Anticancer Activity of Venom Protein Hydrolysis Fraction of Equatorial Spitting Cobra (*Naja sumatrana*)

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Abstract: *Bioactive peptides play an important role in targeting cancer cells. Venom protein from* Naja sumatrana *can be explored as a source of bioactive peptides. This research aims to identify and study the molecular docking of bioactive peptides (BPs) from trypsin hydrolysate of* N. sumatrana *venom protein which was fractionated using an SPE C_{18} column. The venom of* N. sumatrana *was hydrolyzed with trypsin enzyme. The protein hydrolysate was then fractionated using an RP-SPE HyperSep Retain PEP column, and the peptide fractions were tested for their anticancer activity against MCF-7 breast cancer cells using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Identification of peptides in the active fraction was carried out through high-resolution mass spectrometry. The identified peptides were molecularly docked with the EGFR receptor using AutoDock Vina. The results showed that the degree of hydrolysis was 74.7%. The 75% methanol fraction is the active fraction against MCF-7 cells, with an IC_{50} value of 4.80 μg/mL and a selectivity index of 5.00. Peptide-active anticancer fractions with the sequence of NSLLVK, SSLLVK and TVPVKR were successfully identified and exhibited high binding affinity values, good RMSD values, and the most suitable model for the epidermal growth factor receptor.*

Keywords: *bioactive peptides; trypsin; venom;* N. sumatrana*; molecular docking*

■ INTRODUCTION

Globally, cancer is regarded as a major health problem. Based on the Global Cancer Statistics published in 2015, there were around 32.6 million cancer patients worldwide in 2012 [1]. Furthermore, the WHO reported that in 2019 cancer was the leading cause of death before the age of 70 [2]. Breast cancer is the second leading cause of death due to conventional drug resistance [3]. The use of chemotherapy, surgery and radiation therapy using anticancer drugs can cause side effects. This treatment cannot differentiate between normal cells and cancer cells, resulting in systemic toxicity [4]. Thus, the discovery of new drugs with high selectivity and specificity is needed for overcoming cancer.

Bioactive peptides (BPs) derived from animals are able to target cancer cells so that they can function as anticancer agents and are less toxic to normal cells. BPs consist of 2–50 amino acid residues (10^2–10^3 Da), so they easily disrupt cell membranes and result in apoptosis or necrosis [5]. Consequently, BPs become promising alternative candidates for the development of a new generation of anticancer therapies [6]. One source of bioactive peptides can be obtained from snake venom. The therapeutic use of snake venom has been widely carried out by scientists. Protein components, peptides, and chemical compounds in snake venom have a high potential for toxicity. Snake venom contains a complex mixture of peptides, proteins, enzymes, carbohydrates, and minerals with low molecular masses and certain

biological activities [7]. L-amino acid oxidases (LAAOs), phospholipase A_2 (PLA_2), three-finger toxins (3FTxs), the snake venom metalloprotease family (SVMP), cobra venom factor (CVF), and cysteine-rich secretory protein (CRISP) are some of the components that have been isolated from various snake venoms which can be used as anticancer agents [8].

Research [9] reported that crotamine, a short 42-amino acid polypeptide stabilized by cysteine, has shown *in vitro* and *in vivo* toxicity to cancer cells in a melanoma mouse model with concentrations of 5 µg/mL, which was lethal to B16-F10 (murine melanoma cells), SK-Mel-28 (human melanoma cells), and Mia PaCa-2 (human pancreatic carcinoma cells) but not in normal cells. Studies revealed that snake venom contains specific molecules that can slow down cancer cells [10]. The $svPLA_2$ component of the *Bothrops jararacussu* venom shows anticancer activity against MDA-MB-231 triple-negative breast cancer cells [11]. Bioactive peptides from snake venom can contribute significantly to medical treatment and specifically target cancer cell membranes [12]. Bioactive peptides can be encoded in snake venom proteins, and after cleavage, they have more active biological activity as an anticancer [13]. Among several methods of producing bioactive peptides from protein precursors, the most commonly used is enzymatic hydrolysis [14-15].

The enzymatic hydrolysis method uses controlled pH and temperature conditions to reduce the formation of unwanted products [16]. The trypsin enzyme has been widely utilized for the identification of bioactive peptides, which usually cleaves the peptide bond at the C-terminal end of amino acids (R or K), but not when they are next to the P amino acid. Eel protein hydrolysate (EPH) with a molecular weight of 3 kDa showed the highest inhibition of MCF-7 breast cancer cells with an IC_{50} value of 6.50 µg/mL [17]. Studies on protein hydrolysate of oyster (*Saccosrea cucullata*) reported that the peptide sequence Leu-Ala-Asn-Ala-Lys exhibited anticancer activity against human colon carcinoma (HT-29) cell lines [18]. The different structural sequences of the bioactive peptides are unique so that they can interact with the active sites of the substrates. Unique peptide sequences can be used to determine the mechanism of anticancer action, such as being able to destroy cancer cells through apoptosis and necrosis by membrane lysis or pore formation [19]. The mechanism of action of anticancer peptides can be ascertained by the interaction of peptides with inhibitors through molecular docking. Docking is a computational technique capable of predicting the conformation and interaction of a ligand with a particular protein. Molecular docking is an approach utilized to more easily identify the mechanism of peptide action [20].

