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Antibacterial Peptides from Chymotrypsin Hydrolysate of *Jatropha* Seeds with RP-HPLC Fractionation

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ABSTRACT. Antimicrobial peptide (AMP), as a new antibiotic agent, has promising prospects in overcoming the problem of resistance. AMP production can be carried out by proteolytic enzymes. Protein from castor bean *(R. communis)* is toxic, so it can potentially be a source of AMP. This study aims to obtain protein hydrolysate from the castor bean *(R. communis)* using several extraction methods, including SDS dialysis, SDS-gel filtration, trifluoroacetic acid (TFA), and acetone precipitation. Chymotrypsin enzyme was used to hydrolyze the protein, and the peptide hydrolysate was fractionated using RP-HPLC. The peptide fraction was tested for its antibacterial activity by agar diffusion and microdilution methods, and the most active fraction was identified for its amino acid sequence by LC-HRMS. The results showed that the acetone precipitation extraction method was the best method, with a degree of hydrolysis of 83.9%. The active fractions 6 and 10 of RP-HPLC had IC_{50} values of 14.1 and 14.5 μg/mL for *E. coli* and 13.3 and 14.4 μg/mL for *S. aureus*, respectively. NVLRGKGMASL peptides were found in fraction 10, and GIILLSSK, NMIAKR, and LLDILTKK peptides were found in fraction 6 with an alpha helix secondary structure that can cause membrane damage. The peptides NVLRGKGMASL, GIILLSSK, NMIAKR, and LLDILTKK are thought to have potential as antibacterial compounds.

Keywords: acetone precipitation, antimicrobial peptide, chymotrypsin, LC-HRMS, RP-HPLC

INTRODUCTION

Infectious diseases are caused by the growth of bacteria, which are generally harmless but they are able to enter the body and cause infection. Infectious diseases such as diarrhea can result in high morbidity and mortality (Walker et al., 2013). To treat those infectious diseases, antibiotics are utilized. It is a drug that can be used to fight against bacterial infections (Gould & Bal, 2013; Nathan & Cars, 2014). Bacteria, however, develop resistance to antibiotic agents. In 1962 in England, the first reported case of antibiotic resistance was methicillin-resistant *S. aureus* (MRSA) (Anjum et al., 2019; Turnidge et al., 2016). The cause of bacterial resistance to antibiotics is mainly due to the overuse of antibiotics and mutations in the bacterial genome (Schulz et al., 2017; Huan et al., 2020). Antibiotic resistance is a serious global health threat, so it is necessary to develop new antibiotics to overcome this problem. Antimicrobial peptide (AMP) is a promising new alternative against infections caused by multiresistant pathogens (Leon-Buitimea et al., 2020). AMP is a small bioactive peptide that is produced naturally in living organisms (Lei et al., 2019). Natural AMP has a strong and broad spectrum of activity against a variety of pathogens such as viruses, fungi, and bacteria (Luong et al., 2020).

AMP is made up of less than 100 amino acid residues (Pasupuleti et al., 2012). It can kill bacteria in a different mode of action (MOA) than conventional antibiotics. In general, AMP's mechanism of action is by damaging the bacterial cell membrane, causing lysis and cell death, and interfering with intracellular components (Sani & Separovic, 2016). AMP can be isolated from various plant organs and can also be derived from insects, mammals, marine invertebrates, and microbes (Pushpanathan et al., 2013). Several peptides from plants, such as thionin, plant defensins, and lipid transfer, can be used as antibiotics to treat infectious diseases (Hughes et al., 2020; Bogdanov et al., 2016). An AMP with seven amino acids (Lys-Val-Phe-Leu-Gly-Leu-Lys) was isolated from *Jatropha* (Xiao et al., 2011). Peptides such as cecropins (insects), buforins (amphibians), and androctonin (scorpions) have been reported to have antimicrobial activity (Zasloff, 2002). Bacteriocins are a group of small AMPs produced by many bacteria [20]. Lactic acid bacteria are the most well-known bacteria for producing antimicrobial peptides that can be used as a starter for food fermentation (Martínez et al., 2016). Studies have reported that enzymatic hydrolysis of proteins can produce AMP with strong activity (Li et al., 2013).

The results of the polypeptides cleavage by enzymatic hydrolysis produce peptides with different characteristics, namely the composition and sequence of amino acids. Proteases such as trypsin and

chymotrypsin can cleave cationic residues (Lys and Arg) as well as hydrophobic residues (Trp, Tyr, and Phe), which can fulfill the prerequisites for AMP activity. AMP usually contains hydrophobic residues that can interact with microbial cells, causing cell death (Cao et al., 2021). Based on this, the protein can be hydrolyzed by chymotrypsin. Isolation of the peptide fraction from the pepsin hydrolysate of half-fin anchovy (*Setipinna taty)* has antibacterial activity against *E. coli* bacteria, consisting of five cationic peptides (MLTTPPHAKYVLQW, SHAATKAPPKNGNY, PTAGVANALQHA, QLGTHSAQPVPF, and VNVDERWRKL) and three anionic peptides (LAGHLGHLVSNPGAVELCY) (Song et al., 2012). An antibacterial peptide isolated and purified from the whey protein of camel's milk and cow's milk using trypsin has an antibacterial effect against *E. coli* and *S. aureus* (Wang et al., 2020).

