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Research Article

# Discovery of New Antibacterial Peptide from Trypsin Hydrolysate of Monocled Cobra *(Naja kaouthia)* Venom Protein using $C_{18}$ SPE Column and LC-HRMS

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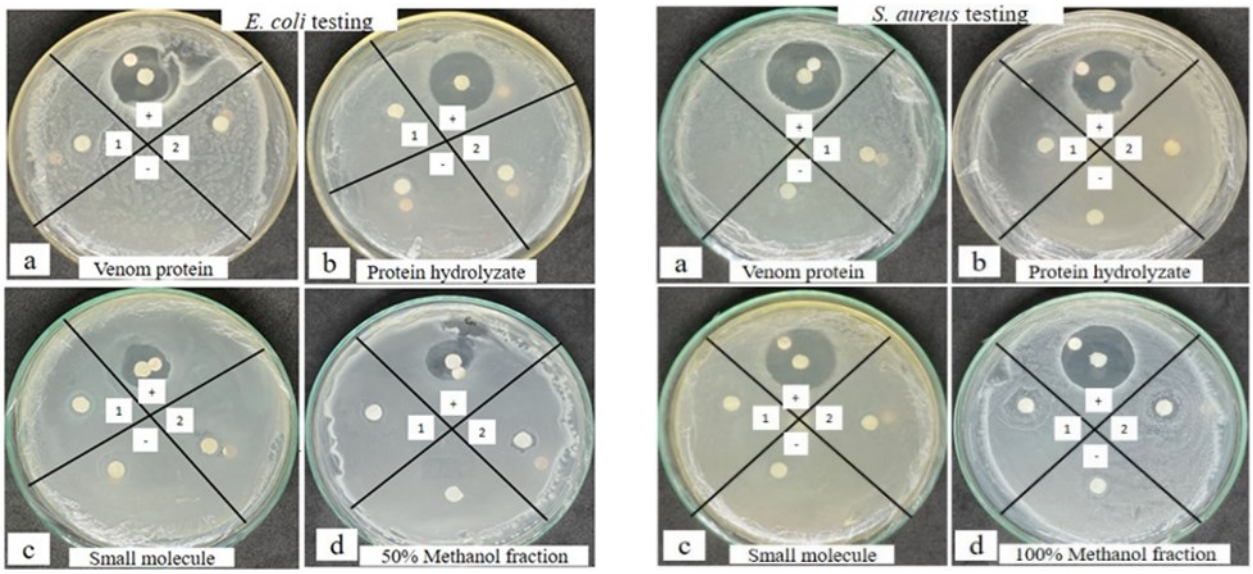
**Abstract**
Antimicrobial peptides (AMPs), as new antibiotic candidates, have the potential to treat infectious diseases. Peptides from *N. kaouthia* venom proteins can be used to produce AMP. The aim of this study was to identify the antibacterial potential of peptides derived from *N. kaouthia* venom protein purified by a reverse-phase solid-phase extraction column (SPE $C_{18}$). *N. kaouthia* venom protein was isolated using Amicon® Ultra-15 with a 3000 Da centrifugal filter and then hydrolyzed using trypsin. The hydrolyzate was fractionated using reverse-phase (SPE $C_{18}$), and the resulting fraction was tested for its antibacterial activity against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria. The most active fraction as an antibacterial was analyzed by high-resolution mass spectrometry (HRMS). The results showed that the 50% methanol fraction was active against *E. coli* bacteria, and the 100% methanol fraction was active against *S. aureus*. There are seven peptides identified, namely TVPVKR, TTMMNMLK, WWSDHR, SSLLVK, NSLLVK, LIPIASK, and GALMLK, which are responsible for the activity of the 50% methanol fraction. Meanwhile, there are two peptides, namely SSLLVK and NSLLVK, responsible for the activity of the 100% methanol fraction. LIPIASK and GALMLK peptides are predicted to show better potential as antimicrobial peptides.

**Keywords:** antimicrobial peptides, HRMS, SPE C18, trypsin, venom protein

## 1. INTRODUCTION

Antibiotics are utilized as the main treatment of infectious diseases. The use of antibiotics, however, faces a problem in the form of antibiotic resistance. According to WHO, antibiotic resistance is one of the leading causes of death due to infectious diseases [1]. One way to deal with antibiotic resistance is to develop new antibiotic compounds. Peptide has a unique structure that varies depending on the amino acid sequence and the size peptide. Low molecular weight leads to wide biological activity including an antimicrobial known as AMP. Melittin from snake venom is the first reported antibacterial ribosomal peptide [2]. Gramicidin, glycopeptide, polymyxin, and bacitracin are non-ribosomal peptide antibiotics of microbial origin [3] [4]. Mammalian peptides belonging to the ribosomal peptide are also reported to have antibacterial activity, one of which is cathelicidins, found in the vertebrate immune system [5].