Based on previous research, this study aims to identify BPs from *N. Sumatrana* venom protein hydrolysate fractionated using an SPE C_{18} column. The anticancer activity of the fractions was tested against MCF-7 breast cancer cells. High-resolution mass spectrometry was then used to identify BPs in the active fraction. After identifying the BPs, a docking study was carried out to examine the interaction of the peptide with the active site of the receptor, which determines the mechanism of action.

■ EXPERIMENTAL SECTION

Materials

Materials and chemicals included in this research were *N. Sumatrana* venom, trypsin USP (G-Bioscience), trypsin sequence grade enzyme (Merck), SPE C_{18} column (HyperSep Retain PEP, Supelco, Thermo Scientific), ammonium bicarbonate 0.05 M (Sigma-Aldrich®), and methanol (Merck). Meanwhile, anticancer activities were carried out by using Dulbecco's Modified Eagle Medium (DMEM) media, MTT solution, 0.05% trypsin, amphotericin B (fungizone), phosphate buffer saline (PBS) solution, penicillin-streptomycin 2%, fetal bovine serum (FBS) from Sigma-Aldrich, a breast cancer cell line (MCF-7), a normal cell line (Vero), and doxorubicin (Kalbe).

Instrumentation

The Instrumentations used in this study were Amicon® ultra-15 (3000 Da MWCO centrifugal filter, Merck), UV-Vis Spectrophotometer (Shimadzu UV 1800), Centrifuge (Biofuge Primo R Centrifuge 7590, Thermo Scientific), Inverted Microscope (Olympus

CKX41), Elisa Reader (Bio-Rad 680 XR), High-Resolution Mass Spectrometry (HRMS, Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer Thermo Scientific), and Proteome Discoverer Ver. 2.5 software (Thermo Scientific).

Procedure

Sample collection of venom and protein extraction

N. sumatrana venom (± 150 mg) was collected from Bhumi Merapi, Yogyakarta, by experienced veterinarians specializing in exotic animals at the Faculty of Veterinary Medicine, UGM. Venom collection was carried out by following the protocol that has been ethically approved according to the letter number 0052/EC-FKH/Ex./2020. The venom was lyophilized and stored in a refrigerator at −20 °C until use. The lyophilized venom (20 mg) was dissolved in 0.05 M ammonium bicarbonate. Venom protein and small molecules were separated with Amicon® ultra-15 with 3000 Da MWCO. The concentration of protein was determined with a UV-Vis spectrophotometer.

Proteomic analysis of N. sumatrana

A trypsin sequence grade enzyme solution was added to the protein venom solution with a ratio of 1:20 (w/w), and then the solution was incubated at 37 °C overnight. After 24 h of incubation, the sample was put into an oven at 80 °C to stop the hydrolysis process, then centrifuged at 5000× g for 45 min. Supernatants were filtered with a 0.22 μm filter membrane, and a total of 5 μL of the sample was transferred into injection vials for analysis using LC-HRMS. Proteome Discoverer software version 2.5 was used to process the raw MS chromatogram data. The database uses *N. naja* because the *N. sumatrana* database is not yet available. The *N. naja* database was downloaded from UniProt.org.

Hydrolysis of venom protein

N. sumatrana protein hydrolysate is produced by hydrolysis of venom protein using the same method as the proteome analysis procedure, but the quantities and grades of the trypsin enzyme are different for preparative purposes. Then the absorbance of the protein hydrolysate obtained was measured with a UV-Vis spectrophotometer at a wavelength of 280 nm to determine the degree of hydrolysis.

Fractionation of venom hydrolysate using reversed-phase SPE column

Fractionation was carried out with a 1 mL column of HyperSep Retain PEP Cartridge in an SPE chamber equipped with a manifold vacuum pump. 2 × 0.5 mL of methanol was used to condition the column, and 2 × 0.5 mL of distilled water was used to balance it. The procedure was terminated when the sample level was just above the adsorbent by progressively pushing the sample into the column using a pump. After washing the column with 2 × 0.5 mL of 5% methanol, methanol at various concentrations of 25, 50, 75, and 100% was used to elute the column. Each obtained fraction's absorbance was measured at a wavelength of 280 nm using the UV-Vis spectrophotometer to calculate the protein concentration in each fraction [21].