Mijiti et al. (2018) succeeded in isolating and purifying antimicrobial peptides from *Cuminum cyminum L.* seeds, showing high levels of antibacterial activity against strains of *E. coli* and *S. aureus* bacteria. *In silico* and *in vitro* hydrolysis of cowpea protein with two enzymes, pepsin, and chymotrypsin, has antimicrobial activity against *S. aureus* and *P. aeruginosa;* bacterial inhibition depends on the dose given (Almeida et al., 2022). The AMP purification methods currently in use include salt deposition, adsorption-desorption, a combination of ion exchange and reverse phase C_{18} solid-phase extraction, reversed-phase high-performance liquid chromatography (RP-HPLC), and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. AMP can be produced by enzymatic hydrolysis of protein from *Jatropha (R. communis),* which is generally known to be toxic. Thus, this study aims to obtain protein hydrolysate from castor bean *(R. communis.)* seeds by employing several extraction methods using the enzyme chymotrypsin. Then, the peptide fraction was obtained by utilizing RP-HPLC fractionation as an antibacterial, and the active fraction was then identified by LC-HRMS. The results of this study are important in finding new antibiotics from peptides to treat infectious diseases caused by bacteria.

EXPERIMENTAL SECTION

Materials

The materials used were castor bean seeds *(R. communis)* (Bantul, Indonesia), USP trypsin enzyme (GBioscience), sodium dodecyl sulfate (SDS; Merck, Germany), distilled water, trifluoroacetic acid Uvasol® Spectroscopy Grade, and HPLC grade aquabidest (Ika Pharmindo). The antibacterial activity test was performed using *S. aureus* (ATCC), *E. coli* (ATCC), resazurin, and LB medium (Liquid Broth). Meanwhile, peptide identification was carried out using acetonitrile (grade LC-MS LiChrosolv®) and water (grade MS, Merck, Germany).

Preparation and Extraction of Castor Seeds Protein

Fine Jatropha seeds obtained from Bantul, Indonesia, which had been aerated and the outer skin removed, were soxhelated with petroleum ether for 19 hours at 75 °C. Jatropha seed protein extraction was carried out using four extraction methods. The first method was extraction with SDS dialysis using 1 g of fat-free castor seeds mixed with 12 mL of 0.01% SDS, which was stirred at room temperature for 24 hours. The solution mixture was then dialysed with 50 mM Tris-HCl buffer overnight at 475 °C. Then, the dialysis membrane was opened and the solution and all the precipitates in it were put into an Erlenmeyer to be sonicated for 15 minutes at room temperature. The solution and precipitate were then centrifuged at 10,000x *g* for 30 minutes, the supernatant was separated from the precipitate, its volume and absorbance were measured, and the concentration and mass of the extract obtained were calculated. Further extraction was performed by SDS-gel filtration. A total of 2 g of fat-free castor seeds were stirred with 24 mL of 0.01% SDS at room temperature for 24 hours. The solution was centrifuged at 3,000 x *g* for 25 minutes. The supernatant obtained was separated and the volume was measured. The supernatant was taken and diluted with a dilution factor of 10x, which was then gelled with 3000 Da MWCO Amicon® Ultra-15 Centrifugal Filter Devices and centrifuged at 4,000 x *g* for 45 minutes.

After that, extraction with TFA was carried out. Fat-free castor seeds were extracted using TFA 1:4 and incubated at room temperature for 2 minutes. The samples were neutralized with 2 M TrisBase using 10x the volume of TFA and centrifuged at 10,000 rpm for 15 minutes before adding 45 mL of supernatant and incubated at 56 °C for 25 minutes. The sample solution (45 mL) was then added to 5 mL of IAD and incubated in the dark for 30 minutes. Furthermore, 45 mL of the sample was added to 5 mL of 50 mM DTT and incubated in the dark for 15 minutes. The absorbance of the sample was measured at a wavelength of 280 nm. The fourth extraction method was carried out by precipitation of acetone. Fat-free jatropha seeds were extracted with 10 mL of aquabidest and vortexed for 3 minutes. The samples were then sonicated for 30 minutes at room temperature and centrifuged at 3,000 x *g* for 5 minutes. A sample of 5 mL of suspension was put into a 15 mL falcon and vortexed for 30 seconds. Protein was precipitated with 5 mL of acetone, vortexed for 1 minute, followed by centrifugation at 4,000 x *g* for 10 minutes. The acetone was removed with a pipette and allowed to stand for several minutes. Thus, the protein pellets were obtained which then were dissolved in 5 mL of 100 mM ammonium bicarbonate and vortexed for 1 minute. After that, the solution was sonicated for 1 hour and denatured by heating at 90 °C for 15 minutes. The sample was cooled at room temperature for 15 minutes.

Protein Hydrolysis

For protein digestion, protein hydrolyzed by chymotrypsin had the best extraction recovery. There were three extraction methods chosen. In the SDS-gel filtration and the TFA method, the samples were hydrolyzed in a ratio of 20:1 (w/w), and incubated for 24 hours at 37 °C. Then it was heated at 80 °C for 15 minutes and cooled at room temperature. The peptides in the hydrolysate were separated from the rest of the proteins using 3000 Da MWCO Amicon® Ultra–15 Centrifugal Filter Devices with filtration gel at 5,000 x *g* for 45 minutes, and the filtrate was separated from the concentrate. The filtrate was then freeze-dried for 48 hours. Subsequently, the sample solution extracted by acetone precipitation was hydrolyzed using the same enzyme ratio as the SDS-gel filtration and TFA extraction methods, which then was incubated for 16 hours at 37 °C. The samples were centrifuged at 5,000 x *g* for 10 minutes. The volume of the three samples obtained was measured using a UV-Vis spectrophotometer at 280 nm, and the degree of hydrolysis was calculated.

Fractionation of Peptide Hydrolysates using RP-HPLC

The filtrate obtained from protein hydrolysis by the TFA method and acetone precipitation was fractionated by RP-HPLC on a Delta-Pak C_{18} column (100 A, 3.9 mm x 150 mm, Interlink Scientific Services Ltd.), equilibrated with trifluoroacetic acid (TFA) at 0.1% for 40 minutes, and followed by 60% acetonitrile in 0.1% TFA for 10 minutes. The sample was injected into 100 μL and eluted gradually with 6% acetonitrile in 0.1% TFA for 10 minutes and 60% acetonitrile in 0.1% TFA for 25 minutes at a flow rate of 1 ml min^{-1}. The fractions were collected within 1 minute and elution peaks were detected at 220 nm and 280 nm.

