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Antibacterial Peptides from Tryptic Hydrolysate of *Ricinus communis* Seed Protein Fractionated Using Cation Exchange Chromatography

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ABSTRACT

Antimicrobial peptide (AMP) has become an interesting target in developing new antibiotics. AMP is possibly generated through the digestion of protein. The protein of castor (*Ricinus communis*) seed is characterized as a ribosome-inactivating protein (RIP) that can be a source of AMP. The objective of this research was to identify antibacterial peptides from *Ricinus communis* seed protein hydrolysate. The seed protein was isolated using sodium dodecyl sulfate and subsequently digested using trypsin. The hydrolysate was fractionated using a strong cation exchange chromatography system, and the resulting fractions were tested for antibacterial activity. The peptides present in the active fraction were identified using high-resolution mass spectrometry. The results showed that the pH 4 and pH 5 fractions of the elution buffer exhibited antibacterial activity, with the pH 4 fraction of the hydrolysate having high activity against both gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacteria. Three peptides that have the sequences EESETVGQR, GQSTGTGQQER, and LDALEPDNR could be responsible for the activity of the pH 4 fraction. The antibacterial activity of these peptides, which is due to their ionic properties and secondary structure, supports the disruption of the bacterial cell membrane. It can be concluded that *Ricinus communis* seed protein hydrolysate contains peptides with sequence EESETVGQR, GQSTGTGQQER, and LDALEPDNR that are potent to be used as AMP lead compounds.

Keywords: peptides, antimicrobial, *R Communis*, SDS extract, protein, HRMS

INTRODUCTION

The treatment of bacterial infections and diseases using antibiotics is currently facing problems because of bacterial resistance to existing antibiotics. In 2015, the United States Centers for Disease Control and Prevention reported that antibiotic-resistant pathogenic bacteria, including *Clostridium difficile*, caused 15,000 deaths annually in the United States (Dodds, 2017). Ironically, the development of new antibiotics is relatively slower than that of other medicines (Aminov, 2016).

Peptides are interesting targets to study for their potential as antibacterial compounds because their structures vary depending on the amino acid sequence and peptide length. Some natural peptides, such as thionin (plant peptides), bombinin, and cecropin (insect peptides) have been used as the basis for the development of antibiotics. Synthetic peptide antibiotics, such as gramicidin and bacitracin, have been successfully developed from natural peptides. Chemically, most antibacterial peptides contain the tip of weakly loaded amino acids, such as arginine (R) and lysine (K) (Hancock *et al*., 2006). This phenomenon is related to the biosynthesis of antibacterial peptides, which involves the enzymatic proteolytic hydrolysis of protease against polypeptides.

Hundreds of antibacterial peptide compounds have been reported in the past 50 years. Peptides are classified into non-ribosomal and ribosomal peptides, such as polypeptides. Antibacterial peptides (i.e., non-ribosome-synthesized peptides) are peptides that are biosynthesized in bacteria and fungi, which contain parts derived from two or more amino acids. Penicillin can be considered a peptide derivative because of the amino acid residues of L-cysteine and D-valine in its structure. Some non-ribosomal

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peptide antibiotics sourced from microbes are gramicidin, bacitracin, glycopeptides, and polymyxins (Xue *et al*., 2018; Zhao *et al*., 2018). The mechanism of action of this antibiotic group mostly follows the cationic mechanism whereby positively charged peptides (e.g. gramicidin can have +15 charge) damage the cell membrane except for streptogramin which inhibits protein synthesis (Hancock *et al*., 2006).

Melittin from snake venom is the first antibacterial ribosome-targeting peptide to be reported (Habermann, 1972). Melittin activity follows the cationic mechanism. The discovery of melittin has triggered the exploration of cationic peptides from other sources, such as mammals (defensins), amphibians (bombinin), insects (cecropin), and plants (thionin) (Habermann, 1972; Berrocal-Lobo *et al*., 2009; Parisi *et al*., 2019). The absence of homologous patterns of specific amino acid sequences for antibacterial ribosome-targeting peptides makes it difficult to develop the peptides synthetically. Mammalian peptides, such as azurocidin, lactoferrin, cathepsin, and cathelicidin, exhibit specificity to mycobacteria; thus, they can be developed into antituberculosis drugs (Padhi *et al*., 2014). Moreover, mammalian endophytic microbes have been reported to be a source of antibacterial peptides. Short peptides with 16 amino acids (ASVVNLKLTGGVAGLLK) are produced by Bacillus bacteria in the pig's intestinal tract. These peptides have been reported to have a low MIC value against *Escherichia coli* (2.5–5 μM) and *Staphylococcus aureus* (10–20 μM) (Xin *et al*., 2017).

