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Original article

Proteome of monocled cobra (*Naja kaouthia)* venom and potent anti breast cancer peptide from trypsin hydrolyzate of the venom protein

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ABSTRACT

Anticancer peptide is one of the target in the development of new anticancer drug. Bioactive peptide can be originated from isolated free peptide or produced by hydrolysis of protein. Protein is the main component of *Naja kaouthia* venom, and due to the toxicity of the venom, it can be assessed as the source of anticancer peptides. This study aims to characterize the venom protein and to identify peptides from the snake venom of *N. kaouthia* as anticancer. Proteome analysis was employed trypsin hydrolysis of *N. kaouthia* venom protein completed with HRMS analysis protein database query. Preparative tryptic hydrolysis of the protein followed by reverse-phased fractionation and anti breast cancer activity testing were performed to identify the potent anticancer from the hydrolysate. Proteomic analysis by high-resolution mass spectrometry revealed that there are 20 enzymatic and non-enzymatic proteins in *N. kaouthia* venom. The 25% methanol peptide fraction had the most active anticancer activity against MCF-7 breast cancer cells and showed promising selectivity (selectivity index = 12.87). Amino acid sequences of eight peptides were identified as potentially providing anticancer compounds. Molecular docking analysis showed that WWSDHR and IWDTIEK peptides gave specific interactions and better binding affinity energy with values of −9.3 kcal/mol and −8.4 kcal/mol, respectively. This study revealed peptides from the snake venom of *N. kaouthia* became a potent source of new anticancer agents.

1. Introduction

Cancer is a disease that occurs due to the uncontrolled growth of body tissue cells. Uncontrolled cell growth is caused by DNA damage as a result of mutations occurring in genes that control cell division due to carcinogens. Based on statistical data, cancer causes almost 10 million deaths each year, and there will be 19.3 million new cancer cases by 2020 globally (Sung et al., 2021). Breast cancer is the most common type of cancer in women worldwide. In 2018, the WHO reported that breast cancer was the second leading cause of death after lung cancer (Bray et al., 2018). More often than not, treatments for cancer, including chemotherapy, have side effects on the normal cells surrounding the cancer cells and are relatively expensive. In addition, cancer cells can develop resistance to chemotherapy (Chong et al., 2020). Cancer leads to a fairly high mortality rate because no drug can cure it completely. Therefore, it is necessary to develop new drugs that can be used as anticancer agents, and animals possessing venom can be explored for anticancer. Several studies have reported that venomous animals have antibacterial, anticancer, and antihypertensive activities (El-Seedi et al., 2020; Valizade et al., 2020; Stepensky, 2018).

Indonesia has a large diversity of snake species. The *N. kaouthia* (monocled cobra) is a very important and deadly species of venomous snake (category 1) (Das et al., 2016). The bites of venomous snakes are commonly lethal. Each year, 2.5 million people are bitten by venomous snakes, and more than 100,000 people die. Nonetheless, snake venom contains important therapeutic properties such as proteins, peptides, carbohydrates, and enzymes (Li et al., 2018). Venoms from snake species contain components such as phospholipase A_2 (PLA_2), cysteine-rich secretory protein (CRISP), vascular endothelial growth factor (VEGF), C-type lectin-like toxin (CTL), L-amino acid oxidase (LAAO), snake venom metalloproteinase (SVMP), snake venom serine protease (SVSP), and three-finger toxins (3FTXs) (Munawar, 2018; El-Aziz et al., 2019). Research that has been carried out on the snake venom of *N. kaouthia* includes venom protein as an anticancer agent and proteomic analysis. Snake venom and the cytotoxin *N. kaouthia* showed cytotoxic activity against lung cancer cells A549, prostate cancer cells (PC-3) and breast cancer cells MCF-7 (Chong

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et al.,2020). The proteomic analysis of *N. kaouthia* venoms identified six protein families, namely 3FTxs, PLA_2, NGF, SVMP, CRISP, and ohanin. Components of 3FTxs and PLA_2 were identified as the most abundant components in the proteomic venom (Xu et al., 2017). Analysis in the field of proteomics has developed significantly, providing a database that can clarify the composition of snake venom in detail (Chen et al., 2018).

Snake venom protein has potential in the development of new anticancer drugs. Anticancer bioactive peptides resulting from protein hydrolysis from *N. kaouthia* have not been thoroughly studied. The latest research on the therapy of cancer cells suggested the utilization of anticancer peptides (ACPs). They are small peptides consisting of 10–100 amino acid residues that contain toxic and selective properties for cancer cells. They provide several advantages, including those against various pathogens such as bacteria, fungi, and viruses. In addition, they also contain high specificity and selectivity against cancer cells, are small in size, are easily synthesized and modified, and have low toxicity, resulting in fewer side effects (Huang et al., 2021). Previous research has shown that ACPs have a variety of mechanisms of action, such as pore-forming peptides, cell-penetrating peptides, and tumor-targeting peptides (Boohaker et al., 2012). Bioactive peptides have electrostatic interactions between cell membranes and peptides that lead to cancer cells, which then lead to necrosis or apoptosis (Montoya et al., 2018). Most of the bioactive peptides are produced in protein and the enzymatic protein hydrolysis method is reliable and well-studied for producing them. Protein hydrolysis may increase biological activities such as antioxidants, antidiabetics, antihypertensive, antimicrobial, and anticancer (Akbarian et al., 2022). The physiological activity of bioactive peptides is influenced by the type, amount, sequence, and properties of amino acids (Daliri et al., 2017).

The hydrolysis process is carried out using enzymes. Protease enzymes are enzymes that can hydrolyze bioactive peptides by cutting peptide bonds. Protein cleavage with trypsin enzyme is in the right mass range for MS and provides an easy-to-understand peptide fragmentation spectrum. Peptides from camel milk hydrolysate hydrolyzed by trypsin are isolated and purified to produce three peptide sequences (RLDGQGRPRVWLGR, TPDNIDIWLGGIAEPQVKR, and VAYSDDGENWTEYRDQGAVEGK) which possess antioxidant activity (Wali et al., 2020). The hydrolyzed peptides were fractionated for easy identification. Peptide fractionation is a separation process to classify chemically similar peptides so that they are easy to analyze with MS (mass spectrometry) (Mostovenko et al., 2013). The reversed-phase SPE method can be used for peptide fractionation. The reversed-phase SPE separates peptides based on their polarity through hydrophobic interactions (Abdah et al., 2014). LC-HRMS is a mass spectrometry that has high sensitivity and selectivity. Atmawati et al. (2022) succeeded in identifying three antibacterial peptides from SPE fractionation of Jatropha seeds *(Ricinus communis)* by LC-HRMS. The results of the analysis with LC-HRMS can be seen in the amino acid sequence of the identified peptides (Atmawati et al., 2022). The identified peptides can be studied in silico. The use of bioinformatics and in silico is a complementary approach that can be applied to predict protein-peptide activity (Diharce et al., 2019). Molecular docking is an in silico method that can estimate the binding affinity between peptides and target molecules (Ferreira et al., 2015).