SPE Pre-concentration using HyperSep™ Retain PEP

The result obtained using the SDS- gel filtration extraction method was concentrated after protein hydrolysis. The result obtained by applying the acetone precipitation extraction method was also concentrated after fractionation with RP-HPLC. The HyperSep™ Retain PEP SPE column was added with 2 mL of 100% methanol, allowed to drop to the SPE bed limit, and added with 2 mL of aquabidest. The sample was loaded into the SPE column and washed twice with 5% methanol. The sample was then eluted with 100% methanol 4 times with 3 mL each. The elution results were collected and evaporated. The concentrated hydrolysate was then measured using a UV-Vis spectrophotometer at a wavelength of 280 nm.

Determination of Antibacterial Activity

An antibacterial activity test was carried out by the disc diffusion and microdilution methods. Peptides from SDS- gel filtration extraction were tested for antibacterial activity by the disk diffusion method with *E. coli* and *S. Aureus* bacteria grown on LB media (NaCl, triptone, and yeast extract) at 37 °C for 24 hours as a bacterial stock solution. Disc diffusion was performed using bacterial stock which was diluted in melted LB agar and then placed on a sterilized petri dish. The discs tested were made by dipping the disc paper into a solution of the peptide fraction, chloramphenicol as a positive control, media, and bacteria as a negative control, followed by placing the disc on the surface of the bacterial culture in a petri dish. The incubation process was carried out at 37 °C for 24 hours. The diameter of the inhibition zone was measured on each paper disc. The peptide fraction with RP-HPLC from TFA extraction and acetone precipitation was tested for antibacterial activity using the microdilution method. The OD value of *E. coli* and *S. aureus* bacteria in liquid media were measured at 600 nm (0.5-0.7) using a UV spectrophotometer. Next, each bacterium was poured into a sterilized petri dish. For each well of the 96-well plates, 80, 75, 70, 65, 60, 55 μl of media were added, then peptide fraction was added to each medium (0, 5, 10, 15, 20, 25 μL) and 10 μL of bacterial culture was added to each well. The 96-well plates were incubated for 20 h at 37°C. Chloramphenicol was used as a positive control for *S. auerus* and *E. coli* bacteria. The samples in the 96-well plates were transferred to an Elisa reader and the OD value was measured at 630 nm. Then the inhibitory concentration 50 (IC_{50}) was determined using the AATBIO.com online tool by first calculating the % inhibition value using Eq. I.

$$\%\text{Inhibition} = (OD\ control - ODsample / ODcontrol) \times 100\% \quad \text{(I)}$$

The % inhibition value was then plotted with the sample concentration values in each well and calculated using the equation in the online tool AATBIO.com (Andriana et al., 2023).

Identification of Antibacterial Peptides

The most active peptide fraction was analyzed by High-Resolution Mass Spectrometry (HRMS). A total of 5 μL of the sample was injected into the AcclaimR PepMap RLSC column (C_{18}, 75 µ m x 150 cm, particle size 2 µ m, size pore 100 A). The mobile phases used were mobile phase A (water and 0.05% TFA) and mobile phase B (water/acetonitrile 20/80 and TFA 0.1%). The flow rate was set at 0.1 mL min^{-1}. Peptide analysis was conducted using the m/z range of 150–2250 with full MS/dd-MS^2 mode. The resolving power of 140,000 (FWHM) was utilized as the full MS parameter, while a resolution of 17,500 (FWHM) was employed as the dd-MS^2 parameter. MS spectra and chromatogram files in the initial format (.raw) were processed by the Proteome Discoverer software ver 2.5. The database used was *R. communis* protein from UniProt.

RESULTS AND DISCUSSION

Castor Seed Protein Extraction and Hydrolysis

A number of protein extraction methods have been applied to various biological samples. Protein extraction can be carried out by several methods, such as SDS, TFA, and acetone precipitation methods. The

SDS method can dissolve proteins by changing their secondary and tertiary structures (Andersen et al., 2009). The SDS method is generally used in polyacrylamide gel electrophoresis (SDS-PAGE), which can separate, characterize, and measure proteins. However, the use of SDS is not compatible with MS and thus is limited in proteomic applications, and the use of high concentrations of SDS can interfere with the enzymatic protein digestion process (Scheerlinck et al., 2015). In addition to the SDS method, protein extraction can be carried out by applying the SPEED method using TFA, which consists of three simple procedures, including acidification, neutralization, and digestion (Doellinger et al., 2020). Protein extraction can also be carried out with acetone precipitation. This method can minimize protein degradation and protease activity and reduce contaminants (Niu et al., 2018).

The results showed that the extraction method with acetone precipitation produced up to 69.8% of protein (**Table 1**). These results are consistent with those of a past study carried out by Huang et al. (2020) who succeeded in extracting protein by the TCA or acetone method and exhibited that this method resulted in better protein extraction. Similarly et al. (2011) reported that TCA or acetone precipitation is the most effective method for protein extraction because it gives a high yield of total protein. The extraction yield with the acetone precipitation method was higher than that of the other methods, which only ranged from 18.5% to 68.3% of protein (**Table 1**). Meanwhile, Raharjo et al. (2021) have succeeded in extracting protein from castor beans using the SDS 0.01% method, yielding 20.9% of protein. Protein extraction by the trifluoroacetic acid (TFA) method from *Jatropha* seeds yielded up to 60.4% of protein (Atmawati et al., 2022). Protein hydrolysis with chymotrypsin resulted in the highest degree of hydrolysis (83.9%) by employing the acetone precipitation extraction method. The number of peptides produced indicates that the degree of hydrolysis is increasing.