The latest development in the study of antibacterial peptides is peptides resulting from the hydrolysis of proteins with proteolytic enzymes. Enzymatic cutting of polypeptides can be done *in vitro* to produce peptides with different characteristics (i.e., size and sequence of amino acids). Peptides with R or K tips can be produced with a specific peptidase enzyme, such as trypsin. Similar to isolated natural peptides, peptides hydrolyzed from proteins vary from those derived from higher organisms. Purification of peptides from the hydrolysis of pepsin protein from *Brucea javanica* fruit produced brucine, an antibacterial peptide with the amino acid sequence HTLCMAGGATY, with activity against *Streptococcus pyogenes* 16 times higher than penicillin (Sornwatana *et al*., 2013). Antibacterial peptides hydrolyzed from plant proteins obtained from the hydrolysis of pepsin against RuBisCO, which was isolated from alfalfa, have three short peptides (i.e., three, five, and six amino acids) with antibacterial activity against both gram-positive and gram-negative bacteria (Kobbi *et al*., 2015). Antibacterial peptides have also been reported to be obtained from the hydrolysis of seed proteins. Song *et al*. (2020) reported three peptides with highly active antibacterial activity identified from cottonseed protein hydrolysate. Antibacterial peptides have also been reported to be obtained from the hydrolysis of animal proteins. Sila *et al.* (2014a) reported eight peptides of three and four amino acid residues resulting from the hydrolysis of barbel fish protein. The mechanism of action of these peptides involves damaging cell membranes. The hydrophobic amino acid sequences in these peptides form a structure of random peptide coil, which is related to antibacterial activity according to Sila *et al.* (2014b). The hydrolysis of cow's milk casein protein with metallocene protease has been reported to produce antibacterial peptides identified using MALDI-TOF as having the amino acid sequence of SSSEESII. This peptide also has a random coil structure and inhibitory activity against gram-positive and gram-negative bacteria (Bougherra *et al*., 2017). The hydrolysis of proteins that produce antibacterial peptides has also been reported for the macroalgae *Saccharina longicruris* hydrolyzed with trypsin. The hydrolysate can inhibit the growth of *S. aureus* at a concentration of 0.31 mg/mL. Nine peptides with sizes ranging from 8 to 16 amino acids, with predominantly arginine at the end, were identified. The mechanism of action of these peptides follows the cationic mechanism (Beaulieu *et al*., 2015).

Ribosome-inactivating protein (RIP) from *Phytolacca dioica* has been reported to be hydrolyzed at the amino acid positions 40–65 to produce a peptide known as PDL440-65, which exhibits antibacterial activity. This peptide has a broad spectrum of activity against *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. In contrast to other antibacterial peptides, PDL440-65 has a β-sheet structure (Pizzo *et al*., 2015). The discovery of PDL440-65 has enabled large-scale research on other RIPs, such as ricin, to potentially produce antibacterial peptides that can be developed into antibiotics. The amino acid sequence pattern of antibacterial peptides reported so far can be said to be erratic, but most antibacterial peptides follow ionic mechanisms that involve damaging bacterial cell membranes.

Jatropha (*Ricinus communis*) seeds contain 1% to 5% of a special protein called ricin, which is toxic because it is a RIP (Wilson *et al*., 2018). RIP inhibits protein biosynthesis, which is one of the mechanisms of action of antibiotics. Peptides with RIP activity that also exhibit antibacterial activity have been reported (Balaban *et al*., 2000). Meanwhile, peptides with antibacterial activity have been successfully obtained from the RIP of the plant *P. dioica* (Pizzo *et al*., 2015). Based on previous findings, ricin and castor seed proteins, which have RIP activity, are hydrolyzed to produce peptides that have antibacterial activity. This study aimed to reveal the antibacterial activity of peptides obtained from the tryptic hydrolysis of castor seed protein.

MATERIALS AND METHOD

The main materials used in peptide preparation were castor beans (*R. communis*) (EcoWell, Indonesia), sodium dodecyl sulfate (SDS; Merck, Germany), trypsin (Bioworld, OH, USA), and cation exchange SPE column (Discovery DSC-SCX; Supelco, PA, USA). The antibacterial activity test used *E. coli* (ATCC), *S. aureus* (ATCC), and a solid medium containing tryptone, yeast extract, yeast agar (Oxoid, Ireland) as the inoculant. Acetonitrile (hyper grade for LC-MS LiChrosolv, Merck, Germany), water (MS grade, Merck, Germany), and trifluoroacetic acid (TFA; Merck, Germany) were used during peptide identification.