This study aims to characterize venom proteome *N. kaouthia* and identify anticancer peptides (ACP) produced from venom protein hydrolyzates against MCF-7 breast cancer cells, which were evaluated by determining IC_{50} and selectivity index (SI). The mechanism of action of bioactive peptides was determined through a docking process by determining the binding affinity and specific interactions of protein-peptide amino acids.

2. Materials and methods

2.1. Snake venom sample collection and protein extraction

Snake venom was collected from a private Zoo (Bhumi Merapi, Yogyakarta). The venom was lyophilized in a sterile flacon and stored in a refrigerator at 20 °C until use. *N. kaouthia* snake venom crystal was dissolved in 0.05 M ammonium bicarbonate solution. The venom solution was then filtered using Amicon® Ultra–15 Centrifugal Filter Devices MWCO 3000 Da and centrifuged at 5,000 g at room temperature. The venom protein was obtained as a concentrated residue, while the filtrate contained small molecules of the venom. The concentration of protein was determined by UV–Vis spectrophotometer.

2.2. Proteome analysis of N. kaouthia venom protein

The venom protein solution was mixed with trypsin sequence grade enzyme in 20:1 (w/w) ratio. The mixture was then incubated for 24 h at 37 °C followed by a hydrolysis stopping process by heating at 80 °C for 15 min. Supernatants were filtered with a 0.22 μm filter membrane and were transferred into injection vials for analysis using LC-HRMS. The MS raw data were processed using the Proteome Discoverer Software ver. 2.5. The database used was a genomic database of *N. naja*, which was downloaded from UniProt.org.

2.3. Hydrolysis of venom protein to produce peptide

Venom protein was hydrolyzed in the same way as proteome analysis using trypsin with different quantities and grades of the enzyme because it was intended for preparative purposes. The hydrolysate obtained was measured for absorbance using a UV–Vis spectrophotometer at a wavelength of 280 nm to determine the degree of hydrolysis. The degree of hydrolysis was calculated according to the formula (Nielsen et al., 2001):

$$\text{Degree, of, hydrolysis,}(\%) = [(\text{Total, protein, mass} - \text{Mass, of, remaining, protein})/(\text{Total, protein, mass})]$$

2.4. Fractionation protein hydrolysate

Fractionation was performed with a HyperSep Retain PEP Cartridge 1 mL column on the SPE chamber device equipped with manifold vacuum pump. The column was conditioned by adding 2 × 0.5 mL of 100% methanol and equilibrated with 2 × 0.5 mL of distilled water, which was aspirated with a manifold pump. The sample was slowly introduced into the column, pushed by a pump, and stopped when the sample level was just above the adsorbent. The column was washed with 2 × 0.5 mL of 5% methanol and then eluted with 3 × 0.5 mL of methanol with various concentrations of 25, 50, 75, and 100%. Each elution was collected into one fraction. The eluate obtained in each fraction was measured for absorbance at a wavelength of 280 nm using a UV–Vis spectrophotometer.

2.5. Cytotoxic bioassay

The activity of the preliminary anticancer test was conducted using a BSLT method that utilized *Artemia Salina*. The eggs of *Artemia salina* shrimp were incubated for about 48 h, then the larvae were separated from the eggs by pipetting into a test tube containing sterile seawater (Supomo et al., 2019). The peptide fractions with 125, 100, 75, 50, and 25 ppm concentrations were added into

96-well plates containing seawater and ten-tail shrimp larvae. It took 24 h of exposure to count the dead shrimp larvae. Test for each concentration was carried out three times and compared with controls. The toxicity data were obtained from the analysis of LC_{50} values, which were performed by Probit analysis (Asmi et al., 2019).

2.6. Anticancer assay

The cell lines MCF-7 and Vero were harvested in DMEM media and added 100 µL to each 96-well plate. To restore cell condition after harvest, MCF-7 cancer cells and Vero cells were incubated at 37 °C for 24 h in 5 % CO_2 flow. 100 µL of each fraction sample with various concentrations that had been prepared by diluting the media tests were also carried out on doxorubicin as a positive control on MCF-7 cancer cells and Vero cells. The 96-well plates were then incubated for 24 h in an incubator with 5 % CO_2 flow at 37 °C. After incubation, the media was removed and 100 µL of MTT solution (0.5 mg/mL in 10 mL of culture medium (DMEM) was added. Finally, 100 µL of 10 % SDS stopper in 0.1 M HCl was filled into each well. The plates were stored at room temperature overnight. An ELISA reader was used to record the absorbance at 595 nm. The percentage of cell inhibition from each sample concentration obtained was calculated using the following formula:

$$\%\text{Inhibition} = [(\text{Absorbance, of, Control-Absorbance, of, Sample})/(\text{Absorbance, of, Control})]$$

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 equation:

$$\text{Selectivity index} = [(IC_{50}\text{Vero, cell}/IC_{50}\text{MCF-7cell})]$$

2.7. Identification of peptides in the most effective anticancer fraction

The peptides of the most effective anticancer fraction were analyzed using LC-HRMS equipped with the Acclaim® PepMap RSLC column (C_{18}, 75 m × 150 cm). The mobile phase consisted of a mobile phase A containing water and 0.05% of TFA, and a mobile phase B containing water, acetonitrile 20:80, and 0.1% of TFA. The mobile phases A and B were then used with a gradient at a flow rate of 0.1 mL/minute. MS/MS peptide analysis used the *m/z* 150–2,250 range with full MS/ddMS^2 mode. The full MS parameter used was the resolving power that was set to 140,000 (FWHM), while the ddMS^2 parameter used was the resolution at 17,500 (FWHM). The MS raw data were processed using the Proteome Discoverer Software ver. 2.5. Peptide identification was performed by processing data using the Fusion Basic Sequest HT workflow and a basic consensus workflow. The algorithm used in peptide analysis was Sequest HT. The database used was a genomic database of *Naja naja*, which was downloaded from UniProt.org. Peptide identification was completed by comparing the research data with the database used (Raharjo et al., 2021).