Peptide Fractionation with RP-HPLC

The separation of peptides by RP-HPLC is based on the hydrophobic interaction between the peptide column material and the mobile phase. More polar peptides elute first, while less polar peptides elute longer due to strong hydrophobic interactions with the stationary phase (Sonklin et al., 2020). Peptide fractionation with RP-HPLC has advantages such as being easy to use and very flexible. The fractions were collected separately using an RP-HPLC column and concentrated before testing for antibacterial activity. Protein hydrolysis from the TFA protein extraction method (**Figure 1A**) and acetone precipitation (**Figure 1B**) with chymotrypsin was further separated into several peaks by RP-HPLC. From the results of the RP-HPLC fractionation using the TFA protein extraction method, 35 components with different retention times were obtained, where the components with the highest peaks were components 21, 31, and 34, with an area of 6.6 and 9.6 and 6.0 mAu, respectively. These components are grouped into 12 fractions based on the peaks that have adjacent retention times, and then the total volume of the fractions obtained after 10 x RP-HPLC cycles is measured, according to (**Table 2**). While the results of fractionation using the acetone precipitation extraction method produced 30 components, the component that has the widest area under the curve is component 29, which is included in fraction 10, and component 11, which is part of fraction 4, with an area of 55.9 and 35.8, respectively. The wider the peak area, the higher the concentration of the sample. From the results of grouping the fractions based on the chromatogram **(Figure 1B)**, 11 fractions were obtained from the acetone precipitation extraction method, which had a retention time and a total volume that can be seen in **(Table 3).**

The extraction method with TFA based on the resulting chromatogram shows that the samples obtained have adjacent retention times which begin to separate at retention times of 10.86 min to 25.92 min in an acetonitrile gradient which increases from 6% to 60%. The extraction method with separate acetone precipitation starts from the retention time to 1.83 min which indicates that the peptide sample is more polar or less hydrophobic at 6% acetonitrile concentration until it is detected at 35.54 minutes at 60% acetonitrile concentration which indicates that the acetone precipitation method acetone precipitation extraction has compounds with more varying degrees of polarity. The peaks produced by the acetone precipitation extraction method were more distributed at the retention time above the 10 minute which indicated that the resulting peptide would be more non-polar or hydrophobic.

Table 1. The percentage of protein content and degree of hydrolysis with different extraction methods

Method	(%) Protein	Degree of hydrolysis (%)
SDS dialysis	18.5	-
SDS-gel filtration	59.3	32.0
TFA	68.3	44.8
Acetone precipitation	69.8	83.9

(**A**)

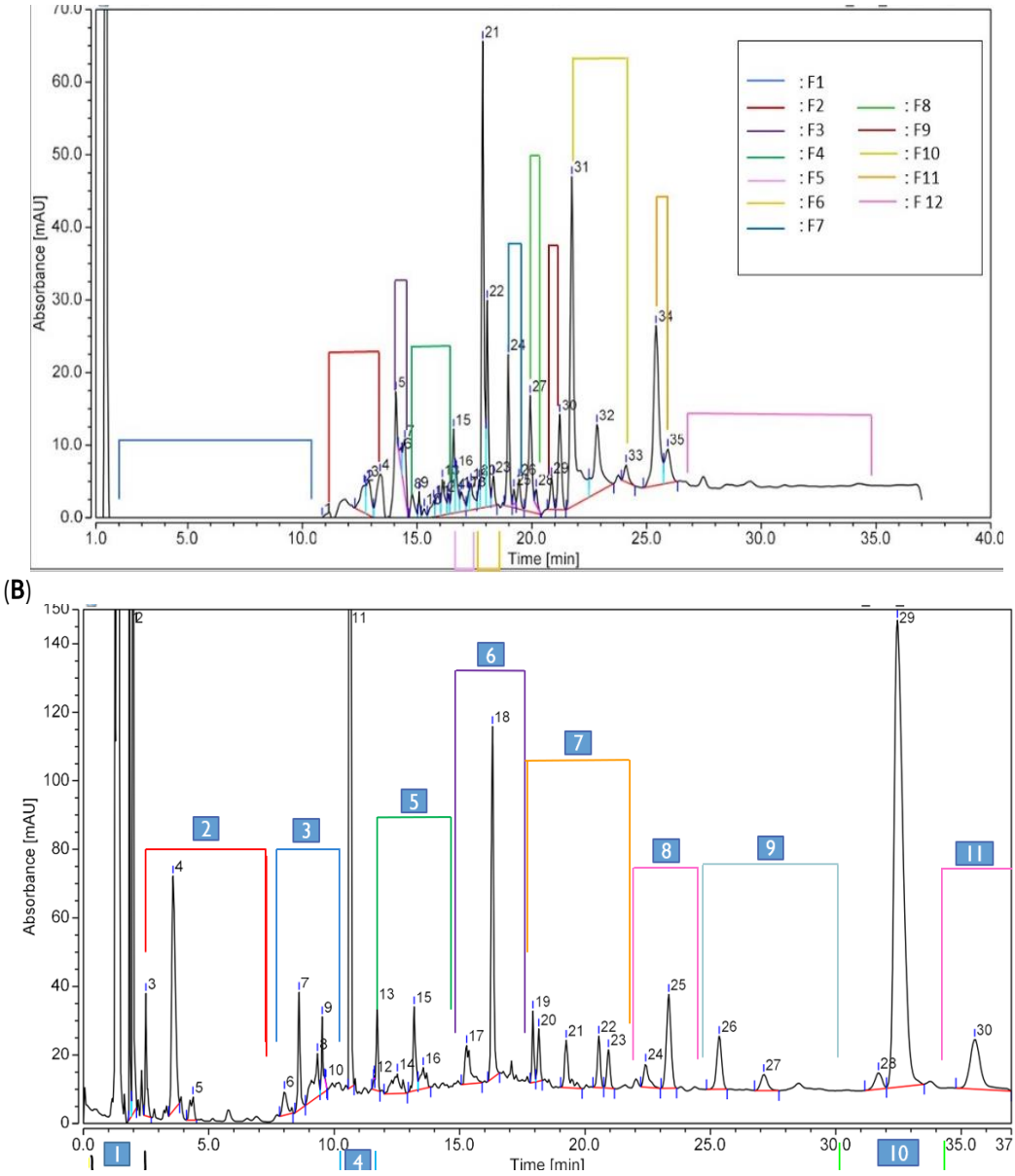

Figure 1. Reversed-phase HPLC profile of protein hydrolysate with TFA (**A**) and acetone precipitation (**B**) method

