtained after getting proton. y_3^+ was formed due to the cleavage of the C - N bond of serine (S) at the N - terminus with phenylalanine (F). The theoretical mass of serine (S), aspartic acid (D), and arginine(R) was 376.1779, which then m/z 377.1812 was obtained after getting proton.

Docking Study

Since the AMP sequences of the hydrolysate do not exhibit strong cationic characteristics in favor of the membrane disruption mechanism, an alternative mechanism should be proposed. One possible mechanism is by preventing bacterial cell division through replication inhibition. One of the key enzymes for replication is DNA gyrase. To determine whether any peptides are active through this mechanism, DNA Gyrase is docked with peptides in molecular docking investigations to discover how the peptides inhibit bacterial growth. Two important parameters to determine the stability of the protein-ligand combination are the

binding energy as the first parameter and the interactions between the ligand and the protein active site as the second parameter. Referring to the previous studies, binding energy had a larger influence in predicting the ideal binding mode than the interactions of the ligand-target protein. Conventional hydrogen bonds and hydrophobic contacts were more effective than the others; the affinity energy, RMSD, hydrogen bonding, and hydrophobic interactions were studied in this research.[20]

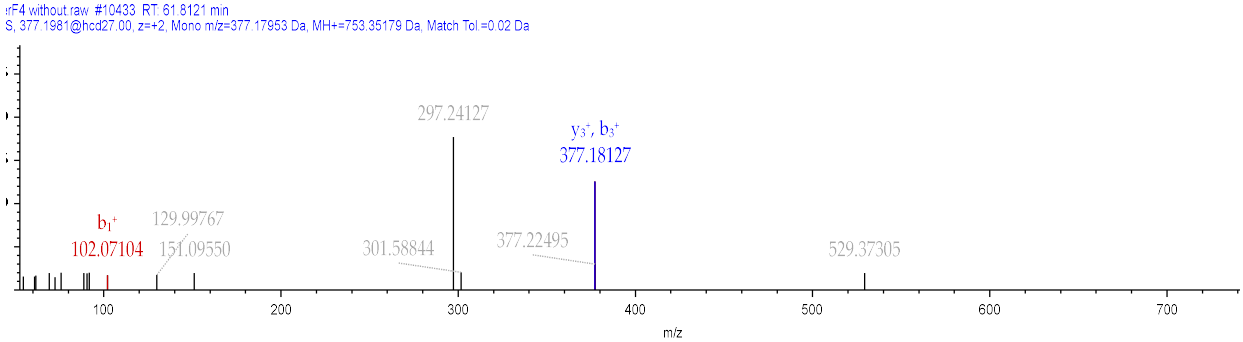
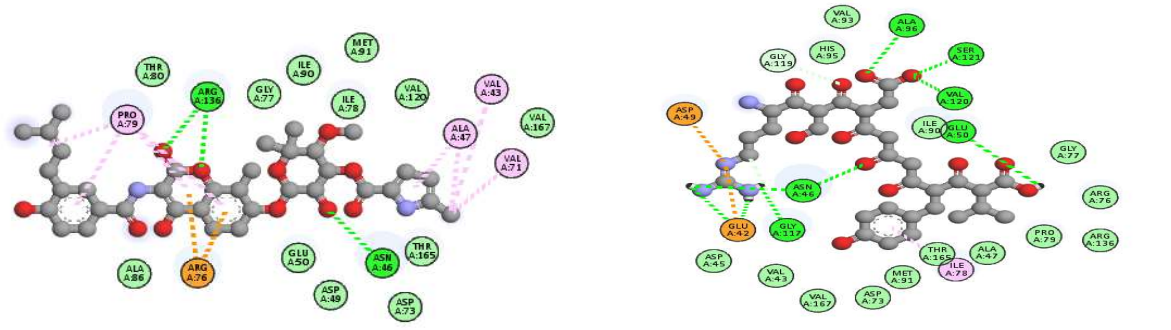