2.8. Molecular docking of the peptides to EGFR

The 3D structures of peptides were designed using ChemBiodraw ultra which then was converted into 3D structures using ChemBiodraw 3D, and the optimization was carried out by employing the MM2 method (Kaur et al., 2017). The crystal structure of complex EGFR (PDB ID: 1 M17) with erlotinib was obtained from the RCSB Protein Data Bank (Erlista, 2022). The docking simulations were carried out with Autodock Vina. A cubic grid box of 40x40x40 size (x, y, z) with a spacing of 1 was created. Docking analysis gave the best result, with an RMSD value of 2. The most suitable conformation was selected from the lowest binding affinity.

3. Results and discussions

3.1. Protein content of the N. kaouthia venom

Protein was isolated from the venom employing gel filtration using Amicon ® Ultra –15 Centrifugal Filter Devices with molecular weight cut-off (MWCO) of 3,000 Da. It is common in protein isolation to claim a molecular weight above 3000 Da as proteins (Muro et al., 2013, Johnsen et al., 2016). The percentage of the protein content of *N. kaouthia* snake venom was 66.78%. The protein content of freeze-dried *N. kaouthia* snake venom determined by the Bradford test resulted in 66.9% of protein (Vejayan et al., 2010). Meanwhile, the venom proteins of *Bothrops asper, Bothrocophias myersi*, and *Crotalus durianssus* were measured by direct measurement with Nanodrop® 280 nm, yielding protein percentages of 6.18%, 97.40%, and 72.57%, respectively (Scovino et al. 2021). Snake venom typically included 70% to 90% of protein (Tu, 1977).

3.2. Proteins component of the venom identified from proteome analysis

Mass spectrometry is the most frequently used technique for proteomic study (Dutta et al., 2017; Chanda et al., 2018), especially high-resolution mass spectrometry (Zhou et al., 2017). Analysis of MS protein must be digested with trypsin since it provides high specificity (Giansanti et al., 2016). The higher number of proteins can be further identified by MS (Dau et al., 2020; Gupta et al., 2010; Laskay et al., 2013). Trypsin enzymes for the proteomic analysis utilized sequencing grade modified trypsin and technical grade trypsin. The proteomic technique can provide information on the protein composition of snake venom. The use of proteomic techniques can analyze as a whole so that it can provide useful data references in research (Cheng et al., 2017). Proteomic analysis using high-resolution mass spectrometry identified 20 proteins from the *N. kaouthia* venom protein. The proteins identified from venom protein were enzymatic protein group and non-enzymatic protein group as presented in Table 1. Previous studies showed that *N. kaouthia* venom contains various components such as three-finger toxins (3FTxs) and phospholipase A_2 (PLA_2), which are the most abundant protein families and minor proteins, including Kunitz-type serine inhibitors, waprin, l-amino acid oxidase (LAAO), cysteine-rich secretory proteins (CRISP), vespyrn, nerve growth factor (NGF), ohanin, and snake venom metallo-protease (SVMP), which were analyzed by high-resolution mass spectrometry (Das et al., 2016; Tan et al., 2017; Xu et al., 2017; Deka et al., 2019).

Based on the importance of the composition of the snake venom protein family, it is divided into five groups, namely the dominant protein family (PLA2, 3FTx, SVMP, and SVSP). Secondary protein family (KUN, LAAO, CTL, CRISP, NP, and DIS). Minor protein families consist of acetylcholinesterase, vascular endothelial growth factor, phosphodiesterase, phospholipase B, hyaluronidase, 50 nucleotidase, nerve growth factor, snake venom metalloprotease inhibitors, and vespryn/ohanin. Rare protein families and unique protein families (defensins, waglerins, maticotoxins, and cystatins) (Tasoulis and Isbister, 2017). The proteomic characterization of the venom of *N. mandalayensis* by mass spectrometry (ESI-IT-TOF/MS) identified venom components such as 3FTxs, which is the main component. SVMP is the most diverse group of enzymes containing components such as phospholipase A_2 (PLA_2), L-amino acid oxidase (LAAO), cysteine-rich venom protein (CRISP), 5-nucleotidase (5 N), and nerve growth factor (NGF). In addition, proteins such as phos-

Table 1
N. kaouthia enzymatic and non-enzymatic protein identified by proteomic analysis using HRMS.

No	Accession number	Protein	MW (kDa)
Enzymatic protein			
Phospholipase A_2 (PLA_2)			
1.	A4FS04	Acidic phospholipase A_2	13.2
Snake venom metalloprotease (SVMP)			
2.	D3TTC2	Zinc metalloproteinase-disintegrin-like atragin	69.1
3.	P82942	Hemorrhagic metalloproteinase- disintegrin-like kaouthiagin	44.5
4.	Q9PVK7	Zinc metalloproteinase-disintegrin-like cobrin	67.6
L-amino acid oxidase (LAAO)			
5.	A8QL58	L-amino-acid-oxidase	57.9
6.	V8P395	Glutathione peroxidase	29.6
Nucleotidase (NT)			
7.	A0A214HXH5	Snake venom 5′-nucleotidase	58.2
Phosphodiesterase (PDE)			
8.	A0A2D0TC04	Venom phosphodiesterase	94.6
Non-enzymatic protein			
Three finger toxins (3FTx)			
9.	P60304	Cytotoxin 1	9.0
10.	P01445	Cytotoxin 2	6.7
11.	P60302	Cytotoxin 3	9.0
12.	P07525	Cytotoxin 5	6.8
13.	P82463	Muscarinic toxin-like protein 2	7.3
14.	P82935	Trytophan-containing weak neurotoxin	9.9
Cysteine-rich secretory proteins (CRISP)			
15.	Q7ZZN8	Cysteine-rich venom protein natrin-2	26.2
16.	P84805	Cysteine-rich venom protein kaouthia-1	26.8
17.	P86543	Cysteine-rich venom protein	3.9
Cobra venom factor (CVF)			
18.	Q91132	Cobra venom factor	184.4
Nerve growth factor (NGF)			
19.	P01140	Venom nerve growth factor	13.0
Ohanin like proteins (OLP)			
20.	P82885	Thaicobrin	12.0

phodiesterase and the related enzyme NADH were also identified (Neto et al., 2021). *O. hannah* snake vemomic proteomics using ESI-LC-MS/MS resulted in 14 different protein types/families including three finger toxin (3FTxs), phospholipase A_2 (PLA_2), cysteine-rich secretory protein (CRISP), cobra venom factor, muscarinic toxin, L-amino acid oxidase (LAAO), hypothetical protein, low cysteine protein, phosphodiesterase, protease, Vespryn toxin, Kunitz, growth factor activator and others (coagulation factors, endonucleases, 5′-nucleotidase) (Danpaiboon et al., 2014).