Protein extraction

Dry seeds were ground into powder and defatted before extraction. Castor seed powder was extracted using 0.01% SDS at room temperature for 12 h and dialyzed against a 50mM Tris–HCl buffer (pH 7.5) overnight at 4℃. After sonication for 15min, the mixture was centrifuged. The protein extract was obtained from the supernatant. Then, the protein content was measured using a UV–Vis spectrophotometer at a wavelength of 280nm (Utami, 2019).

Protein hydrolysis

The extracted protein was hydrolyzed with trypsin using an enzyme-to-protein ratio of 1:10 (w/w). The mixture was incubated for 24 h at 37℃ and further incubated for 15 min at 80℃. Protein hydrolysate was collected from the supernatant after 10,000×g centrifugation for 10 min at room temperature. The degree of hydrolysis that occurred in 900μL of protein hydrolysate mixed with 100μL of 10% trichloroacetic acid solution was determined. The solution was incubated for 10min at room temperature, followed by 8,000×g centrifugation for 10min. The supernatant was separated, and its absorbance was measured using a UV–Vis spectrophotometer at a wavelength of 280 nm. The level of hydrolysis was expressed as the ratio of the percentage of absorbance after and before enzymatic hydrolysis (Sornwatana *et al*., 2013).

Peptide fractionation

The column was pretreated with methanol and distilled water at a flow rate of 1–3mL/min. After washing with distilled water, the column was conditioned using a pH 3 buffer. Protein hydrolysate pH 3 was applied to the column slowly. Then, the sample was propelled at a flow rate of 1–3mL/min and stopped when the sample level was directly above the adsorbent. Elution was performed with pH 4, 5, 6, 7, and 8 buffers in sequence at a flow rate of 1–3mL/min to obtain eluate as much as twice the column volume. Each elution of the same pH was collected into one fraction and neutralized to pH 7. The eluate obtained was freeze-dried and redissolved in distilled water. Each fraction was used to determine the amount of peptide that was eluted by measuring its absorbance at 280nm (Fajr, 2019).

Antibacterial activity test of the protein hydrolysate fraction

The antibacterial activity test was performed using the disk diffusion method. The inoculants (i.e., *E. coli* and *S. aureus*) were made by growing test bacteria from the stock solution on LB media (made from a mixture of tryptone, yeast, and NaCl at a ratio of 2:1:1 in distilled water). Bacteria were grown until their turbidity (OD) reached 0.5–0.7, which was determined by measuring their absorbance at a wavelength of 600 nm. The inoculants were mixed with the melted agar medium at a ratio of 1:100 (1%), homogenized, and poured into a sterile Petri dish. Disk paper was diluted with a test solution (peptide fraction, 15 μg) and affixed to the frozen media. The positive control used was amoxicillin (10 μg), and the negative control used was sterile distilled water. The disk paper was incubated overnight at 37℃. Then, the diameter of the clear zone or zone of resistance that formed around the disk paper was measured.

Identification of antibacterial peptides in hydrolysate fractions

Peptides in the fraction were separated using a liquid chromatography system equipped with the Acclaim® PepMap RSLC column (C18, 1mm × 150cm, with a particle size of 3µm). The peptides in the column were eluted using gradient mobile phases composed of 0.05% TFA in water (mobile phase A) and water/acetonitrile at a ratio of 20:80 and 0.1% TFA (mobile phase B). Mobile phase containing 96% A and 4% B flowed at 0.3µL/min from 0 min to 2.99min, changed to 90% A and 10% B at the 3rd min, changed linearly to 80% A and 20% B at the 30th min, and changed to 5% A and 95% B at the 70th min and maintained until the 90thmin. MS and tandem mass spectrometry (MS/MS) analyses were conducted using the electrospray ionization method. The positive ion mode, which is operated with high resolution and accurate mass mode, was used for MS detection. MS/MS peptide analysis used the m/z range of 350–1,800 with full-MS/dd-MS2 mode. The resolving power was set at 140,000 full width at half maximum (FWHM) for the full-MS scan. Meanwhile, the resolution was set at 17,500 FWHM for the dd-MS2 scan.