Anticancer activity test

A total of 100 μL of cell culture medium was added into 96-well plates where three wells were kept specific for media control and three other wells for control cells. The cell's plate was incubated at 37 °C for 24 h in a 5% CO_2 flow to recover cell conditions after culturing. The media in the test well was emptied, and 100 μL of the solution was added in a series created by diluting the media. Each series of concentrations, whether cancer cells or normal cells, was repeated three times. The test was also conducted on doxorubicin as a positive control and then incubated for 24 h in an incubator with a 5% CO_2 flow at 37 °C. After incubation, the media sample was removed and 0.5 mg/mL of MTT solution was prepared in a 10 ml media culture with 100 μL added to each well, including control media (without cells). They were incubated again for 4 h until a formazan (purple) color appeared. It was then observed under an inverted microscope at 20× magnification, and a 100 μL stopper SDS 10% was added. The 96-well plates were wrapped in paper and incubated in the dark at room temperature overnight. After that, those plates were put into the Elisa Reader to be measured by the absorbance at 595 nm and the half-maximal inhibitory concentration (IC_{50}) value was calculated. The percentage of cell inhibition obtained from each sample concentration was calculated using the following Eq. (1):

$$\% \text{ inhibition} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \quad (1)$$

The selectivity index was obtained from the IC_{50} ratio of Vero cells compared to the IC_{50} of MCF-7 cancer cells. The selectivity index was calculated using the following Eq. (2):

$$\text{Selectivity index} = \frac{IC_{50}\text{Vero cell}}{IC_{50}\text{MCF}-7\text{ cell}} \quad (2)$$

Identification of anticancer peptides

An Acclaim® PepMap RSLC column was used for HRMS analysis of the active anticancer fraction (C_{18}, 75 m × 150 cm). The mobile phase was divided into two categories: mobile phase A containing water and 0.05% trifluoroacetic acid (TFA), and mobile phase B containing water, acetonitrile 20:80, and 0.1% TFA. Then, a gradient was applied using both mobile phases at a flow rate of 0.1 mL/min. By using full MS/ddMS^2 mode and *m/z* range of 150–2250, peptides were examined by MS/MS. Split power was set to 140,000 (FWHM) for the complete MS parameter, and the resolution was set to 17,500 for the ddMS^2 parameter (FWHM). The MS data were examined by utilizing Proteome Discoverer Software ver. 2.5 using the *N. naja* genome downloaded from UniProt.org.

The peptide's molecular docking with EGFR

Peptides from the active fraction were docked with the EGFR receptor to examine the interaction of bioactive peptides with the EGFR receptor. The EGFR receptor was downloaded from the RCSB Protein Data Bank (PDB ID: 1M17). Peptide structures were drawn in a Gaussian view and semiempirically optimized with Gaussian. AutoDock Vina was employed to simulate docking, and Discovery Studio was used to visualize the results. AutoDock Vina has up to twice the speed compared to AutoDock 4. In addition to increased speed, AutoDock Vina has increased accuracy and significantly more predictable binding modes than AutoDock 4. Then, the value with the lowest binding affinity was chosen.

■ RESULTS AND DISCUSSION

Proteomic of *N. sumatrana* Venom

Analyses of proteomics were performed using HRMS. The results showed that there were 14 identified proteins, consisting of enzymatic and non-enzymatic proteins, in *N. sumatrana* venom originating from Sumatra, which is presented in Table 1. The main protein

Table 1. *N. sumatrana* enzymatic and non-enzymatic protein identified in proteome analysis

No.	Accession number	Protein	Length (Amino acid residues)	MW (kDa)	Calc. pI
		Enzymatic protein			
1	Q9PVK7	Zinc metalloproteinase-disintegrin-like cobrin	600	67.6	6.3
2	A4FS04	Acidic phospholipase A2 natratoxin	119	13.2	5.1
3	D3TTC2	Zinc metalloproteinase disintegrin-like atragin	613	69.1	6.4
4	V8P395	Glutathione peroxidase (Fragment)	264	29.6	8.1
5	A0A2I4HXH5	Snake venom 5'-nucleotidase (Fragment)	529	58.2	7.3
6	A8QL58	L-amino-acid oxidase (Fragment)	507	57.9	8.5
7	Q10749	Snake venom metalloproteinase-disintegrin-like mocarhagin	609	68.1	6.7
8	A0A2D0TC04	Venom phosphodiesterase	830	94.6	7.8
		Non-enzymatic protein			
9	P60309	Cytotoxin SP15d	60	6.6	9.4
10	P60308	Cytotoxin SP15c	60	6.8	9.1
11	P82885	Thaicobrin	108	12.0	9.1
12	P60306	Cytotoxin SP13b	60	6.8	9.2
13	P01140	Venom nerve growth factor	116	13.0	6.1
14	Q91132	Cobra venom factor	1642	184.4	6.4

families of *N. sumatrana* were detected, such as phospholipase A_2 (PLA_2) and three-finger toxins (3FTxs). Previous studies also reported that the two main categories of *N. sumatrana* proteins identified by LC-MS were phospholipase A_2 and three-finger toxins (5 neurotoxins and 9 cardiotoxins, or cytotoxins). In addition, proteins such as thaicobrin, aminopeptidase, zinc metalloproteinase-disintegrin (cobrin), CRISP, cobra venom factor, cobra serum albumin, and natriuretic peptide were also identified [21]. *N. sumatrana* venom from four different regions, namely Penang, Negeri Sembilan, Southern Thailand, and Sumatra, showed that the three-finger toxins (3FTxs) components followed by phospholipase A_2 (PLA_2) were the main proteins present in all *N. sumatrana* venoms. Seven protein families were consistently identified from the four regions, including three-finger toxins (3FTX), phospholipase A_2 (PLA_2), phosphodiesterase (PDE), L-amino acid oxidase (LAAO), venom nerve growth factor (vNGF), snake venom metalloproteinase (SVMP), and cobra venom factor (CVF) [22].