Proteomic analysis using a combination of a shotgun, gel filtration chromatography, and tandem mass spectrometry in *C. rhodostoma* and *O. hannah* snakes produced 114 and 176 proteins, respectively. Both snake venoms contain components such as phospholipase A_2, flavin monoamine oxidase, phosphodiesterase, metalloproteinase snake venom, and serine protease toxin family (Kunalan et al., 2018). The proteins of the *N. kaouthia* venom ranged in masses from 3,9 to 184.4 kDa (Table 1). The composition of the adult and juvenile *N. kaouthia* snake protein found about 18 protein bands with sizes ranging from 4 to 100 kDa (Modahl et al., 2016). Based on the research by Danpaiboon et al. (2014), the protein from *O. hannah* venom had masses ranging from 10 to 130 kDa. Meanwhile, *N. naja oxiana* venom produced a range of mass between 5 to more than 116 kDa (Samianifard et al., 2021). Enzymatic proteins had a smaller percentage of coverage ranging from 4 to 16% compared to that non-enzymatic proteins which ranged from 7 to 67%. This indicates that the percentage of protein sequences covered by peptides from non-enzymatic proteins was higher. The greater the percentage, the more adequate the protein identity.

3.3. The reversed-phase peptide fraction of trypsin hydrolyzed venom protein

Since the production was preparative work, the use of sequence-grade trypsin was not efficient. However, the use of technical-grade trypsin could reduce the effectiveness of the hydrolysis process. The ratio of trypsin to the protein in this study was 1:20 (w/w) which is relatively high compared to the common ratio for proteomic analysis which is 1:40 (w/w) (Garikapati et al., 2022). A higher ratio of enzyme to protein is expected to produce a larger number of peptides. The degree of hydrolysis (DH) is usually used to determine the extent of protein hydrolysis. The degree of hydrolysis of the venom protein by technical grade trypsin was 77.5%. It shows that more than half of the total protein was successfully hydrolyzed by trypsin. Cow's milk whey hydrolyzed with trypsin obtained a hydrolysis degree of 9.5% (Duan et al., 2014), while jatropha seed protein hydrolyzed with trypsin resulted in 82.15% (Raharjo et al., 2021). The higher the enzyme concentration used, the higher the degree of hydrolysis and the more peptide bonds released (Ghosh et al., 2017; Salwanee et al., 2013).

Peptide fractionation was carried out using a reverse phase solid phase extraction (RP-SPE) column. In several investigations, SPE can be used for fractionation and has shown excellent results (Fauland et al., 2013). Therefore, the SPE fractionation of *N. kaouthia* snake venom protein hydrolyzate was carried out using the HyperSep Retain PEP column, and fractions were collected based on methanol concentrations varied from 25, 50, 75, and 100%. Methanol, which was less polar than water, was used to make different polarity variations in the solvent to elute the peptide. At 25% methanol concentration, peptides containing high polarity were eluted. Furthermore, by using 50, 75, and 100% methanol concentrations, the higher the methanol concentration used, the slower the peptide polarity would decrease. The total eluted peptide was 3000 μg. The mass of fractionated peptides is shown in Table 2.

Compared to other fractions, the 50% methanol fraction produced the most peptides, with a mass of 414.50 μg. The trypsin hydrolyzed peptides of the *N. kaouthia* venom were dominated by peptides with 50% methanol percentage. The mass of fractionated peptides shown in Table 2 exhibits that not all eluted peptides could be eluted by methanol solvent with several concentration variations. This was influenced by the protein hydrolysis process with the trypsin enzyme which lead to various number of amino acids that make up the peptide.

3.4. Anticancer activity of the peptide fraction

3.4.1. The toxicity of the venom protein and venom protein hydrolysate

The initial cytotoxic testing was performed by employing the Brine Shrimp Lethality Test (BSLT) which was an easy, simple, and quick method using shrimp larvae (*Artemia salina*) (Thangapandi and Pushpanathan, 2014). The toxicity was determined as LC_{50} of the sample to shrimp larvae. The LC_{50} value of venom protein and protein hydrolysates were 1.23 μg/mL and 398.10 μg/mL, respectively, each of which was categorized as very

Table 2
Mass of fractionated peptides from *N. kaouthia* venom protein hydrolysate.

Fractions	Total mass of peptides (μ g)
25% Methanol	361.15
50% Methanol	414.50
75% Methanol	307.83
100% Methanol	304.50
Total of peptides	1,387.98

toxic and toxic, respectively. A sample is classified as toxic if the LC_{50} value is 1000 μg/mL and is declared non-toxic if the LC_{50} value > 1000 μg/mL (Supomo et al., 2019). The smaller the LC_{50} value, the higher the toxicity of the test compound. Venom protein is categorized as very toxic while protein hydrolysate is categorized as toxic.

The result showed that the protein of *N. kaouthia* venom contained toxins such as three-finger toxin (3FTxs) and phospholipase A_2 (PLA_2). These toxins can damage *A. salina* cell membranes. Three-finger toxin (3FTxs) also exhibits cytolytic properties such as disruption of the bilayer membrane by forming pores on the cell surface (Hus et al., 2018). Protein hydrolysate is categorized as toxic so that the hydrolysate can be tested for further anticancer. The lower toxicity of the hydrolysate, compared to the protein, could be due to the disruption of some parts of the toxin protein caused by the hydrolysis. This lower toxicity is expected to increase the selectivity of the toxicity which is crucial in the development of anticancer.

3.4.2. Anticancer activity

Samples were tested against MCF-7 breast cancer cells and Vero cells obtained IC_{50} value (Table 3). Based on data from the United States National Cancer Institute (NCI), there are several categories of cytotoxicity in the anticancer sample such as IC_{50} 20 μg/mL which possesses high cytotoxicity, IC_{50} 21–200 μg/mL which contains moderate cytotoxicity, and IC_{50} 201–500 μg/mL (Tanamatayarat et al., 2003). However, a potential anticancer compound must also be selective. The selectivity of anticancer compounds can be measured by calculating the selectivity index by dividing the IC_{50} value of normal cells by the IC_{50} value of MCF-7 cancer cells. It was reported that compounds with selectivity index values higher than 6 have high selectivity, selectivity index values between 3 and 6 have moderate selectivity, and selectivity index values lower than 3 are non-selective (Amin et al., 2013). The results obtained showed the cytotoxic effect of some of the investigated methanol fractions on the MCF-7 cancer cell line in a concentration-dependent manner (Fig. 1, Fig. 2). The results showed that the 25% methanol fraction had the lowest percentage of viability and showed significant antitumor activity against MCF-7 and selective at the same time.