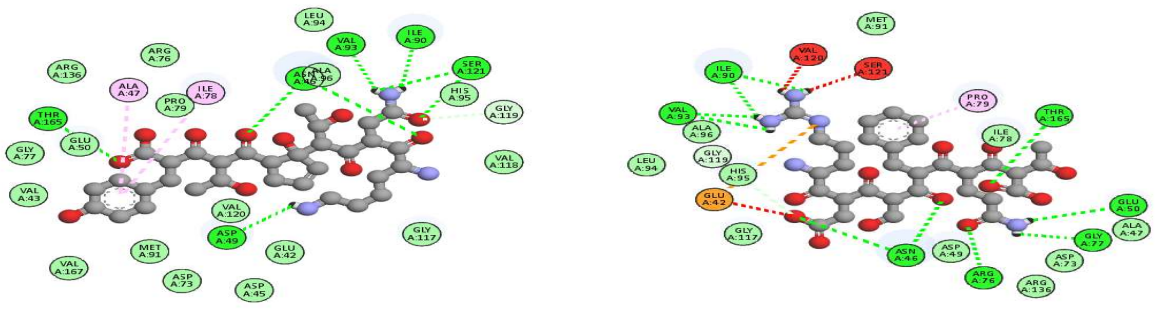
Fig.-2: MS Spectra of the TQFSDR Peptide

Table-1: Binding Affinity of Bioactive Peptides that Shows Activity Against *Gyrase*

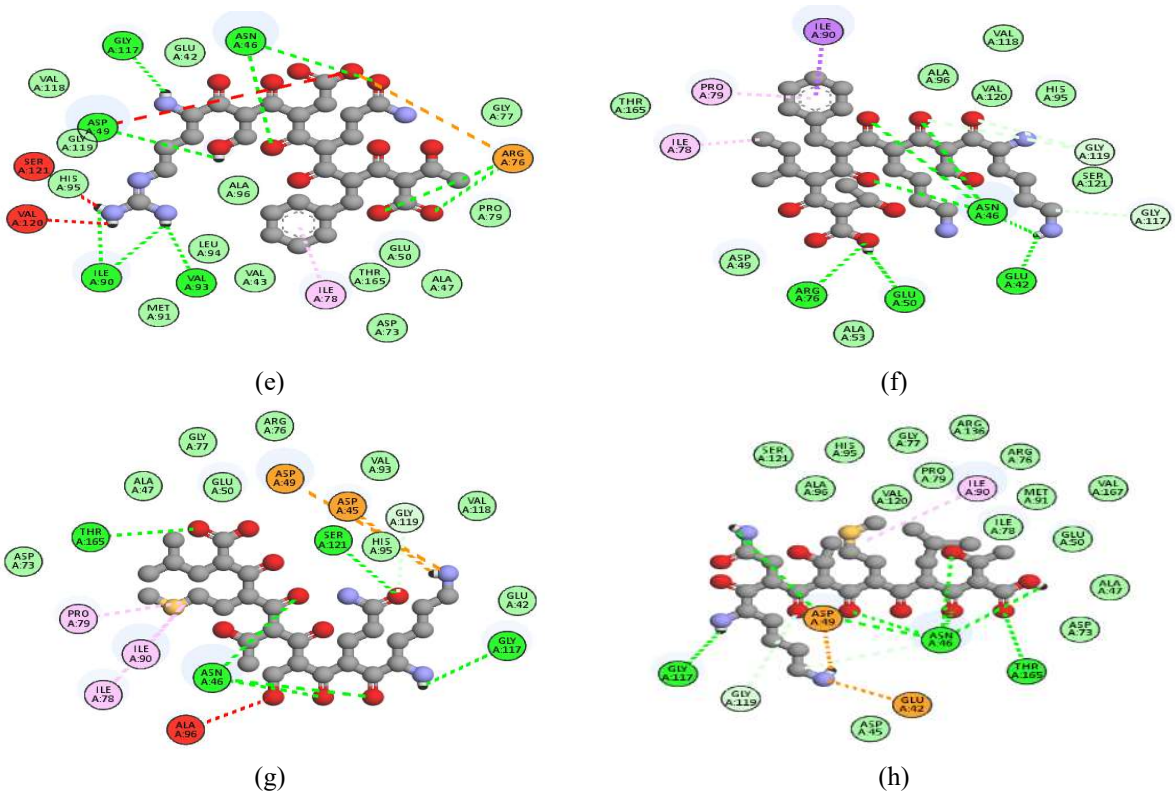
No	Peptides Sequence	*Escherichia coli*		*Staphylococcus aureus*	
		(affinity) kcal/mol)	RMSD	(affinity) kcal/mol)	RMSD
1	VYGGDSR	-7.7	1.757	-6.0	1.943
2	YTPTNK	-7.0	1.491	-6.1	1.822
3	TQFSDR	-7.0	1.980	-6.2	1.455
4	TFQDSR	-6.6	1.822	-5.9	1.994
5	TIFKSK	-6.0	2.857	-5.2	1.986
6	LMTSQK	-5.7	1.615	-5.8	2.314
7	TLMTNK	-5.7	1.716	-5.7	1.802