Table 2. Retention time of TFA protein extraction method

Fraction	Retention time (minute)	Total volume fraction (mL)
F1	2.00-10.50	82.5
F2	10.75-14.00	29.5
F3	14.01-14.75	4.2
F4	14.75-16.50	12.6
F5	16.51-17.50	6.0
F6	17.51-18.50	6.0
F7	18.51-19.75	5.6
F8	19.76-20.75	6.0
F9	20.76-21.75	7.6
F10	21.76-21.75	21.0
F11	24.51-26.35	14.2
F12	26.36-35.00	69.2

Table 3. Retention time of acetone precipitation protein extraction method

Fraction	Retention time (minute)	Total volume fraction (mL)
F1	0.00-2.25	22.5
F2	2.26-7.50	52.5
F3	7.51-10.00	25.0
F4	10.01-11.25	12.5
F5	11.26-14.75	37.5
F6	14.76-17.50	25.0
F7	17.51-21.50	40.0
F8	21.51-24.50	30.0
F9	24.51-30.00	55.0
F10	30.01-34.75	47.5
F11	34.76-37.00	22.5

The mobile phase of RP-HPLC is generally made of a strong acid such as trifluoroacetic acid (TFA). TFA can maintain pH, increase the peak shape of the peptide, and have an effect on increasing the hydrophobicity of the peptide. TFA acts as a counterion. Acidic counter ions can prevent ion exchange interactions by preventing the silanol ionization process (Madhurkar et al., 2021). The peaks produced from the two protein extraction methods tend to be narrow and sharp, which indicates that at the retention time there is only one peptide compound. Peptides that have similar polarity properties will be eluded at a close period of time. Peptides identified in the initial fraction tend to be polar. Fractions 12 and 11 resulting from both protein extraction methods contained the most hydrophobic peptides. Fraction 6 and fraction 10 of the acetone precipitation protein extraction method showed antibacterial activity against gram-positive (*S. aureus*) and gram-negative *(E. coli)* bacteria. The active fraction is consistent with findings of previous studies suggesting that the sequence of bioactive peptides is based on the hydrophobic nature of the amino acid residues (Shu et al., 2011). Both fractions were included in the LC-HRMS analysis aimed at identifying their peptide sequence.

Antibacterial Activity

An antibacterial activity test was carried out on gram-positive *(S. aureus)* and gram-negative *(E. coli)* bacteria. Protein extraction using the SDS-gel filtration method was tested for antibacterial activity using the agar diffusion method presented in (**Figure 2**). Agar diffusion is the most familiar and basic method. In the agar diffusion method, agar plates were inoculated with *S. aureus* and *E. coli* inoculums. Disc paper that had been given the test compound was placed on the agar medium. Then it was incubated at 37 °C for 24 h, which was then measured as the diameter of the inhibition zone (mm) (Espinel-Ingroff et al., 2011). Use the agar diffusion method on SDS-gel filtration as a preliminary study to see whether the extract from jatropha seeds has activity as an antibacterial. The protein extraction method with SDS-gel filtration resulted in an inhibition zone diameter of 10 mm against *S. aureus* bacteria, whereas in *E. coli* bacteria an inhibitory zone was not formed. The diameter of the inhibition zone was greater than that of chloramphenicol as a positive control at 17 mm. According to Davis and Stout (1971), an inhibition zone with a diameter of 10–20 mm is included in the strong category, so the protein extraction method with SDS-gel filtration is also included in the same category. Based on the results of testing the antibacterial activity of the SDS-gel filtration extract, it is known that the castor oil seed extract has antibacterial activity.

Diffusion agar cannot determine the minimum inhibitory concentration because it cannot measure the amount of antimicrobial agent that is diffused in the agar medium. In addition, the agar diffusion method is only limited to qualitative data. Meanwhile, protein extraction using the TFA method and acetone precipitation were tested for antibacterial using the microdilution technique. The microdilution technique was chosen by considering several factors such as time efficiency and the use of an Elisa reader so that the results obtained were more accurate. The antibacterial activity test in this study used the antibiotic chloramphenicol as a positive control. Chloramphenicol is an antibiotic with bacteriostatic activity (Kostopoulou et al., 2015). The microdilution technique belongs to quantitative analysis by measuring the IC_{50} value. The IC_{50} value is defined as the concentration of the fraction in which the bacterial inhibitory activity is 50%. The resazurin dye and Luria-Bertani (LB) medium in the microdilution technique were used to grow the test bacteria. Resazurin dye (Alamar blue) was applied to observe the growth of bacteria through a blue-to-pink color change. The higher bacterial growth was indicated by the more

concentrated pink color formed. The results of the IC_{50} value by applying the protein extraction method using TFA produced 12 fractions and the acetone precipitation method produced 11 fractions. Based on this, fractions 6 and 10 of the protein extraction method using acetone precipitation produced IC_{50} values with no major difference, meaning that both fractions were able to inhibit gram-positive and negative bacteria with the same ability (**Table 4**) and could be considered potential fractions compared to other fractions.