Ebrahim et al. (2016) reported that snake venom (SVT) from the Caspian Cobra *(Naja naja oxiana)* resulted in IC_{50} values for the cancer cell lines HepG2, MCF-7, and DU145 of 26.59, 28.85, and 21.17 μg /mL, respectively. SVT induces mitochondrial and caspase-3 dependent apoptosis in cancer cell lines. Toxin NN-32 showed cytotoxic activity against MCF-7 and MDA-MB-231 with IC_{50} values of 2.5 and 6.7 μg/mL, respectively (Attarde and Pandit, 2017). Previous studies reported that the crude venom *N. kaouthia* showed cytotoxic activity against lung cancer cells A549, prostate cancer cells (PC-3), and breast cancer cells MCF-7 with IC_{50} values for each of 1,22 ± 0,09 μg/mL, 4,46 ± 0,36 μg/mL, 12,23 ± 0,76 μg/mL. Only the A549 lung cancer cell line showed cytotoxins to have promising selectivity (selectivity index = 2.26) (Chong et al.,2020). Debnath et al. (2007) reported that the crude venom of *N. kaouthia* showed cytotoxic activity in U937 and K562 cells. Several factors affect the anticancer peptide activity, including the amino acid composition and physicochemical characteristics such as charge, aliphatic index, and amphipathic structural or confirmation form (E-Kobon et al., 2016). So, this is the first time in this study that a peptide from the trypsin hydrolysis of the snake venom of *N. kaouthia* was carried out as an anticancer.

As can be seen in Fig. 3, after the addition of MTT to MCF-7 control cells (Fig. 3a) and Vero control cells (Fig. 3d), many formazan crystals were formed so that many living cells appeared clustered, which was marked by the dark purple color. The administration of 25% methanol fraction to MCF-7 cells (Fig. 3b) showed that not many formazan crystals were formed so fewer cells survived than control MCF-7 cells, whereas in Vero cells (Fig. 3e) purple formazan crystals were formed, but there was space between cells and the cell density was reduced.

This suggests that the administration of the 25% methanol fraction did not kill all Vero cells, meaning that there was no significant difference between control Vero cells and the active fraction of living cells. The addition of doxorubicin to MCF-7 cells showed that fewer purple formazan crystals were formed compared to control MCF-7 cells (Fig. 3c). Thus, it can be concluded that after the addition of doxorubicin, many cells died. Meanwhile, in Vero cells treated with doxorubicin (Fig. 3f), purple formazan crystals were formed but only small in number, and there were spaces between cells compared to control Vero cells. This indicates that doxorubicin did not kill all Vero cells.

3.5. Identification of the peptide fraction against anticancer activity by HRMS

Peptide identification was carried out using high-resolution mass spectrometry (HRMS), which was connected to the Proteome Discoverer version 2.5 with the *N. naja* database obtained from Uniprott. Table 4 summarizes the identified peptides from the active anticancer fraction.

One example of the use of HRMS data in identifying peptide sequences is displayed in Fig. 4. The figure exhibits MS/MS spectra of parent ion of $(MH)^+$= 904.47692 Da, which corresponds to the mass of the IWDTIEK peptide. The IWDTIEK peptide was eluted at 13.1321 min with an *m/z* value of 452.74210 Da and (z = +2). In the confirmed MS^2 spectrum, b_2^+, y_1^+, y_2+, y_2^{2+}, y_3^+, y_4^+, y_5^+, y_6^+, and y_6^{2+} were obtained. The b ion is the result of cutting the peptide bond from the N-terminal end, while the y ion is the result of cutting the peptide bond from the C-terminal end. The b_2^+ fragment ion represents the W amino acid (Trp); the y_1^+ fragment ion represents the K amino acid (Lys) which lost the NH_3 group so that it had a fragment ion mass of 130.08640; the y_2^+ fragment ion represents the E amino acid (Glu); the y_2^{2+} ion represents glutamate amino acid, which lost the NH_3 group with a larger charge of 2+ and produced a fragment ion value of 130.08640; the y_3^+ ion represents amino acid I (Ile); the y_4^+ ion represents T amino acid (Thr); y_5^+ ion value was 130.08640; the y_3^+ ion value was 130.08640; the y_3^+ ion represents the amino acid D (Asp); y_6^+ ion represents the amino acid W (Trp); and y_6^{2+} ion represents the amino acid Tryptophan, which lost the NH_3 group with a fragment ion value of 387.68649.

The dominant amino acids in peptides with anticancer abilities are glycine, lysine, and leucine (Shoombuatong et al., 2018). Peptides containing positive hydrophobic amino acids lysine and argi-

Table 3
Anticancer activity of venom, hydrolyzed venom protein, and peptide fractions.

Sample	Toxicity		Selectivity index
	Cell line	IC_{50} (μ g/mL)	
Venom protein	MCF-7	3.02	66.05
	Vero	199.50	
Protein hydrolysate	MCF-7	199.53	2.08
	Vero	416.87	
Fraction 25% methanol	MCF-7	4.17	12.87
	Vero	53.70	
Fraction 50% methanol	MCF-7	1.66	1.47
	Vero	2.45	
Fraction 75% methanol	MCF-7	1.02	0.14
	Vero	1.17	
Fraction 100% methanol	MCF-7	1.05	0.97
	Vero	1.02	
Doxorubicin	MCF-7	7.59	6.75
	Vero	51.29	