Although some free peptides have been reported as AMP [6], the discovery of AMPs through protein hydrolysis became common due to their high probability to obtain new AMP. Several previous studies have discovered active peptide used this approach. For example, purification from laba *garlic (Allium sativum L.)* hydrolysate driven by pepsin and trypsin hydrolysis produces antibacterial peptides (YNHNF, WPTSFT, and AVDRAV) that could inhibit the mycelial growth of *E. coli* and *S. aureus in vitro* with MIC of 100 µM, respectively [7]. In the other study, AGLAPYKLKPIA was isolated from egg white ovotransferrin via pepsin hydrolysis with Gram-positive and Gram-negative antibacterial activities [8]. GITDLRGMLKRLKKMK that isolated from turbot viscera hydrolysate also showed antibacterial activity against Gram-positive and Gram-negative bacteria [9]. Another study discovered an antibacterial peptide (SSSEESII) from bovine casein hydrolysate with serine metalloprotease [10]. Protein hydrolysis that produces antibacterial peptides was also reported in the macroalgae *Saccharina longicrucis* hydrolyzed with trypsin. Nine peptides with a size of 8-16 amino acids were identified as AMPs [11].




**(i)** **(ii)**

**Figure 1.** Growth inhibition zone of *N. kaouthia* toxin against (i) *E. coli* and (ii) *S. aureus* with chloramphenicol, and distilled water were used as positive (+) and negative (-) controls. The active used samples are: (a) venom protein, (b) protein hydrolyzate, (c) small molecules, and (d) active methanol fraction (50%).

*Naja kaouthia* snake venom consists of protein components and non-protein components. The venom proteins of *N. kaouthia* include neurotoxins, cardiotoxins, thaicobrins, natriuretic peptides, and aminopeptidases. Some proteins play a role in antibacterial activity such as phospholipase $A_2$ ($PLA_2$) in venom which can damage the structure of bacterial cell membranes by breaking down bacterial membrane lipids [12]. The antibacterial activity of the protein is definitely related to the structure of one of the parts or domains of the protein molecule. If hydrolysis of proteins is carried out enzymatically, peptides will be produced from the protein part, among which are the domains responsible for the antibacterial activity of proteins so that they can be categorized as AMP. There are no specific patterns when coming out of the amino acid sequence of antibacterial peptides. Most antibacterial AMP contains hydrophobic amino acids that make up the coil of the secondary structure. Generally, AMP's mechanism of action is to damage cell membranes.

In addition to damaging the membrane's integrity by disrupting membrane proteins and lipids, the presence of hydrophobic amino acid sequences in peptides forms a random coil structure related to the antibacterial activity [13]. The structure of the random coil will damage the lipid integrity of the phospholipid bilayer of the bacterial cell membrane. This peptide has a random coil structure with antibacterial activity against Gram-positive and Gram-negative bacteria. Isolation of the peptide from protein hydrolysates is a crucial step in AMP finding. Based on the fact that most AMPs rely on the hydrophobic structure of amino acid to form their structure, reversed system such as reverse-phase column can be used as a choice for peptide purification. Brucin, the AMP isolated from *Brucea javanica* fruit protein, was isolated using reverse-phase semi-preparative high-performance liquid chromatography system [14]. The peptide fractionation of hydrolysis results is usually performed by using filtration gel chromatography, semipreparative, and preparative high-performance liquid chromatography [15]. Based on previous studies, this study aims to identify the antibacterial potential of peptides derived from *N. kaouthia* venom protein purified by the reverse-phase SPE column. The fraction obtained was then tested for its antibacterial activity, and the highest fraction was used to determine the sequence of its constituent amino acids by high-resolution mass spectrometry.

## 2. MATERIALS AND METHODS

### 2.1. Material

Materials and chemicals used in the current research were *N. kaouthia* venom (Bhumi Merapi, Yogykarta, Indonesia), SPE $C_{18}$ column (HyperSep Retain PEP; Supelco, USA), and trypsin (USP grade; G-Bioscience, USA). *E. coli* (ATCC 23522) and *S. aureus* (ATCC 23523) were utilized for the antibacterial activity test. Meanwhile, acetonitrile (hyper grade for LC-MS LiChrosolv, Merck, Germany), distilled water (MS grade, Merck, Germany), and high-resolution mass spectrometry (HRMS; Thermo Scientific, USA) were used to identify peptides.

### 2.2. Methods

#### 2.2.1. Snake Venom Collection

*N. kaouthia* venom was collected from two male snakes aged 2 years and 8 months. The snake venom was collected from a private zoo (Bhumi Merapi, Yogyakarta, Indonesia) by an expert from the Faculty of Veterinary Medicine, Universitas Gadjah Mada. The venom collection process was carried out by following the protocol approved by the Faculty of Veterinary Medicine, Gadjah Mada University (letter number 00103/EC-FKH/Eks./2021).

#### 2.2.2. Protein Extraction

Before usage, the venom was lyophilized in a clean flacon and kept in a refrigerator at 20 °C. Crystals of *N. kaouthia* venom were dissolved in 0.05 M solution of ammonium bicarbonate. The venom solution was then centrifuged at 5,000× g for 45 min at room temperature after being filtered with Amicon® Ultra-15 Centrifugal Filter Devices (MWCO 3000 Da, Merck, Germany). A UV-Vis spectrophotometer (Shimadzu UV-1800, Thermo Scientific, Japan) set to 280 nm was used to measure the protein concentration.