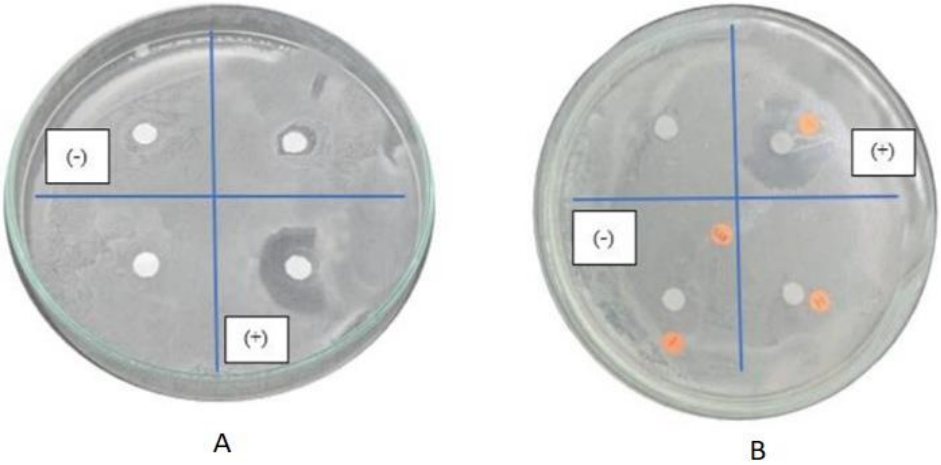

Figure 2. Results of an antibacterial activity test against (**A**) *S. aureus* bacteria (**B**) *E. coli* bacteria

Table 4. The reported inhibition zone diameters and IC_{50} values of all different protein extraction methods

Method	Sample	IC_{50} (µg/mL)	
		S. aureus	*E. coli*
TFA	Fraction 1	29.4	33.4
	Fraction 2	47.7	42.8
	Fraction 3	277.5	25.4
	Fraction 4	43.3	38.3
	Fraction 5	130.2	6.1
	Fraction 6	73.2	34.1
	Fraction 7	65.9	76.2
	Fraction 8	6.9	4.8
	Fraction 9	324.5	328.1
	Fraction 10	369	29.0
	Fraction 11	45.0	56.4
	Fraction 12	60.4	339.9
	Positive control	3.1	32.0
Acetone precipitation			
	Fraction 1	17.9	18.2
	Fraction 2	10.0	7.4
	Fraction 3	9.6	12.5
	Fraction 4	15.0	44.2
	Fraction 5	15.0	10.8
	Fraction 6	13.3	14.1
	Fraction 7	21.2	38.9
	Fraction 8	9.0	17.3
	Fraction 9	11.7	22.1
	Fraction 10	14.4	14.5
	Fraction 11	18.5	14.9
	Positive control	14.6	14.7

positive control = chloramphenicol, negative control = aquadest

Peptide Analysis of Antibacterial Active Fraction

The peptide fraction of the protein hydrolysate with the most potential antibacterial activity was determined by the amino acid composition by LC-HRMS analysis. The most active fraction of RP-HPLC was obtained from the extraction method using acetone precipitation. The active fraction consisted of fractions 6 and 10. In this study, one peptide sequence was obtained from fraction 10, which corresponds to the cut point of the chymotrypsin enzyme. Fraction 10 identified 1 peptide consisting of 11 amino acids, namely the NVLRGKGMASL peptide. The NVLRGKGMASL peptide had m/z = 581.32794 Da (z = 2), indicating several low-intensity isotopic ions (**Figure 3A**).

The molecular mass (MH+) of the peptide was 1161,64861. The MS^2 spectrum yielded four fragment ions, including b_2^+, b_3^+, y_2^+, and y_8^+ (**Figure 3B**). The b ion was the result of cutting the peptide bond from the N-terminal end, while the y ion was the result of cutting the peptide bond from the C-terminal end. The difference in mass between b_3^+ ions (327,20268) and b_2^+ ions (214,11862) indicates the presence of L (113,08406), leading to the VL amino acid sequence at the N-terminus. The y_2^+ fragment ion represents the S amino acid (Serine) and the y_8^+ fragment ion represents the A amino acid (Arginine). The NVLRGKGMASL peptide had a pI value of 11.00, a hydrophobic ratio of 45%, a gravy value of 0.182, and a total charge of 2. The NVLRGKGMASL peptide has a positive charge due to the large amount of lysine (K), arginine (R), and glycine (G) residues, so that the peptide produced has good binding ability with the membrane of negatively charged microbes.

In this study, the HRMS results for fraction 6 were obtained and have peptide ends with different characteristics from the dots cut off from the enzyme chymotrypsin. This can be caused by the level of specificity of the chymotrypsin enzyme used; an enzyme with high specificity will cut at tryptophan (W), tyrosine (Y), and phenylalanine (F) residues, whereas an enzyme with lower specificity can cut on residues of leucine (L), methionine (M), and histidine (H), as well as to a certain degree, according to Keil (1992). Cuts on arginine (R) and lysine (K) residues may occur. Fraction 6 identified 3 peptides, namely GIILLSSK, NMIAKR, and LLDILTKK, with theoretical pI values of 10.1, 11.5, and 9.9, respectively. The peptides GIILLSSK and LLDILTKK have GRAVY values of 1.34 and 0.49, respectively, indicating that they are hydrophobic. An example of HRMS data in the identification of fraction 6 peptide sequences can be seen in **(Figure 4).**