Components of snake venom, such as PLA_2, are enzymatic proteins that have various pharmacological activities and are usually present in snake venom [23]. The PLA_2 enzymatic protein detected in *N. sumatrana* venom is diverse and contains acidic PLA_2 in almost every cobra [24]. Cytotoxin SP15d, cytotoxin SP15c, and cytotoxin SP13b consist of 60 amino acids with molecular weights of 6.6, 6.8 and 6.8 kDa, respectively, and isoelectric points of 9.45, 9.14 and 9.29, respectively. Cytotoxin SP15d mostly shows cytolytic activity in various cells and functions to bind heparin with high affinity and create pores in the lipid membrane of organisms [25]. Cytotoxin SP13b and SP15c have their isoelectric points in the hydrophobic zone. Cytotoxin SP13b is an S-type cytotoxin that exhibits a high isoelectric point so that this protein can penetrate the membrane [26]. Cobra cytotoxins (CTX) can also be known as cardiotoxins, which are generally present in the cobra venom of *Naja* spp. [27]. This type of venom is involved in the pathogenesis of cytotoxicity and tissue necrosis [28]. Thaicobrin consists of 108 amino acids, has a molecular weight of 12 kDa, and an isoelectric point of 9.11. Venom phosphodiesterase consists of 830 amino acids with a molecular weight of 94.6 kDa and an isoelectric point value of 7.8. L-amino oxidase consists of 507 amino acids with a molecular weight of 57.9 kDa, has a relatively high isoelectric point of 8.5, and exhibits hydrophobic properties. The minor protein components identified in the *N. sumatrana* venom, such as SVMP, CVF, vNGF, PDE, and SVSP, generally have high molecular weights. These components play a role in the inflammatory response as well as facilitate the spread of venom during venomous snake bites [29].

Hydrolysis of Venom Protein

Bioactive peptides are obtained by hydrolyzing them from protein sources. The enzymatic hydrolysis method is easy to carry out, controllable and can maintain the amino acid structure. In this study, *N. sumatrana* venom protein was hydrolyzed with the trypsin enzyme to obtain bioactive peptides. The enzyme hydrolyzes the peptide bonds on the carboxylic side of lysine and arginine [30]. In the trypsin enzyme, there is a negatively charged aspartic acid catalytic bag that is responsible for binding the basic amino acids with a positive charge [31]. This enzyme works optimally in the pH range of 7.5–8.5 and at a temperature of 37 °C. Combinations such as temperature conditions, type of enzyme, pH, and concentration of protein substrates are needed for enzyme activity to maximize the degree of hydrolysis [32]. The degree of hydrolysis is the ratio of the number of peptides cleaved during hydrolysis to the total number of peptide bonds in the protein mass [33]. In the current study, the degree of hydrolysis was 74.7%. In previous research [34], trypsin was utilized to hydrolyze Jatropha seed protein and achieved a degree of hydrolysis of 82.1%. Protein hydrolysis with trypsin can hydrolyze up to 65.9% of proteins [35]. Protein from epiphytic bacteria associated with the brown algae *Sargassum* sp. was successfully hydrolyzed with the trypsin enzyme, resulting in a degree of hydrolysis of 27% in 9 h [36]. Soy protein was hydrolyzed using trypsin, resulting in a degree of hydrolysis of 20.4% [37]. The high percentage of hydrolysis value indicates that the hydrolysis is going well.

Anticancer Activity

A cytotoxicity test of venom protein, protein hydrolysate, and anticancer peptide fraction was performed on MCF-7 breast cancer cells and normal cells (Vero) with IC_{50} as a parameter. The smaller the IC_{50} value, the higher the potential of the test compound. Tests on Vero cells were conducted to determine the selectivity index value of the samples tested. The anticancer activity was tested using the MTT assay, where the reduction of tetrazolium salt to formazan produced a purple color. The more purple formazan crystals formed indicate that the cells are alive. The IC_{50} values of venom protein, protein hydrolysate, and peptide fractions are presented in Table 2. The venom protein produced a smaller IC_{50} value for MCF-7 cells or Vero cells compared to that of protein hydrolysate and had a selectivity index of 9.12. The selectivity index was calculated by dividing the IC_{50} value of normal cells by the IC_{50} value of MCF-7 cancer cells. If the selectivity index is higher than 2, this sample can be used as an anticancer agent [38]. The greater selectivity index, the higher the selectivity. Samples with high selectivity can kill cancer cells but are safe for normal cells.