#### 2.2.3. Hydrolysis of Venom Protein

The ratio of the venom protein solution to trypsin enzyme solution is 20:1 (w/w). In order to obtain 5 mL of liquid, 0.05 M ammonium bicarbonate was added. The mixture was first incubated for 24 h at 37 °C, then the hydrolysis was stopped by heating the mixture for 15 min at 80 °C, cooling it, and then centrifuging it for 10 min at 1,500× g at room temperature. To measure the level of hydrolysis, the acquired absorbance was measured using a UV-Vis spectrophotometer at 280 nm.

#### 2.2.4. Fractionation of Protein Hydrolysate

In the SPE chamber device with a manifold vacuum pump, fractionation was carried out using a 1 mL HyperSep Retain PEP Cartridge column. The column is conditioned with 1 mL of 100% methanol

**Table 1**. The diameter of the inhibition zone of peptide of *N. kaouthia* venom protein against *E. coli* and *S. aureus*.

| Sample | Diameter of inhibition zone (mm) | |
|---|---|---|
| | ***E. coli*** | ***S. aureus*** |
| Venom protein | 0 | 6.2 |
| Protein hydrolyzated | 6.5 | 5.3 |
| Small molecule | 8.8 | 7.0 |
| Chloramphenicol | 18.6 | 22.9 |
| Aquadest | 0 | 0 |
| 25% methanol fraction | 6.6 | 5.8 |
| 50% methanol fraction | 7.2 | 8.6 |
| 75% methanol fraction | 6.6 | 6.7 |
| 100% methanol fraction | 5.4 | 9.2 |
| Chloramphenicol | 14.0 | 23.7 |
| Aquadest | 0 | 0 |

**Table 2.** Characteristics of peptides methanol fraction 50% and methanol fraction 100% protein hydrolyzate of *N. kaouthia* venom.

| Methanol fraction (%) | Peptide Sequence | Theoritical $MH^+$ (Da) | Observed $MH^+$ (Da) | Protein | pI | Gravy Index | Hydrophobic ratio (%) |
|---|---|---|---|---|---|---|---|
| 50 | TVPVKR | 699.45119 | 699.45087 | Cytotoxin 2 | 11.00 | -0.38 | 33.3 |
| | TMTMNMLK | 1001.44644 | 1001.45423 | NADH-ubiquinone oxidoreductase chain 5 | 8.41 | 0.08 | 50.0 |
| | WWSDHR | 886.39546 | 886.39551 | Cobrotoxin-b | 6.74 | -2.30 | 33.3 |
| | SSLLVK | 646.41340 | 646.41344 | Cytotoxin 1 | 8.47 | 1.05 | 50.0 |
| | NSLLVK | 673.42430 | 673.42406 | Cytotoxin 2 | 8.75 | 0.60 | 50.0 |
| | LIPIASK | 741.48690 | 741.48693 | Cytotoxin 1 | 8.75 | 1.18 | 57.1 |
| | GALMLK | 632.37999 | 632.37682 | Cobra venom factor | 8.75 | 1.16 | 66.6 |
| 100 | SSLLVK | 646.41340 | 646.41246 | Cytotoxin 1 | 8.47 | 1.05 | 50.0 |
| | NSLLVK | 673.42430 | 673.42333 | Cytotoxin 2 | 8.75 | 0.60 | 50.0 |

and equilibrated with 1 mL of distilled water. A total of 3 mL of protein hydrolyzate samples were added to the column. The column was washed with 1 mL of 5% methanol, and methanol in various concentrations of 25%, 50%, 75%, and 100% was used to elute the column. All fractions obtained were collected, and their absorbance was measured at a wavelength of 280 nm using a UV-Vis spectrophotometer.

### *2.2.5. Determination of Antibacterial Activity*

All bacteria used in current study were coming from Department of Microbiology, Faculty of Biology, Universitas Gadjah Mada collection. *S. aureus* and *E. coli* bacteria were grown in lactose broth media (NaCl, tryptone, and yeast extract) at 37 °C for 24 h. The microbial suspension ($OD_{600}$ = 0.5–1.0 a.u.) that had grown was inoculated into a petri dish (15 cm in diameter) containing sterile agar medium. Samples were tested for antibacterial activity using the same volume. Paper discs 5 mm in diameter were impregnated in each sample solution as much as 10 µL, including the positive control (chloramphenicol 3 µg/mL), negative control (sterile distilled water), venom protein solution (15 µg/mL), hydrolysate (small molecules (14 µg/mL), the peptide fraction (15 µg/mL). The venom protein sample was replicated by placing a paper disc that had been impregnated with the sample solution. By putting two impregnated paper discs with the same volume on top of each other, it was possible to replicate hydrolyzed samples, small molecules in venom, and the methanol fraction. Paper discs that have been treated with sample solution are then placed on the surface of the agar medium, which has been sub cultured with bacteria, and incubated for 24 h at 37 °C. The diameter of the inhibition zone around the paper disc was measured using a digital calliper to determine the antibacterial activity.