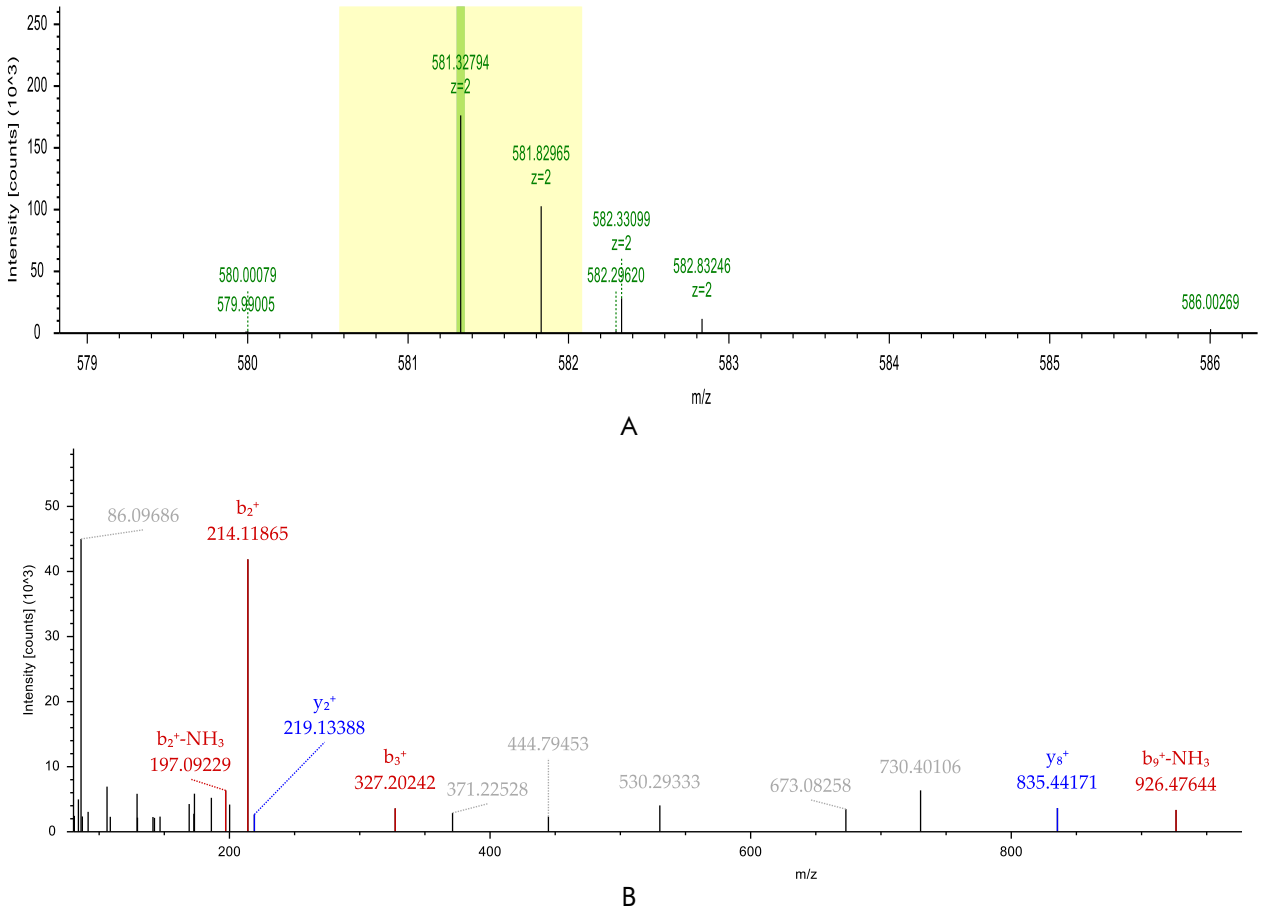

Figure 3. MS^1 peptide spectrum from NVLRGKGMASL peptide of the 10^{th} fraction identified the isotopic pattern of the peptide ion (**A**), whereas MS2 identified peptide fragmentation data (**B**)