(a) (b)



(c) (d)



(e) (f)

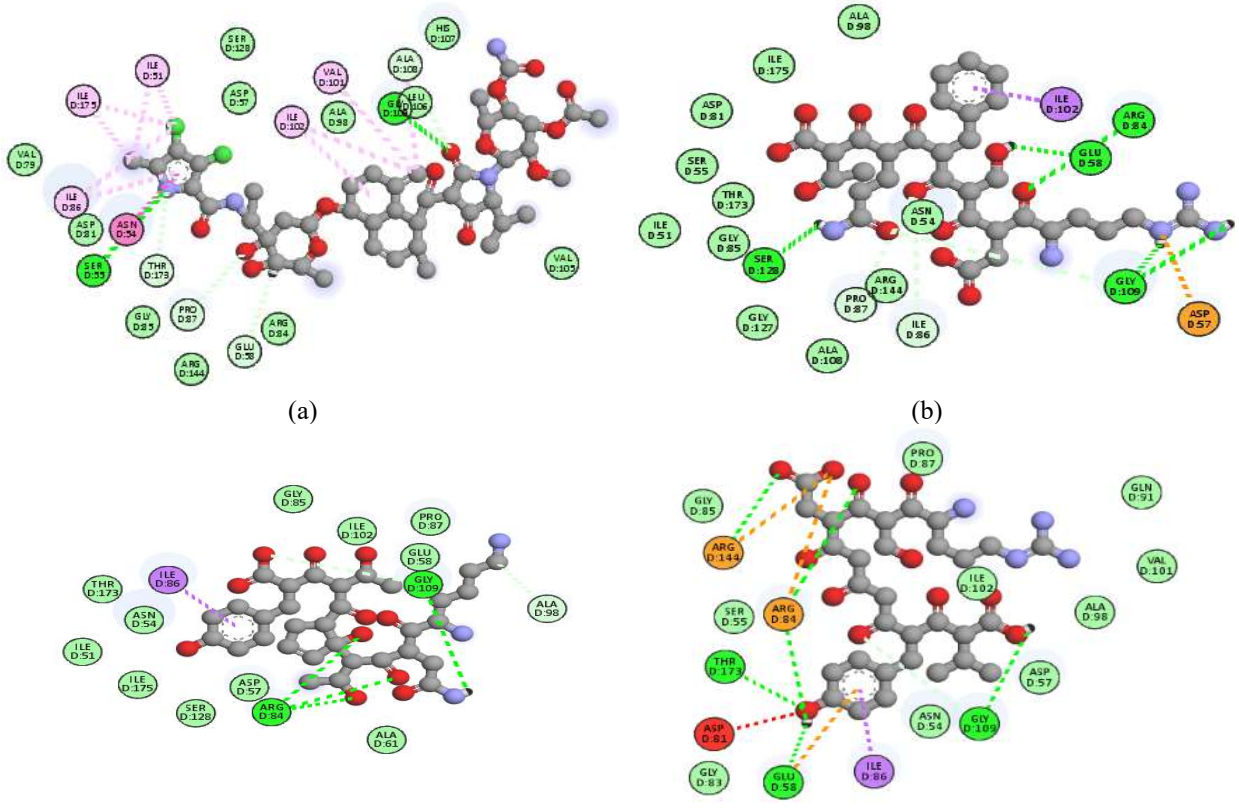
(g) (h)