### *2.2.6. Identification of Antibacterial Active Fraction Peptide*

The active antibacterial fraction was analyzed using high-resolution mass spectrometry (HRMS; Acclaim® PepMap ($C_{18}$, 75 µm × 150 cm) RSLC column, Thermo Scientific, USA) operated at 25 ° C. There were two types of mobile phase used, namely mobile phase A consisting of water and

0.05% trifluoroacetic acid, and mobile phase B consisting of water, acetonitrile 20:80, and trifluoroacetic acid 0.1%. Mobile phase containing 95% A and 5% B flows at 0.1 mL/min from 0 min to 50 min, converts to 50% A and 50% B at 51 min to 53 min, changes to 95% A and 95% B at 53.1 min, and stops running at 60 min. For MS and MS/MS, it is done using the method of electrospray ionization set at 3800 V while collision energy was set at 35 V for $MS^2$. MS detection uses positive ion mode, which is operated in high mode with accurate resolution and mass. Peptides were analyzed by MS/MS with an m/z range of 150-2250 in full MS/ddMS$^2$ mode. The full MS parameter applied was the split power set to 140,000 full width at half maximum (FWHM), while the ddMS$^2$ parameter utilized was the resolution at 17,500 (FWHM). The *N. naja* genome was downloaded from UniProt.org and it was used to analyze MS data with Proteome Discoverer Software ver. 2.5. The findings of the current study were compared to the peptide database [16]. The identified peptides were searched for physicochemical properties such as hydrophobicity ratio, pI, and gravy index by writing the amino acid sequence of the peptide online through the ProtParam tool (https://web.expasy.org/protparam/).

## 3. RESULTS AND DISCUSSIONS

### *3.1. Hydrolysis of Venom Protein using Trypsin*

Enzymatic hydrolysis is carried out with the help of enzymes, one of which is trypsin (protease). Trypsin can cleave the C-terminus of arginine or lysine residues, releasing positively charged peptides with better solubility [17]. This study resulted in the hydrolysis of up to 77.5% of the venom protein of *N. kaouthia* using trypsin. Previous research on *Naja sumatrana* venom protein showed that it was successfully hydrolyzed by reduction and alkylation prior to trypsin digestion, resulting in a degree of hydrolysis of 49.71% [18]. The degree of hydrolysis value of the starry triggerfish (*Abalistes stellaris*) muscles obtained using the trypsin enzyme was 60% [19]. The enzymatic hydrolysis method is an effective way to produce bioactive peptides [20]. Enzymatic hydrolysis can increase bioactivity and is superior

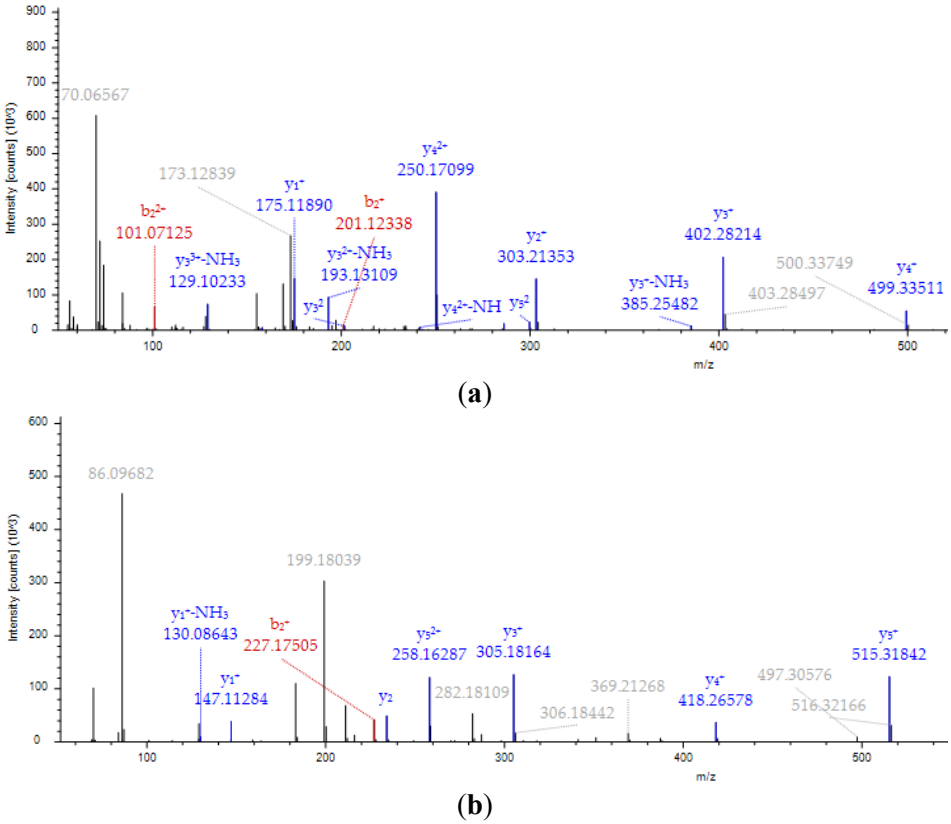



**Figure 2.** MS2 spectra of (a) TVPVKR peptide of 50% methanol fraction and (b) spectra LIPIASK peptide of 50% methanol fraction.