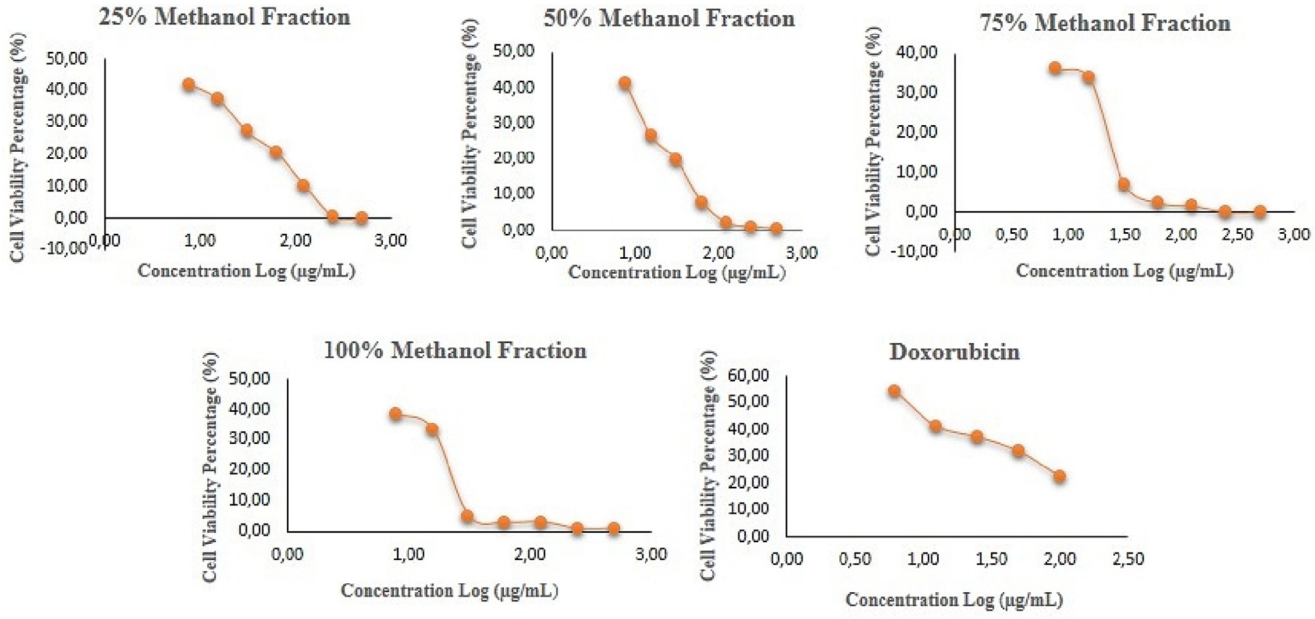

Fig.1. Cytotoxicity of methanol fractions and doxorubicin on MCF-7 cancer cell.

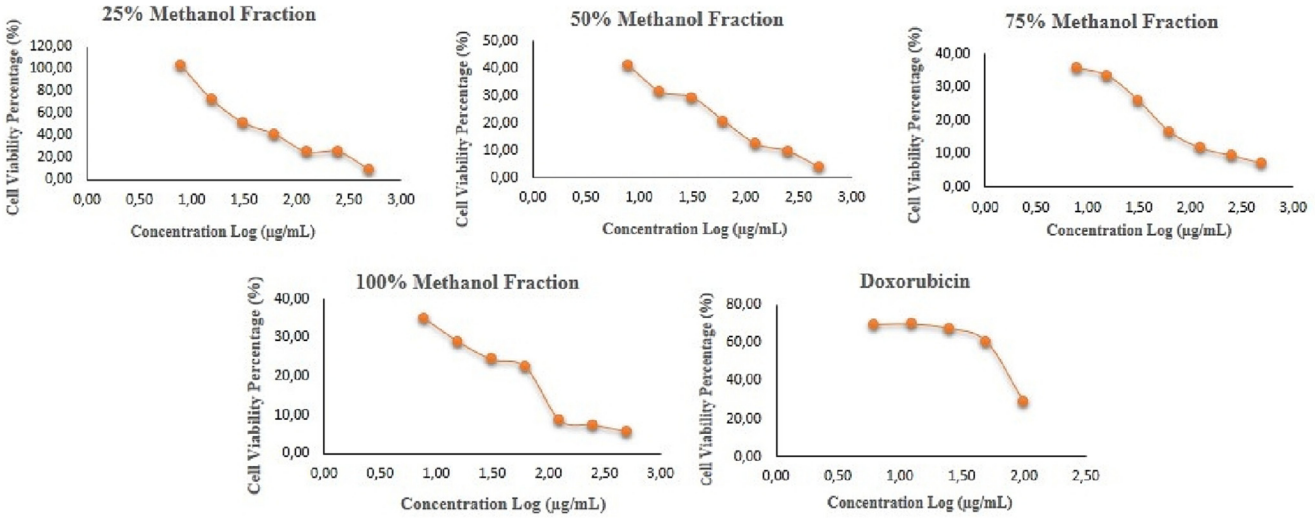

Fig.2. Cytotoxicity of methanol fractions and doxorubicin on normal cell (Vero cell).

nine may function as cationic peptides and interact with cancer cell membranes, disrupt cell membrane integrity, penetrate membranes, and potentially cause cancer cell toxicity. Peptides containing the amino acid histidine may induce cancer cytotoxicity through membrane permeability under acidic conditions. The combination of glutamic acid and aspartic acid amino acid residues has antiproliferative activity, suggesting that it may be effective against tumor cells (Yamaguchi et al., 2016). Peptides containing amino acid residues in the form of tyrosine, phenylalanine, or proline can increase cytotoxic activity (Ahmaditaba et al., 2017). Anticancer peptides must contain cationic and hydrophobic amino acid residues, which can further form secondary structures that affect cancer cells. The peptide sequences obtained (Table 4) show variations in the number of amino acids consisting of 6–13 amino acids. This value can be categorized as short-chain peptides (composed of 2–20 amino acids). Short-chain peptides are known to have high bioactivity (Priya, 2019). Anticancer activity has been reported for lunasin (a peptide found in soybeans), RLQLQGVN, GLTSL, LSGNL, GEGSGA, MPACGSS, and the peptide MTEEY (Fernandez-Tome et al., 2017). The venom of a spider (*Lachesana tarabaevi*) contains a short cationic linear α-helical peptide called latarcin 2a (Ltc2a), which is cytotoxic to human erythroleukemia K562 cells (Vorontsova et al., 2011). The amino acid sequence of the pure peptide SCAP1 (Leu-Ala-Asn-Ala-Lys) isolated from oyster protein hydrolyzate (*Saccostrea cucullata*) contains anticancer activity against the human colon carcinoma cell lines (HT-29) (Umayaparvathi et al., 2014).

3.6. Docking study of the peptide to EGFR protein

EGFR receptors are involved in apoptosis, proliferation, angiogenesis, invasion, and metastasis in the development of cancer cells (Baselga 2002). One of the mechanisms of action of anticancer peptides is that they can inhibit the migration of breast cancer cells by interfering with the interaction of CD151 and EGFR signaling (Deepak et al., 2020). The EGFR receptor is a well-known target