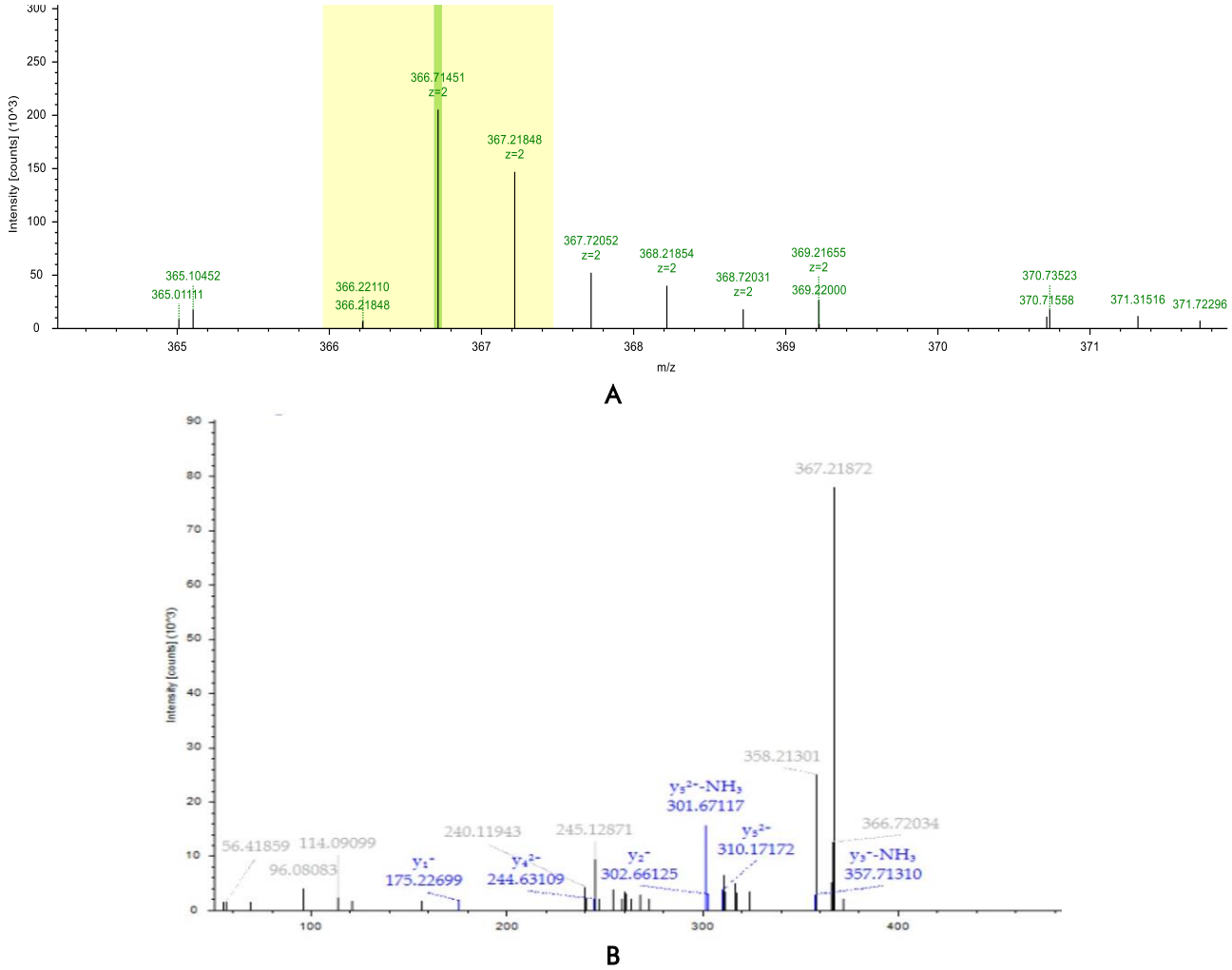

Figure 4. MS^1 peptide spectrum from NMIAKR peptide of the 6 fraction identified the isotopic pattern of the peptide ion (**A**), whereas MS^2 identified peptide fragmentation data (**B**)

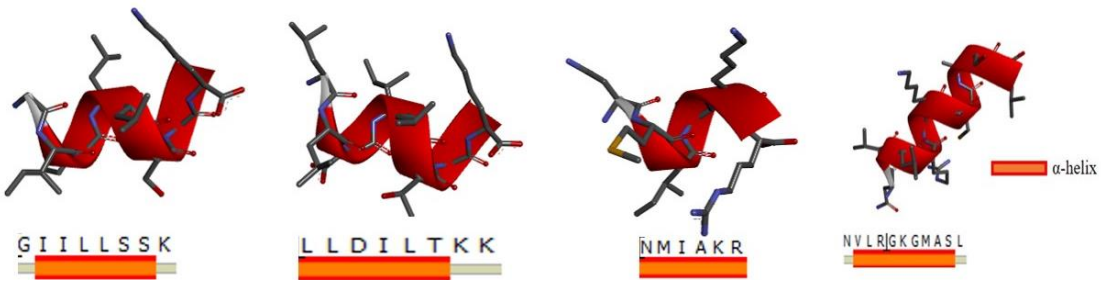

Figure 5. Peptide secondary structure modeled by Chimera 1.16 software

The NMIAKR peptide has a GRAVY value of -0.62, which indicates that the peptide has low hydrophobicity or is more hydrophilic. The NMIAKR peptide eluted at 21.9492 minutes with a value of m/z = 366.71451 and an ionic charge of +2, and a precursor ion mass of 732.42174 Da, which is presented in (**Figure 4A**). In the MS^2 spectra, it was confirmed that the ions y_1^+, y_2^+, y_3^+, y_4^{2+}, and y_5^{2+} were displayed in the theoretical fragment (**Figure 4B**). The y_1^+ fragment ion represents the R amino acid (arginine), the y_2^+ ion represents the K amino acid (lysine), and the y_3^+ ion represents the A amino acid (alanine), which has lost the NH_3 group, so it has a fragment ion value of 357.71310. The y_4^{2+} ion represents an acid amino I (isoleucine) with a greater charge of 2+, and the y_5^{2+} ion represents the M amino acid (methionine) with a larger charge of 2+, and the same y_5^{2+} ion represents methionine, which has lost the NH_3 group, so it has a fragment ion value of 301.67117.

Besides the positive charge of peptides, another important factor in antimicrobial activity is hydrophobicity. Generally, antimicrobial peptides have about 50% hydrophobic residue. Antimicrobial peptides have both hydrophobic and cationic parts. The hydrophobic part is used to enter the cell

membrane so that it becomes unstable and causes lysis. Meanwhile, the cationic part can bind to the negatively charged phospholipid heads on the bacterial cell membrane. The gravy peptide value was 0.182. The positive value indicates that the peptide tends to be hydrophobic. Kadri et al. (2022) reported that peptides with a positive gravy value are hydrophobic, while a negative gravy value means more hydrophilic. The secondary structure of the peptides modeled with the Chimera 1.16 software shows that the peptides from fractions 6 and 10 have an α-helix conformation associated with the antimicrobial activity of AMP (**Figure 5)**. Peptides with α-helix secondary structure are important cytolytic AMPs and can increase antibacterial potential because they can interact with membranes (Khara et al., 2017).

CONCLUSIONS

Castor seed protein extraction (*R. communis*) with acetone precipitation gave better results than SDS dialysis, SDS-gel filtration, and TFA extraction methods. Protein digestion with chymotrypsin followed by fractionation using RP-HPLC from the acetone precipitation method showed that fractions 6 and 10 had antibacterial activity. It is active against gram-positive (*S. aureus*) and gram-negative *(E. coli)* bacteria. The peptides found in fractions 6 and 10 have antibacterial potential. The antibacterial activity is a result of the secondary structure of the peptide that forms the α-helix so that it plays an important role in interaction with the cell membrane.

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