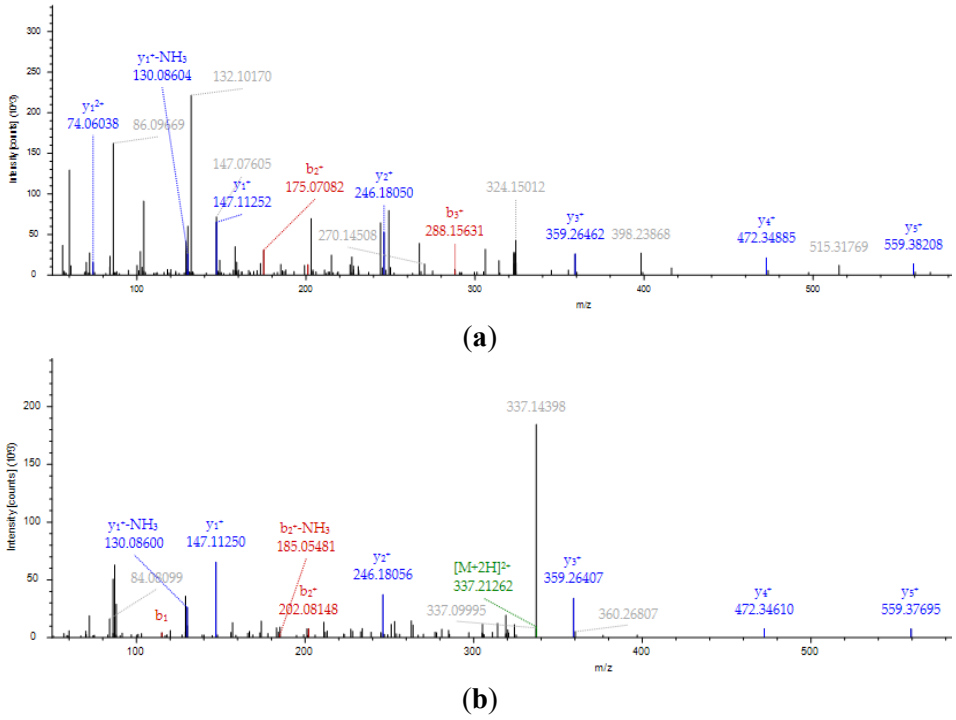



**Figure 3.** MS2 spectra of (a) SSLLVK peptide of 100% methanol fraction and (b) NSLLVK peptide of 100% methanol fraction.

to native proteins [21]. The degree of hydrolysis is influenced by several factors, including enzyme-substrate ratio, pH, temperature, hydrolysis time, protein source, and concentration [22].

### *3.2. Antibacterial Activity of Reverse-phase SPE Peptide Fractions*

Peptides from *N. kaouthia* venom were fractionated using a reverse-phase SPE column. The solvent for the mobile phase in the SPE method is distinguished by its concentration. This is conducted in order to provide different interactions between the peptide molecules and the eluted mobile phase. The fraction was eluted using methanol-water solvent variations with methanol concentrations of 25%, 50%, 75%, and up to 100%. The use of the SPE method in fractionation obtained better results for the separation [23]. The methanol fractions of 25%, 50%, 75%, and 100%, respectively, produced peptides with masses of 361.15, 414.50, 307.83, and 304.50 µg. The highest yield of total peptides in the hydrolysate was shown in the 50% methanol fraction.

The antibacterial test was carried out using the agar diffusion method, and the zone of inhibition of bacterial growth is shown in Figure 1. The agar diffusion method is the most commonly used in vitro method because the technique is simple and inexpensive [24]. Chloramphenicol was used as a control because it belongs to a class of broad-spectrum antibiotics that are capable of inhibiting the growth of Gram-positive and Gram-negative bacteria [25]. Aquadest was used as a negative control because it has a neutral pH and good solubility but does not have antibacterial properties and is unable to influence antibacterial activity [26].

Antibacterial testing was carried out on *S. aureus* and *E. coli*. The two bacteria represent Gram-positive and Gram-negative bacteria, respectively. *N. kaouthia* venom protein, hydrolyzate, and small molecule tests were aimed at early screening for antibacterial activity prior to fractionation. There are criteria for strength-based antibacterial activity. The inhibition zone diameter of >20 mm was classified as very strong, an inhibition zone diameter of 10–20 mm was classified as strong, an inhibition zone diameter of 5–10 mm was classified as moderate, and an inhibition zone diameter <5 mm was classified as weak [27]. The data showed that hydrolyzate, small molecule, and peptide fractions had antibacterial activity against *S. aureus* and *E. coli* bacteria, respectively while the venom protein only show activity against *S. aureus* (Figures 1(a) and 1(b)).

The methanol fractions of 25%, 50%, 75%, and 100% produced inhibition zone diameters of 6.6, 7.2, 6.6, and 5.4 mm, respectively, which belonged to the moderate category against *E. coli* bacteria. The 50% methanol fraction produced the highest diameter value of 7.2 mm compared to the other fractions, so it can be said to be stronger in inhibiting *E. coli*. Whereas for *S. aureus*, the methanol fractions of 25%, 50%, 75%, and 100% produced inhibition zone diameters of 5.8, 8.6, 6.7, and 9.2 mm, respectively, as shown in Table 1. The 100% fraction produced the highest diameter value of 9.2 mm, so it can be said to be stronger at inhibiting *S. aureus* bacteria. The positive control (chloramphenicol) had a higher inhibition zone and belonged to the stronger category than the active peptide fractions 50% and 100%, these results indicated that after peptide fractionation, the diameter of the inhibition zone was better, as shown by the standard drug, chloramphenicol. Negative control did not show the diameter of the inhibition zone for both bacteria.