For peptide identification, the MS raw data were processed using the Proteome Discoverer Software ver. 2.2. Peptide identification was done by processing data using the Fusion Basic Sequest HT workflow and a basic consensus workflow. The algorithm used in peptide analysis is Sequest HT. The database used is a genomic database of castor bean plantations (*R. communis*), which was downloaded from UniProt.org (The UniProt Consortium, 2019). Peptide identification was completed by comparing the research data with the database used.

RESULTS AND DISCUSSION

Production of the jatropha seed protein hydrolysate

SDS, a biomolecule-solubilizing agent is widely used to treat protein before polyacrylamide gel electrophoresis. This is known as the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. The use of SDS to extract protein is preferable to strong acid and base because it disrupts the secondary, tertiary, and quaternary structures of protein without any hydrolytic side effects (Zhou *et al*., 2012). The extraction of protein from castor beans using 0.01% SDS yielded up to 20.94% of fat-free seed powder. This yield was quite high considering that the seed is not only composed of protein, and the concentration of SDS used was low. These results are consistent with those obtained by Lacerda *et al*. (2014) who were able to extract protein from Jatropha seed, with up to 11.4% to 27.3% yield. The increase in SDS concentration increased the yield of the extraction process. However, because the protein would be subjected to downstream processes, including enzymatic digestion and MS analysis, the use of a high concentration of SDS was avoided. At a concentration of 0.1%, SDS could inhibit protease enzymes such as trypsin, because it disrupts the secondary and tertiary structures of trypsin. A higher concentration of SDS could lead to ion suppression that interferes with ion exchange fractionation and MS analysis of the peptides (Scheerlinck *et al*., 2015).

Dialysis was performed to remove small molecules including SDS, obtained from the extract before tryptic digestion. A clean extract could ensure the success of the digestion process, as indicated by the degree of hydrolysis. Trypsin hydrolysis was able to digest up to 82.15% of Jatropha seed protein, as indicated by the hydrolysis data. This high result was supported by the quality of trypsin that exhibited an activity of up to 2,500 U/mg. A high degree of hydrolysis is unnecessary in peptide identification. Sornwatana *et al*. (2013) succeeded in identifying active peptides from protein hydrolysate, with a 3.7% degree of hydrolysis. However, a large amount of unhydrolyzed protein present in hydrolysate could hinder peptide identification. Therefore, extra preparation is essential before MS analysis.

Antibacterial activity of the protein hydrolysate fraction

The peptides obtained from trypsin hydrolysis were fractionated using a cation exchange SPE column. SPE is mainly used to concentrate trace organic compounds to enable detection using certain analytical tools. However, ion exchange SPE, where pH or ionic strength gradient could be employed during elution, makes fractionation possible. Several reports proposed the use of SPE for fractionation (Fauland *et al*., 2013; Cutignano *et al*., 2015) and obtained satisfying results. SPE fractionation of the Jatropha seed protein hydrolysate was performed using a strong cation exchange SPE column and fractions were collected on the basis of the pH of the elution buffer varying from pH 4 to pH 8.

The highest yield of 9.14% of the total peptides present in the hydrolysate was obtained from the pH 4 fraction, whereas the lowest yield was obtained from the pH 8 fraction. Cation exchange fractionation was chosen because it is compatible with the separation process. All peptides could be separated because it is easy to achieve a low pH that renders peptides as cations. Cation exchange fractionation also provided better resolution for peptides than other fractionation techniques, such as IEF and SDS-PAGE (Mostovenko *et al*., 2013).

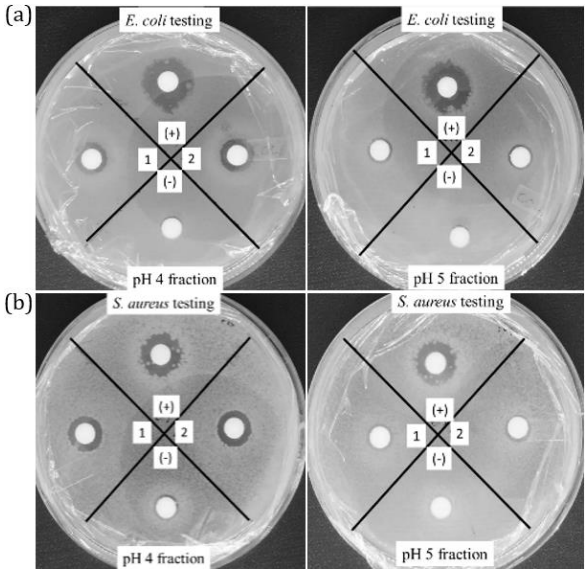

Figure 1. Growth inhibition zones of the pH 4 and pH 5 fractions of *R. communis* seed protein hydrolysate for *E.coli* (a) and *S. aureus* (b). Amoxicillin and distilled water are used as positive control (+) and negative control (−), respectively, and each fraction was replicated (1 and 2)