Fig.-3: (a) is an Active Site of Native Ligand, while (b), (c), (d), (e), (f), (g), and (h) are the active sites of VYGGDSR, YTPTNK, TQFSDR, TFQDSR, TIFKSK, LMTSQK, and TLMTNK Peptides, respectively, against Escherichia coli Receptor

Table-4 shows the affinity energy and RMSD values of the peptides when docked to *E. coli*, DNA Gyrase subunit B (PDB ID: 1KZN). As can be seen in Fig.-3a, there were interactions between the native redocked ligand and protein. Figure-3 proves that the native ligand had conventional hydrogen bonds at Arg A:36 and Asn A:46, while the van der Waals bound to various amino acids. Figure-3b shows the VYGGDSR interaction site; VYGGDSR interacted with protein at Asn A:46, proving that these peptide interactions were at the correct active site. Besides, it had hydrogen bonds with Val A:120 and Gly A:117 which is highly hydrophobic and, ultimately, makes it a potential candidate for the antibacterial agent. This is also supported by affinity energy of -7.7 kcal/mol while the RMSD value was 1.757. All this evidence supports the antibacterial strength of VYGGDSR against *E. coli*. YTPTNK bound deeply into the active site of protein at Asn A:46, proving very tight binding and inhibiting the enzyme's access to the substrate (Fig.-3c). At the same time, its affinity score was -7.0 kcal/mol and its RMSD value was lower than 2, which was 1.491, proving that it is a potential antibacterial candidate against *E. coli*. Figure-3d displays TQFSDR interaction with the receptor, while its affinity was -7.0 kcal/mol and the RMSD value was 1.980. Its interactions at Asn A: 46 proves that the bond was in the right position. Meanwhile, the other conventional bonds, Gly A: 77 and Val A: 93, show high hydrophobicity. All peptides (TFQDSR, TIFKSK, LMTSQK, and TLMTNK) demonstrated interactions at the active site of Asn A: 46 and prove that all these peptides interacted in the right position. Their affinity energy was -6.6 kcal/mol, -6.0 kcal/mol, -5.7 kcal/mol, and -5.7 kcal/mol, while the RMSD value was, 1.822, 2.85, 1.615, and 1.716, respectively. Apart from Asn A:46 for TFQDSR, the hydrogen bonds between the protein and the above ligand peptides were Asp A:49, Gly A:117, Ile A:90, and Val A:93, which make it a more hydrophobic and become the best antibacterial agent (Fig.-3a). For the TIFKSK, the hydrogen bonds between the protein and the above ligand peptides were Glu A:42 and Arg A:76 (Fig.-3f), while the LMTSQK was Gly A:117, Ser A:121, and Thr A:165. As exhibited in Fig.-3g, it can be seen that van der Waals bonds were present. Meanwhile, TLMTNK hydrogen conventional bonding other than Asn A:46 were Gly A:117 and Thr A:165 (Fig.-3h). For *E. coli*, almost all the above peptides had good docking pose and hydrophobicity, but VYGGDSR, YTPTNK, TQFSDR, and TFQDSR had the most appropriate affinity scores, RMSD, and model scores. They can easily penetrate