Research on peptides isolated from *N. kaouthia* venom has not been widely reported. Previous research reported the antibacterial activity of the crude venom of several types of snakes. Venom *Naja haje* and *Naja pallida* produced antibacterial activity with inhibition zone diameters of 7.6 ± 0.5 mm and 6.3 ± 0.8 mm against *E. coli* and 12.1 ± 1.7 mm and 9.7 ± 0.5 mm against *S. aureus*, respectively [28]. Crude common snake venom from Iraq-Iran showed antibacterial activity against Gram-negative bacteria (*Proteus mirabilis*) with an inhibition zone of about 10 mm and 8 mm, at snake concentrations of 500 and 250 µg/mL, respectively [29].

The selection of active peptide fractions 50% (*E. coli*) and 100% methanol (*S. aureus*) is because these two fractions produce the largest diameter of the inhibition zone and are close to the highest value in the medium category, namely 5–10 mm compared to the other fractions, making them the most active peptide fraction in inhibiting bacteria. The venom protein *N. kaouthia* has no antimicrobial activity against *E. coli*, as indicated by the absence of an inhibition zone. The results obtained were the same as previous studies on *Naja naja* crude venom, which produced antibacterial activity with an inhibition zone diameter of 27.8 ± 1.10 mm against *S. aureus*, while *E. coli* did not produce antibacterial activity [30].

### *3.3. Identification of Antibacterial Active Fraction Peptide*

The antibacterial active fractions peptide, the 50% and 100% methanol fractions, were targeted by high-resolution mass spectrometry in the positive mode to determine the molecular mass and the sequence of the amino acids that compose them. The correlation of peptide tandem mass spectral data with amino acid sequences in databases was compiled using Sequest HT [31][32]. The complete protein sequence database of *N. kaouthia* was not yet available, so it could be identified based on similarities with proteins that have been reported from the nearest organism, namely the *N. naja* database, with a de novo approach. The data showed that the 50% methanol fraction identified 7 peptides with 6-8 amino acid sequences (Table 2). Meanwhile, 2 peptides consisting of 6 amino acids were identified in the 100% methanol fraction (Table 2). The identified peptides had a C-terminus, terminal arginine (R), and lysine (K).

This is because the trypsin enzyme plays a role in selectively cutting polypeptides. The four best bioactive peptides from the hydrolysis of *N. sumatrana* trypsin protein against *E. coli* and S. bacteria yielded the amino sequences VYGGDSR, YTPTNK, TQFSDR, and TFQDSR [18]. The active peptide fractions 50% and 100% methanol have similar peptides; this is in line with the previous research [33], who stated that 89% of the proteins identified from the proteome of 12 combined fractions were also identified in the proteome of 24 fractions, which illustrated good reproducibility, thereby proving that there was a possibility of similar peptides in different fractions.

The $MS^2$ data of the first peptide threonine, valine, proline, valine, lysine, arginine (TVPVKR) obtained from 50% methanol fraction confirmed the presence of $b^+$ and $y^+$ fragment ions. The confirmed fragment ions consisted of $b_2^+$, $y_1^+$, $y_2^+$, $y^{3+}$, and $y_4^+$, which are shown in (Figure 2(a)). The $b_2^+$ ion represents the amino acid V = 201.12338, and the fragment $y^+$ ion (175.11890) represents the amino acid R at the C-terminal carboxyl of the peptide. The mass difference between the $y^{3+}$ ion (402.28214) and $y_2^+$ ion (303.21353) represents the

amino acid V = 99.06861 and confirmed the $y_3^+$-$NH_3$ ion (385.25482), $y_2^+$-$NH_3$ (193.13109), $y_3^{2+}$-$NH_3$ (129.10233). Meanwhile, that the mass difference between the $y_4^+$ ion (499.33511) and the $y_3^+$ ion confirmed the presence of the amino acid P = 97.05297. The amino acid confirmed the amino acid sequence of PVKR. The peptide TVPVKR is part of the protein cytotoxin 2.

The $MS^2$ data from the second peptide leucine, isoleucine, proline, isoleucine, alanine, serine, lysine (LIPIASK) with 50% methanol fraction confirmed the presence of $b^+$ and $y^+$ fragment ions. The confirmed fragment ions consisted of $b_2^+$, $y_1^+$, $y_3^+$, $y_4^+$, and $y_5^+$, which are shown in (Figure 2(b)). The $b_2^+$ = 227.17505 represents amino acid I, while the fragment ion $y_1^+$ = 147.11284 represents amino acid K at the C-terminal carboxyl and confirmed the $y_1^+$-$NH_3$ ion (130.08643). The difference in mass between $y_4^+$ ion (418.26578) and the $y_3^+$ ion (305.18164) confirmed the presence of amino acid I = 113.08414. In addition, the difference between $y_5^+$ ion (515.31842) and the $y_4^+$ ions confirmed the presence of amino acid P = 97.05264. The LIPIASK peptide is part of the protein cytotoxin 1.