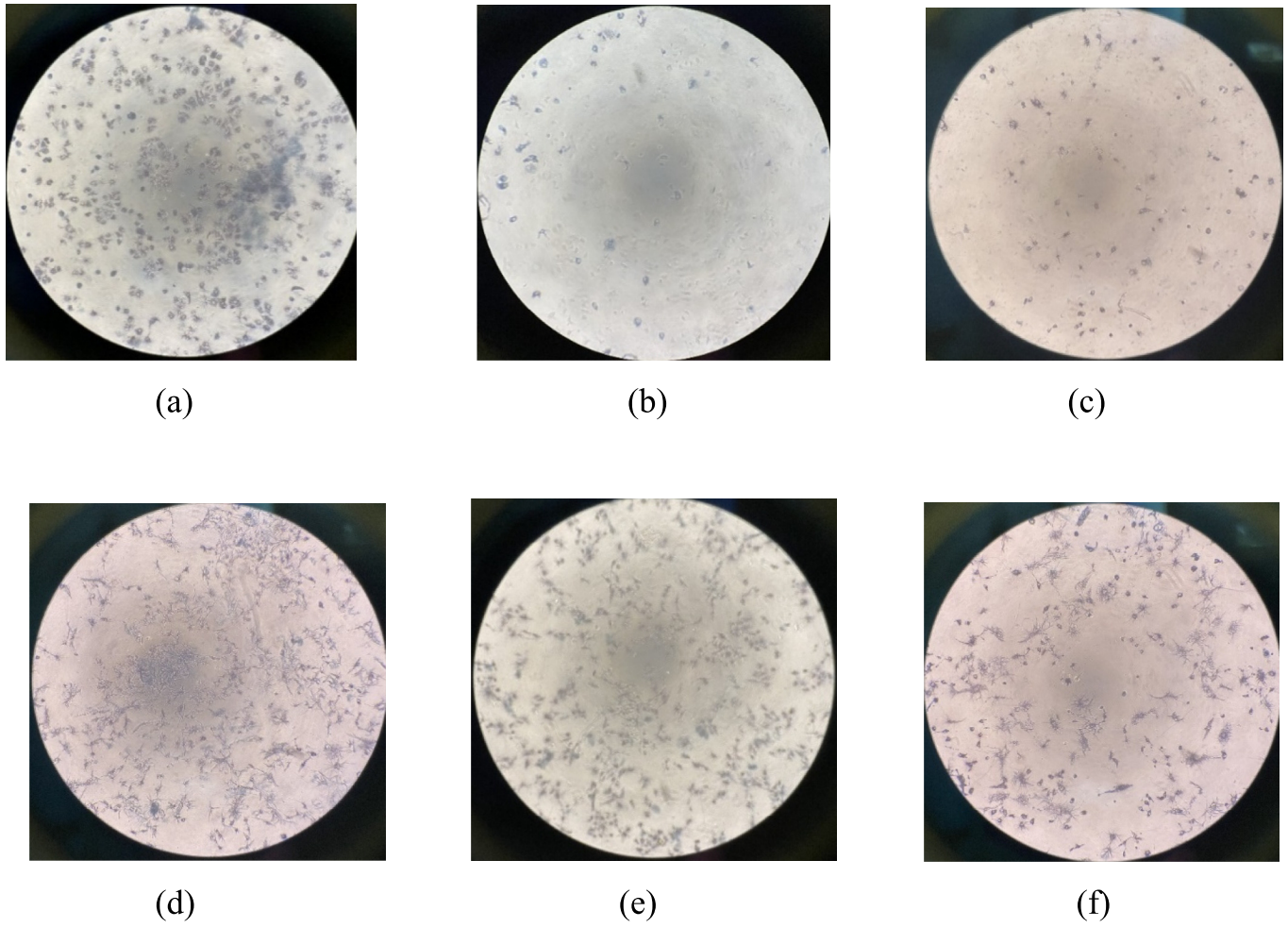

Fig. 3. Microscopic image of untreated MCF-7 cell (a), MCF-7 cell treated with the 25% methanol peptide fraction (b), MCF-7 cell treated withdoxorubicin (c), untreated Vero cell (d), Vero cell treated with 25% methanol peptide fraction (e), and Vero cell treated with doxorubicin.

Table 4
Peptide sequence identified from anticancer active petpide fraction.

No.	Peptide sequences	Theoretical $[MH]^+$ (Da)	Observed $[MH]^+$(Da)	The protein of peptide origin
1.	GDNLIQMPGAAMK	1345.66031	1345.65727	Cobra venom factor
2.	NSLLVK	673.42434	673.42327	Cytotoxin 2
3.	SSLLVK	646.41345	646.41179	Cytotoxin 1
4.	GGSGTPVDDLDR	1188.54916	1188.54717	Acidic phospholipase A_2
5	GVGGTQLEVIK	1100.63104	1100.62993	Cobra venom factor
6	IWDTIEK	904.47750	904.47692	Cobra venom factor
7	WWSDHR	886.39550	886.39450	Cobrotoxin-b
8	MFMVSNK	856.40560	872.39911	Cytotoxin 2

of antitumor agents. Overexpressed EGFR may cause malignancy in breast cancer cells (Song et al., 2020). The anticancer mechanism can be demonstrated by docking the molecule with the EGFR protein. Through the use of AutoDock Vina, docking investigations were conducted to determine the binding mode of peptide compounds. Redocking was performed first before docking the receptor protein with the proposed compound (peptide). The erlotinib ligand anchored into EGFR protein (PDB ID: IM17) showed the lowest binding energy of −7.3 kcal/mol and the best RMSD value of 1.53 Å. Those outcomes revealed that AutoDock was reliable under testing conditions since the RMSD values were less than 2. The binding site of the erlotinib ligand to the EGFR protein was at the amino acid Gln767 acid residue. The active binding site is a macromolecular region where the ligand binds and can inhibit disease (Sharma et al., 2021). Hydrogen interactions that occur in the erlotinib ligand with the EGFR protein are hydrogen bonds in Met769, Gln767, and Thr830 (Abourehab et al., 2021). Lys721 at the binding site EGFR is considered one of the key residues to determine the biological activity of EGFR. Molecular docking peptides with EGFR proteins are arranged at the same position from the redocking analysis. The peptides with the lowest binding energy were WWSDHR and IWDTIEK, with the lowest binding affinity energies of −9.3 kcal/mol and −8.4 kcal/mol, respectively (Table 5). The binding affinity energy of the two peptides was lower than the binding affinity value of the native erlotinib ligand, which was −7.3 kcal/mol.