Based on the results of the four fractions, the 75% methanol fraction produced a relatively smaller IC_{50} value with a selectivity index of 5.00 compared to the other fractions Table 2. This proves that the 75% methanol fraction has high selectivity. BPs can inhibit the active site of the MCF-7 cancer cell line and cause anticancer activity [39]. Crude venom of *N. sumatrana* showed concentration-dependent cytotoxic activity against lung cancer cells (A549), prostate cancer cells (PC-3), and MCF-7 cancer cells with an IC_{50} value of 0.88 ± 0.06, 3.13 ± 0.58, and 9.10 ± 0.56 μg/mL, respectively. The highest selectivity was produced by lung cancer cells (A549), which was 2.17 [28]. Even though the anticancer activity of *N. sumatrana* venom has not been widely reported, proteomics of *N. sumatrana* venom has identified anticancer proteins such as PLA_2, neurotoxin, and cardiotoxin. A previous study has found that phospholipase A_2 (PLA_2) purified from *Daboia russelii siamensis* venom contains anticancer activity [40]. However, peptides derived from the hydrolysis of *N. sumatrana* trypsin venom as anticancer agents have never been studied before. The current study is the first to conduct this topic.

The appearance of purple formazan crystals could be seen after the addition of MTT to control MCF-7 cells (Fig. 1(a)) and control Vero cells (Fig. 1(c)). Both control cells formed a lot of formazan crystals, which indicates that many cells were alive. The concentration determines the amount of purple formazan crystals formed. This study used a variety of concentrations: 7.8125; 15.625; 31.25; 62.5; 125; 250; and 500 μg/mL. The greater the concentration is given, the smaller of cell viability percentage and the more cells die (no purple formazan is formed). Meanwhile, adding 75% methanol

Table 2. Anticancer activity of venom, hydrolyzed venom protein, and the peptide fractions

Sample	Toxicity		Selectivity index
	Cell line	IC_{50} (μg/mL)	
Venom protein	MCF-7	6.16	9.12
	Vero	56.23	
Protein hydrolysate	MCF-7	398.10	2.29
	Vero	912.01	
Fraction of 25% methanol	MCF-7	20.42	2.60
	Vero	52.50	
Fraction of 50% methanol	MCF-7	16.10	1.16
	Vero	18.60	
Fraction of 75% methanol	MCF-7	4.80	5.00
	Vero	24.00	
Fraction of 100% methanol	MCF-7	12.02	0.46
	Vero	5.50	

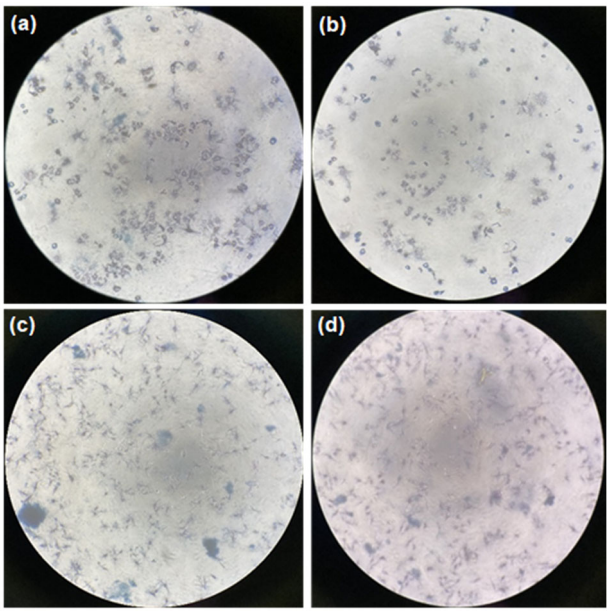

Fig 1. Microscopic images of untreated MCF-7 cell (a), MCF-7 cell treated with 75% methanol fraction (b), untreated Vero cell (c) and Vero cell treated with 75% methanol fraction (d)

fraction to MCF-7 cells (Fig. 1(b)) caused fewer purple-colored formazan crystals to form, implying that many cells died when treated with the active fraction. When 75% methanol fraction was added to Vero cells (Fig. 1(d)), the number of purple formazan crystals was not much different from that of the control Vero cells. Thus, it can be said that the administration of the active fraction did not kill all the Vero cells.

Identification of Anticancer Peptides

HRMS was employed to identify the peptide sequence of the active fraction as an anticancer. Using HRMS for peptide identification has been successful in determining the active fraction of peptides [34-35,41]. The *N. sumatrana* database is not yet available in the transcriptome database with Proteome Discover version 2.5, which includes the Sequest and Mascot search algorithms. The peptide sequence of the active fraction consisting of four peptides is presented in Table 3.

According to the database of peptides based on the genome of *Ophiophagus hannah*, the NSLLVK peptide was identified, and it was eluted at a retention time of 7.0383 min. The result obtained $[M-H]^+$ = 673.42290 Da, with a z = + 2, MS^2 fragmentation with mono *m/z* = 337.21509 Da. Its GRAVY Score was 0.600, and the pI was 8.75. It was lysed from protein-tyrosine-phosphatase, EC 3.1.3.48, at a location of 562–567 amino acids. The mass spectra of the NSLLVK are displayed in Fig. 2(a).