deeply into the *Escherichia coli* cell membrane due to their high hydrophobicity. All peptides inside the active fractions against *S. aureus* were covalently docked into the *S. aureus* DNA Gyrase subunit B active site. The best docking pose was shown by TQFSDR with an affinity score of -6.2 kcal/mol. Its RMSD value was 1.455 while its interactions side had a resemblance to a native ligand. For the TQFSDR, the hydrogen bonds between the protein and the ligand peptides were Gly D:109, while the other hydrogen bonds were with Glu: D:58, Arg D:84, and Ser D:128 (Fig.-4b). Besides, there was a Vander Walls bond between ligan and Ser D:55, Asp D:81, Arg D:144, and so forth, indicating that it can be a potential candidate for an antibacterial agent against *S.aureus*. YTPTNK and VYGGDSR had -6.1 kcal/mol and -6.0 kcal/mol affinity scores, respectively, while the RMSD score were 1.822 and 1.943, respectively. Gly D:109 for both of the peptides indicates that both were in the correct pose of docking, while another hydrogen bonding of YTPTNK was Arg D:84 (Fig.-4c). In addition, it also had van der Waals as an alkyl bond. The hydrogen bonds of VYGGDSR other than Gly D:109 were Thr D:173 and Glu D:58. Figure-4d displays interactions of VYGGDSR. The formation of the covalent complex suggested that the enzyme would be permanently damaged. In addition to the hydrogen bond, it also had a van der Waals bond with various amino acids, alkyl groups, and pi-cation bonds. TFQDSR and LMTSQK resembled the native ligand at Gly D:109 and Ser D:55 in the interaction site respectively. TFQDSR also had other conventional covalent bonds with Asn D:54 (Fig.-4e) and another various van der Waals bonds. Their affinity scores were -5.9 kcal/mol and -5.8 kcal/mol, respectively, while their RMSD values were 1.994 and 2.314 respectively; the RMSD value of LMTSQK was higher than the two, which proved that peptides did not fit tightly with the binding site. The interactions of LMTSQK are displayed in Fig.-4f. LMTSQK also had some other hydrogen bonds besides Ser D:55, including Asn D:54 and Arg D:84. Furthermore, there were some Vander walls bonds as well as a pi covalent bond.

TLMTNK peptide resembled the native ligand interaction site at Ser D 55 (Fig.-4g). Its affinity score was -5.7 kcal/mol and its RMSD value was 1.802. For the TLMTNK, the hydrogen bonds shown other than Ser: D:55 were Alan D:98, Asp D:81, THR D:173, Arg D:84, and Asp: D:89; another remaining bond was the van der Waals bond. The TIFKSK affinity score was -5.2 kcal/mol, and its RMSD value was 1.986. The interaction site of TIFKSK was Ser: D:55, similar to the native ligand (Fig.-4h).



(a) (b)

(c) (d)