This indicates that the WWSDHR and IWDTIEK peptides have interactions at the binding site of EGFR which are more stable than

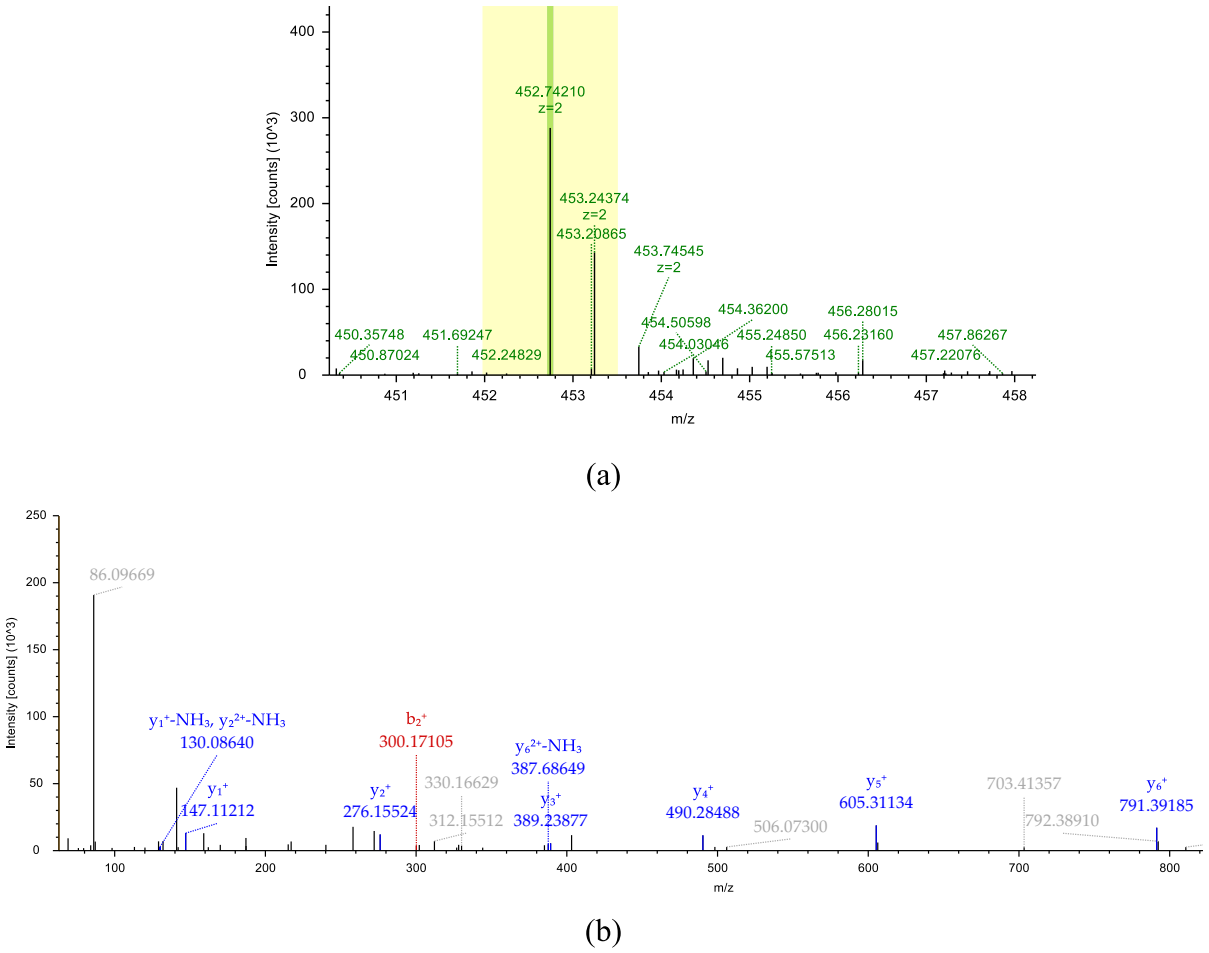

Fig. 4. MS spectra of IWDTIEK peptide. MS^1 identify the isotopic pattern of the peptide ion (a), whereas MS^2 identifies the b^+ and y^+ ions from the peptide fragmentation data (b).

Table 5
Docking results of identified peptides to EGFR protein.

Ligand/peptides	Binding affinity (kcal/mol)	Amino acid residues form hydrogen bond with the peptides
Erlotinib	−7.3	Gln767
GDNLIQMPGAAMK	−7.3	Asp831, Lys721, Glu734, Cys773, Asn818, Asp813, Arg817
NSLLVK	−6.7	Lys721, Asp831, Glu738
SSLLVK	−7.4	Leu764, Thr766, Arg817
GGSGTPVDDLDR	−7.5	Met769, Lys851, Glu734, Arg817, Asn818, Thr830, Cys751
GVGGTQLEVIK	−7.1	Asp831, Arg817, Glu734, Cys773, Glu738
IWDTIEK	−8.4	Lys721, Asp831, Leu764, Ala719, Glu738, Glu734
WWSDHR	−9.3	Met769, Leu694, Arg817, Asp813, Ala698
MFMVSNK	−7.3	Met769, Leu694, Arg817, Asp813, Ala698

the interactions of the native erlotinib ligands and possess potential anticancer activity. These two peptides bind to the amino acid residues Met769 and Lys721, which played an important role in determining the biological activity of the EGFR protein (Table 5). Hydrogen bonds located around the binding site can affect the strength of the compound binding to receptor proteins (Głowacki et al., 2013). The shorter the distance of the hydrogen bond, the stronger the hydrogen bond, which in turn leads to a more stable protein bond with the ligand. The bond distance, or bond length, is the average distance between a molecule's nuclei of two bonded atoms. The hydrogen bond formed in the WWSDHR peptide to the amino acid residue Met769 has a bond length of 2.70 Å., and the IWDTIEK peptide to the amino acid residue Lys721 has a bond length of 1.94 Å. Hydrogen bonds have strong bonds with bond lengths of less than 3.00 (Fikrika et al., 2016). Therefore, the hydrogen bonds formed are important for the interaction of the peptide with the EGFR protein receptor. These results indicate that the WWSDHR and IWDTIEK peptides have adequate interaction properties and more potent anticancer activity than erlotinib.

4. Conclusion

Proteomic analysis revealed that there are 20 enzymatic and non-enzymatic proteins distributed in 10 protein families in the snake venom of *N. kaouthia*. The bioactive peptide isolated from the snake venom protein of *N. kaouthia* can be hydrolyzed by the enzyme trypsin. The snake venom protein of *N. kaouthia* can be used as a source of anticancer peptides. Protein digestion with trypsin enzyme followed by RP-SPE fractionation showed that the 25% methanol fraction had anticancer activity against MCF-7 cancer cells with IC_{50} value of 4.17 μg/mL and a selectivity index value of 12.87. The sequences of eight peptides (GDNLIQMPGAAMK, NSLLVK, SSLLVK, GGSGTPVDDLDR, GVGGTQLEVIK, IWDTIEK, WWSDHR, and MFMVSNK) were identified in the fraction of 25% methanol and were responsible for the activity. WWSDHR

and IWDTIEK peptides have high potential as breast anticancer agents as they bind to the active site of EGFR.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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