The $MS^2$ data from the first peptide serine, serine, leucine, leucine, valine, lysine (SSLLVK) with 100% methanol fraction confirmed the presence of $b^+$ and $y^+$ fragment ions. The confirmed fragment ions consisting of $b_2^+$, $b_3^+$, $y_1^+$, $y_2^+$, $y_3^+$, $y_4^+$, and $y_5^+$ are shown in (Figure 3(a)). The mass difference between $b_3^+$ ion (288.15631) and $b_2^+$ ion (175.07082) confirmed the presence of amino acid L = 113.08549. The $y_1^+$ ion (147.11252) represents the K amino acid at the C-terminal carboxyl of the peptide, confirmed by the $y_1^+$-$NH_3$ ion (130.08604) and the $y_1^{2+}$ ion (74.06038). The difference in mass between $y_{2+}$ ions and $y_1^+$ ions confirmed the presence of amino acid V = 99.06798. The difference in mass between the $y_3^+$ ion (359.26462) and the $y_2^+$ ion (246.18050) confirmed the presence of the amino acid L = 113.08412. The difference in mass between the $y_4^+$ ion (472.34885) and the $y_3^+$ ion confirmed the presence of the amino acid L = 113.08423. Meanwhile, the mass difference

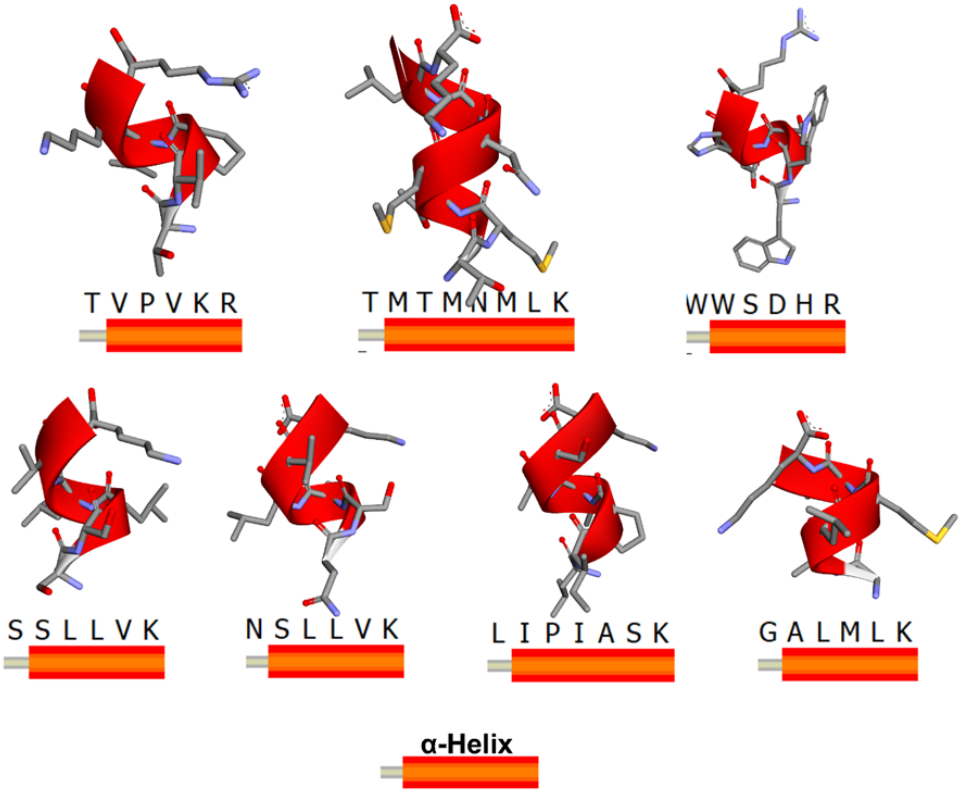



**Figure 4.** Secondary structure 100% methanol fraction peptides (SSLLVK and NSLLVK) and 50% methanol fractions peptides (TVPVKR; TMTNMLK; WWSDH; LIPIASK and GALMLK) predicted by Chimera 1.16.

between the $y_5^+$ ion (559.38208) and the $y_4^+$ ion confirmed the presence of the amino acid S = 87.03323. The amino acid confirmed the amino acid sequence of SLLVK. The SSLLVK peptide is part of the cytotoxin 1 protein.