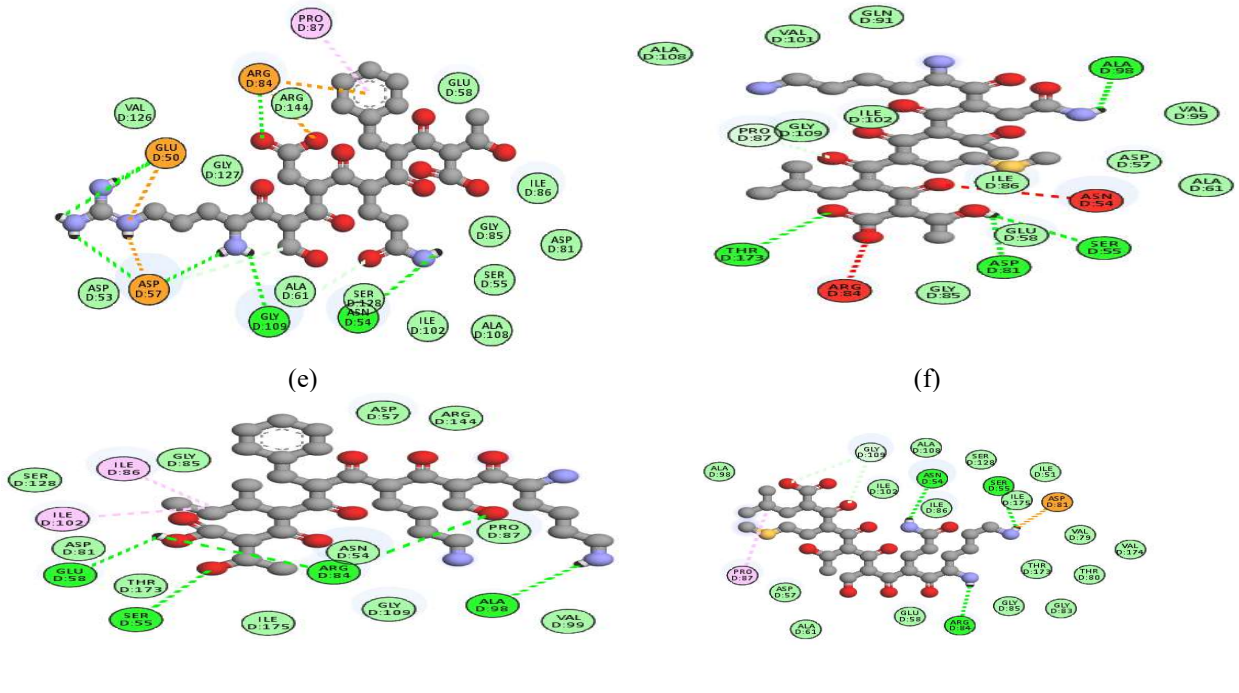


Fig.-4: (a) and (b) are the Active Sites of Native Ligand and TQFSDR peptide; (c), (d), (e), (f), (g), and (h) is the Active Sites of YTPTNK, VYGGDSR, TFQDSR, TLMTNK, TIFKSK, and LMTSQK, Respectively, against *Staphylococcus aureus* Receptor

The other hydrogen bonds shown were Arg D:84, Ala D:98, and Glu D:58. Two alkyl bonds with Ile: D:86 and Ile: D:102 remaining was the van der Waals bond.TQFSDR, YTPTNK, VYGGDSR, and TFQDSR had the most potent antibacterial activities of all the peptides due to their high-affinity score, hydrophobicity, strong RMSD values, most suitable site interactions, and stable amphiphilic structure, which were all essential for antimicrobial activity.[33] The biological activity of drugs was improved by the addition of hydrophobic and aromatic amino acids to the molecules. The side-chain functions, characteristics, and hydrophobicity of each of the twenty amino acids were unique. The hydrophobic activity of the molecule was found to be involved in the rupture of the cell membrane, which resulted in cell content leakage. The activity of curcumin has been linked to *S. aureus* and *E. coli* membrane damage, and the hydrophobic nature of curcumin nature made it a biocidal agent.[34]

CONCLUSION

Venom protein digestion by alkylation and reduction showed a high degree of hydrolysis compared to that digested without the alkylation and reduction process. Venom protein hydrolysate and venom protein provided antibacterial activity against *E. coli* but showed no activity against *S. aureus*, a Gram-Positive positive bacterium. A strong cation exchange solid-phase extraction column showed that several fractions had antibacterial activity against both *S. aureus* and *E. coli*. The peptides with sequences of TQFSDR, YTPTNK, VYGGDSR, and TFQDSR were found in all active fractions. The affinity score of docking and the hydrophobicity suggested that TQFSDR, YTPTNK, VYGGDSR, and TFQDSR were potentially classified as antimicrobial peptides against both *E. coli* and *S. aureus,* respectively. High pI is responsible for hydrophobicity, good affinity, and RMSD score. The interactions of those amino acids in a specific position lead to a high probability of membrane disruption.

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