Ion y_1^+ = 147.11237 confirmed the presence of K amino acids at the end of the C-terminal peptide. The mass difference between the y_2^+ ion (246.18051) and the y_1^+ ion confirmed the presence of V (99.06814), leading to a sequence of VK at the C-terminus. Sequentially, the difference in mass between y_3^+ (359.26419) and y_2^+ ions was 113.08368 (L), and between y_4^+ (472.34811) and y_3^+ ions was 113.08392 (L). In addition, y_4^{2+} ions = 236.67761 was also confirmed. Meanwhile, the difference in mass between y_5^+ (559.37933) and ions y_4^+ was 87.03122 (S), thereby confirming the SLLVLK amino acid sequence. The mass of the b_1^+ ion was 115.05013, confirming the presence of N amino acids. The mass difference between

Table 3. Peptide sequence identified from anticancer active peptide fractions

No.	Peptide sequences	$[M-H]^+$ Da	The protein of the peptides origin*	Amino acid position in the protein	GRAVY	Predicted pI
1	NSLLVK	673.42229	Protein tyrosine phosphatase, EC 3.1.3.48, putative	562–567	0.600	8.75
2	SSLLVK	646.41240	Methyl cytosine dioxygenase TET, EC 1.14.11.n2, putative	1229–1234	1.050	8.47
3	TVPVKR	699.45096	Cytotoxin 2, CX2 (Toxin CM-7A), putative	31–36	−0.383	11.00
4	MFMVSNK	872.39893	Cytotoxin 3, CX3 (Toxin CM-7), putative	24–30	0.371	8.50

*) based on *Ophiophagus hannah* and *Naja siamensis* protein database

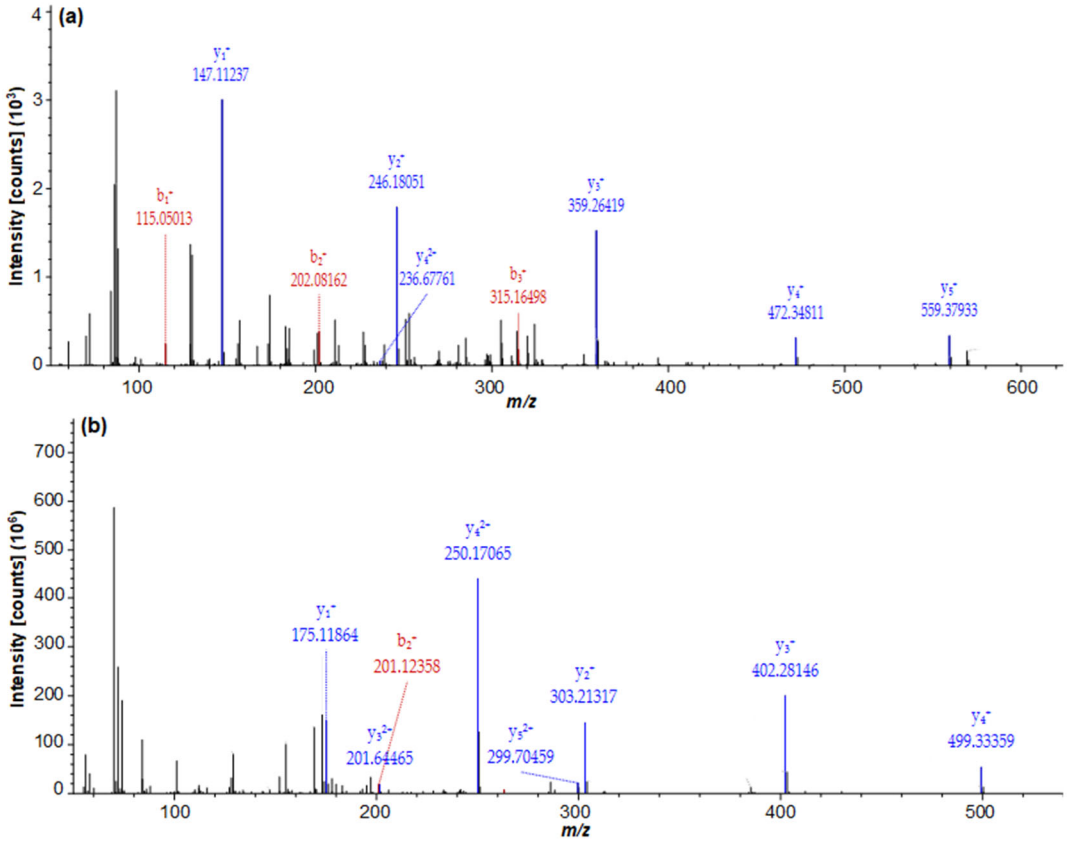

Fig 2. The MS/MS spectra of NSLLVK peptide (a) and TVPVKR peptide (b)

the b_2^+ (202.08162) and b_1^+ ions confirmed the presence of S (87.03149), and the mass difference between the b_3^+ (315.16498) and b_2^+ ions confirmed the presence of L (113.08336).