The MS2 data from the second peptide asparagine, serine, leucine, leucine, valine, lysine (NSLLVK) with 100% methanol fraction confirmed the presence of $b^+$ and $y^+$ fragment ions. The confirmed fragment ions consisted of $b_2^+$, $y_1^+$, $y_2^+$, $y_3^+$, $y_4^+$, and $y_5^+$, which are shown in (Figure 3(b)). The $b_2^+$ ion represents the amino acid S = 202,08148 and the confirmed $b_2^+$-$NH_3$ ion (185.0581). The $y_1^+$ ion (147.11250) represents the K amino acid at the C-terminal carboxyl of the peptide. The difference in mass between the $y_2^+$ ion (246.18056) and the $y_1^+$ ion confirmed the presence of the amino acid V = 99.06806. The difference in mass between the $y_3^+$ ion (359.26407) and the $y_2^+$ ion confirmed the presence of the amino acid L = 113.08351. The difference in mass between the $y_4^+$ ion (472.34610) and the $y_3^+$ ion confirmed the presence of the amino acid L = 113.08203. Meanwhile, the mass difference between the $y_5^+$ ion (559.37695) and the $y_4^+$ ion confirmed the presence of the amino acid S = 87.03085. The amino acid confirmed the amino acid sequence of SLLVK. The NSLLVK peptide, is part of the protein cytotoxin 2.

Physicochemical properties such as hydrophobicity ratio, positive net charge, and secondary structure need to be considered in predicting antimicrobial peptide activity [34][35]. The characteristics of the active fraction peptides are shown in (Table 2), which were calculated using the ProtParam tool. Cationic and hydrophobic amino acid residues are generally associated with AMP activity. Cationic residues such as arginine (R), lysine (K), and histidine (H) can interact with bacterial lipids. Arginine and lysine amino acids have the ability to disrupt membranes and cause bacterial lysis [36]. Hydrophobic amino acid residues such as tryptophan (W), leucine (L), and phenylalanine (F) can be associated with membrane damage [37]. The identified peptides from the active fractions of 50% and 100% methanol produced cationic amino acid cleavage end such as R or K so that they can be advantageous in antibacterial activity. The grand average of hydropathicity (GRAVY) can predict the hydrophobicity of peptides.

The more positive the gravy value, the more hydrophobic the peptide; the more negative the gravy value, the more hydrophilic the peptide will be [38]. TVPVKR and WWSDHR peptides from the active fraction of 50% methanol produced negative gravy values of -0.38 and -2.30, indicating that the peptides tended to be polar (hydrophilic). Meanwhile, the TMTMMNMLK, SSLLVK, NSLLVK, LIPIASK, and GALMLK peptides had positive gravity values of 0.08, 1.05, 0.60, 1.18, and 1.16, respectively which indicated that these peptides tended to be non-polar (hydrophobic), as can be seen in Table 2. SSLLVK and NSLLVK peptides from 100% methanol fraction have positive gravity values of 1.05 and 0.60, indicating that these peptides tend to be hydrophobic (non-polar), as presented in Table 2. The solvent of 50% methanol, which is polar, produced peptides that are polar and tend to be non-polar. The LIPIASK and GALMLK peptides from the active 50% methanol fraction have a positive gravy value (hydrophobic) compared to the other peptides, so their hydrophobic ratio is also higher, namely 57.1% and 66.6%, respectively.

The hydrophobic residue of 50% AMP can form an amphipathic conformation with the bacterial cell membrane so that bacteria are easily inhibited [39]. The solvent of 100% methanol produced peptides that tend to be nonpolar. The value of the hydrophobic ratio of the two peptides was 50%. Peptides with a high hydrophobic ratio (30%) displayed typical AMP properties [40]. Peptides from both 50% and 100% methanol fractions were predicted to form the α-helical structure (Figure 4). Peptides with α-helical structure are common structures in AMP and have a strong correlation to antibacterial activity [41]. AMP α-helical can cause membrane destabilization and the formation of non-bilayer lipid structures [42]. The activity of antimicrobial peptides is determined by their physicochemical properties (peptide charge and hydrophobic characteristics) [43]. Based on the physicochemical properties of the peptides, which include gravy value, hydrophobic ratio, and secondary structure, LIPIASK and GALMLK peptides have more potential to support bacterial cell disruption.

## 4. CONCLUSIONS

The venom protein of *N. kaouthia* was successfully hydrolyzed with trypsin and followed by fractionation using a reversed-phase SPE column. The results showed that the 50% and 100% methanol fractions were the most active peptide fractions as antibacterials against *E. coli* and *S. aureus* bacteria with moderate inhibition zone diameters, while the positive control produced strong inhibition zone diameters. Peptide fractions have more than one compound or peptide, and not all their components are active, so the diameter of the inhibition zone of the most active peptide fraction can be lower than that of chloramphenicol. The 50% methanol fraction identified seven peptides, while the 100% methanol fraction identified two peptides. Due to their hydrophobicity and secondary structure that were similar to AMP typical physicochemical properties, GALMLK and LIPIASK are potential to be AMP candidates. To determine peptide's structure activities relationship, further study like single peptide activity and mechanism of action assay are needed.

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### Author Contributions

T. J. R. and S. R. conceived the research idea and designed the methodology; S. R. also carried out the venom collection. The majority of the experimental work, including protein hydrolysis, peptide fractionation, and antibacterial assays, was conducted by G. P. E.; T. J. R. analyzed the LC-HRMS raw data to identify the peptides. The original draft of the manuscript was prepared by G. P. E., with T. J. R. and S. R. providing intensive review and editing before submission.

### Conflicts of Interest

The authors declare no conflict of interest.

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## DECLARATION OF GENERATIVE AI

Not applicable.

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