The TVPVKR was eluted at a retention time of 1.4765 min and this peptide was identified from the peptide database based on the genome of *Naja siamensis*. MS^2 fragmentation of TVPVKR was detected with *m/z* value of 233.82184 Da. It obtained $[M-H]^+$ = 699.45096 Da and z = + 3. Its GRAVY score was –0.383, while the pI value recorded was 11.00. It was isolated from one of the *N. sumatrana* venom proteins Cytotoxin 2, CX2 (Toxin CM-7A), at the location of 31-36 amino acids. The mass spectra of the TVPVKR are displayed in Fig. 2(b). The y_1^+ ion was formed due to the cleavage of the C-N bond between lysine (K) at the N-terminus with the arginine (R). The mass difference between the y_2^+ (303.21317) and y_1^+ ions confirmed the presence of K (128.09453), which leads to a KR sequence at the C-terminus. Sequentially, the difference in mass between y_3^+ (402.28146) and y_2^+ ions was 99.06829 (V), and between y_4^+ (499.33359) and y_3^+ ions was 97.05213 (P). Moreover, y_3^{2+} ions (201.64465), y_4^{2+} ions (250.17065), and y_5^{2+} ions (299.70459) were also confirmed, thereby confirming the PVKR amino acid sequence. In addition, Ion b_2^+ (201.12358) confirmed the presence of amino acid V.

Study of Molecular Docking

Molecular docking studies are useful for drug discovery and development. Similar significant advances have been made in the field of peptide therapy [42]. Molecular docking was used to determine the interaction between peptides and receptors in breast cancer anticancer peptides produced from the active fraction. The receptor used as EGFR, which was overexpressed in nearly half of triple-negative and inflammatory breast cancer patients. The EGFR protein was overexpressed by 16–36% in breast cancer [43]. The binding affinity and interactions of amino acid residues (hydrogen bonds and hydrophobic interactions) with

the ligand and the active site are important parameters in determining the stability of the protein-ligand. Both parameters have a significant impact on predicting the mode of protein-ligand interaction. The initial step was redocking between the native ligand (erlotinib) and the EGFR receptor to avoid blind docking and study the receptor interaction of the original ligand. Thus, data concerning the active site and RMSD value will be obtained. The original ligand interactions can be seen in Fig. 3.

The native ligand (erlotinib) formed conventional hydrogen bonds, namely the Cys773 amino acid residue. In addition, three cations consisting of Met742, Lys721, and Asp831 formed hydrophobic interactions, similar to the three alkyl interactions at the amino acid residues of Leu821, Leu764, and Val702. Some of the electrostatic interactions found were Glu738, Thr830, Phe699, Asp776, Phe771, Gly772, Leu694, Thr766, Met769, Leu768, Ala719, and Gln767. After redocking, the peptide was docked to the EGFR receptor. The interactions of the peptide with the EGFR receptor are shown in Fig. 4. NSSLVK peptides produced a good bonding affinity of −6.7 kcal/mol with an RMSD value of 1.953. NSSLVK demonstrated the best-matched model to the EGFR-receptor breast cancer cell line. NSSLVK peptides exhibited conventional hydrogen bonding consisting of Cys773, Lys721, Glu738, and Cys751 amino acid residues. These hydrogen interactions were more numerous than those of the original ligand (erlotinib) when the redocking process made the NSSLVK peptide's interaction with the receptor more stable. The peptide has the same position as the original ligand, which was located on the Cys773 amino acid residue. This proves that NSSLVK interacts with the active site of the EGFR receptor. There were also several electrostatic bonds consisting of Arg17, Leu820, Met769, Leu694, Thr830, Ala719, Ile720, Thr766, Ile765, Leu764, Phe832, Val702, Asp827, Phe699, Gly695, Asn818, Phe771, and Gly772. Moreover, one hydrophobic interaction was also found, which was the pi-ionic Asp776 bond presented in Fig. 4.

The SSLLVK peptide formed hydrogen bonds with Cys773, Asp831, Asp776, and Arg817 amino acid residues, including the same Cys773 amino acid residues

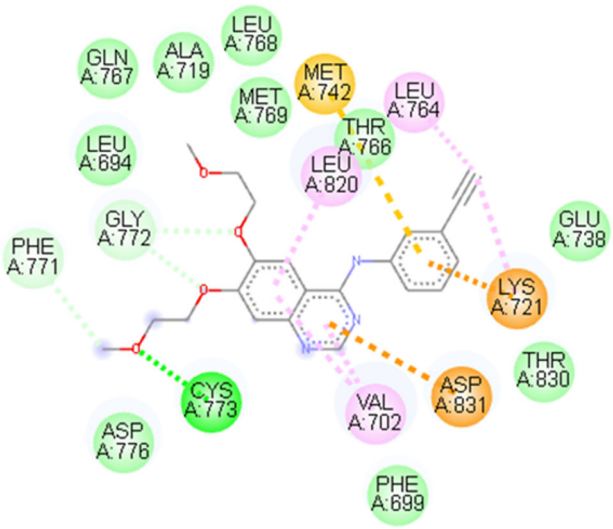

Fig 3. Interaction of the erlotinib (native ligand) to the EGFR

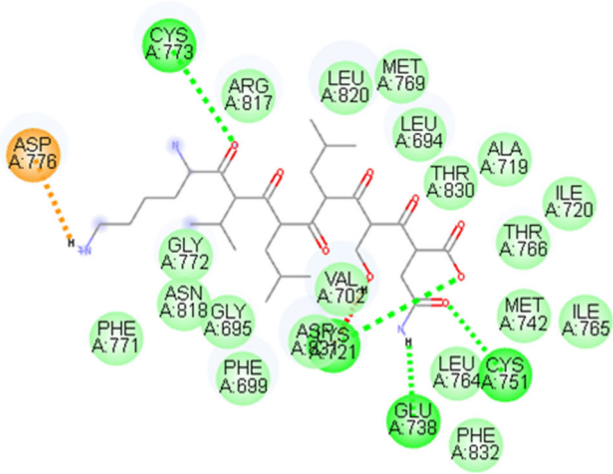

Fig 4. Interaction of NSSLVK peptide to the EGFR

as the original ligand. These hydrogen bonds were more numerous than those of the docking ligands. Therefore, they had a higher negative bond affinity value because more hydrogen bonds were formed with the receptor. The binding affinity value obtained was −6.65 kcal/mol, and the RMSD showed a value of 1.890 Å, confirming that the SSLLVK peptide has a stable interaction with the active site and is the most suitable model with the EGFR receptor. In addition, SSLLVK also had electrostatic interactions on the amino acid residues of Leu694, Leu820, Met769, Thr830, Asn818, Gly772, Gly695, Phe699, and Tyr766. Several other interactions were also present, i.e., the hydrophobic alkyl interactions at the

amino acid residue Ala719, Lys721 and Val702, which is shown in Fig. 5.

The affinity value of the TVPVKR peptide bond was −6.70 kcal/mol, and the RMSD score was 1.900 Å. The TVPVKR peptide exhibited hydrogen bonding with the receptor consisting of Cys773, Met769, and Gln767 amino acid residues, which were more than the redocking of the native ligand. Cys773 amino acid residues were similar to the native ligand, which proves that TVPVKR interacts with the same active site as the original ligand (erlotinib). In addition to hydrogen bonds, several electrostatic interactions were also shown by TVPVKR peptides, namely Phe699, Glu738, Thr766, Val702, Ala719, Leu768, Leu820, Asn818, Leu694, Gly772, Arg817, Asp776, and Glu780, which were fewer in number than those of the original ligands. Several hydrophobic interactions were also shown by the TVPVKR peptide, and there were three pi-ionic interactions found at Lys721, Asp831, and Asp813 residues. In addition, three alkyl interactions were present, including Tyr777, Phe771, and His781 (Fig. 6). Previous studies on the docking of peptides with AutoDock Vina from black soybean showed that the purified Leu/Ile-Val-Pro-Lys (L/I-VPK) peptide effectively binds to four proteins (XIAP, caspase-3, caspase-7, and Bcl-2) via hydrophobic effect and hydrogen bonding [44]. Peptides from the sea cucumber (*Cucumaria frondose*) were docked with four proteins (EGFR, PI3K, AKT1, and CDK4) using AutoDock Vina, indicating that the twelve peptides bind to the active sites

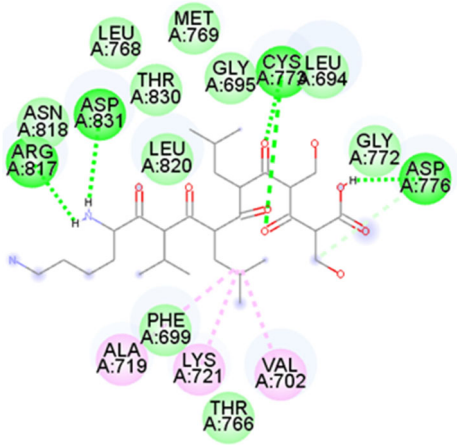

Fig 5. Interaction of SSLLVK peptide to the EGFR

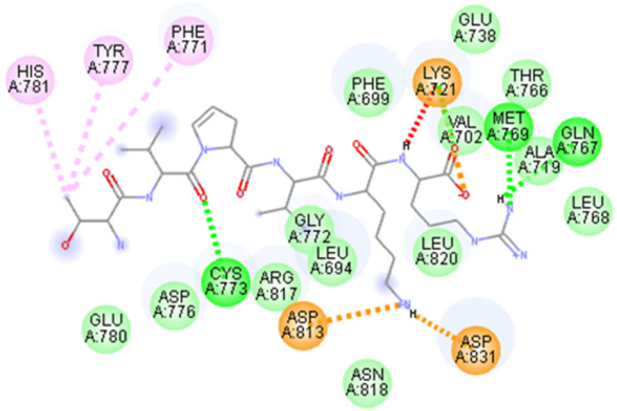

Fig 6. Interaction of TVPVKR peptide to the EGFR

of the four proteins. WPPNYQW and YDWRF peptides bind to proteins with lower binding affinity values than inhibitors (positive control) [45].

CONCLUSION

The trypsin enzyme successfully hydrolyzed *N. sumatrana* venom, resulting in a relatively high degree of hydrolysis. Hydrolysis of trypsin from venom followed by fractionation using an SPE C_{18} column showed that the 75% methanol fraction had anticancer activity against breast cancer (MCF-7) with a selectivity index of 5.00. The peptides identified by HRMS from the active fraction produced four peptide sequences, namely MFMVSNK, NSLLVK, SSLLVK, and TVPVKR. According to the docking study, the NSLLVK, SSLLVK, and TVPVK peptides possessed high binding affinity values and good RMSD values, making them the most suitable models for 1M17, the breast cancer EGFR receptor.

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