Table I. Inhibition zones of the peptide fractions of *R. communis* seed protein hydrolysate for *E.coli* and *S. aureus*

Sample	Diameter of the inhibition zone (mm)	
	E. coli	***S. aureus***
pH 4 fraction	4.0	4.5
pH 5 fraction	2.0	2.0
pH 6 fraction	0	0
pH 7 fraction	0	0
pH 8 fraction	0	0
Positive control	9.6	6.4
Negative control	0	0

The data show that the pH 4 and pH 5 fractions had antibacterial activity against both *E. coli* and *S. aureus* as representatives of gram-negative and gram-positive bacteria, respectively (Figure 1 and Table I). The disk diffusion method was used because it is a simple and low-cost technique, particularly for compounds with low concentrations, such as natural products or peptides present in the fraction (Das *et al*., 2010; Caron *et al*., 2012; Balouiri *et al*., 2016). Furthermore, the method has been established as a standard method for determining antimicrobial activity (CLSI, 2012). Peptides of the pH 4 fraction exhibited higher antibacterial activity than the pH 5 peptide fraction, as indicated by the diameter of the inhibition zone. The pH 4 fraction has a 4.0 and 4.5mm inhibition zone for *E. coli* and *S. aureus*, respectively, whereas the pH 5 fraction has a 2.0mm inhibition zone each for both bacteria. However, the activities in the negative control were lower than that in the positive control, even though the amount of peptides (15 µg) in the disk was more than the amount of amoxicillin (10 µg). The peptide fraction might consist of more than one compound or peptide and not all of the components were active and thus, the actual amount of the active peptides could be lower than that of amoxicillin.

Identification of the antibacterial peptides

Given that only pH 4 fraction of Jatropha seed protein hydrolysate exhibited interesting antibacterial activity, peptide identification of the fractions was further conducted. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) equipped with an HRMS using *de novo* peptide sequencing is one of the gold standard methods used for proteomic studies (Syka *et al*., 2004; Coon *et al*., 2005; Hunt *et al*., 2015; Tran *et al*., 2017). Protein identification could be performed using the peptide sequence tag obtained by MS/MS analysis and the protein and genome database search engine (Nesvizhskii *et al*., 2007; Cottrell *et al*., 2011). Meanwhile, the available genome and protein databases of certain species could be used to characterize certain peptides, even though the fragmentation data obtained using MS/MS peptide analysis are incomplete. The genome sequence of *R. communis* is available in GenBank, making peptide identification of pH 4 fraction using the UniProt database possible.

The results of LC-MS/MS analysis of pH 4 fraction using the Orbitrap HRMS are plotted in the total ion chromatogram (TIC) (Figure 2).

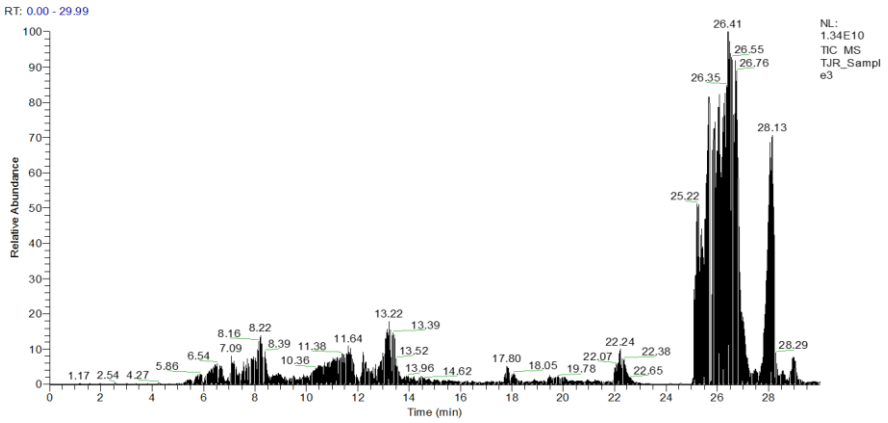

Figure 2. Total ion chromatogram of the pH 4 fraction of *R. communis* seed protein hydrolysate analysis obtained using high-performance liquid chromatography coupled with high-resolution mass spectrometry.

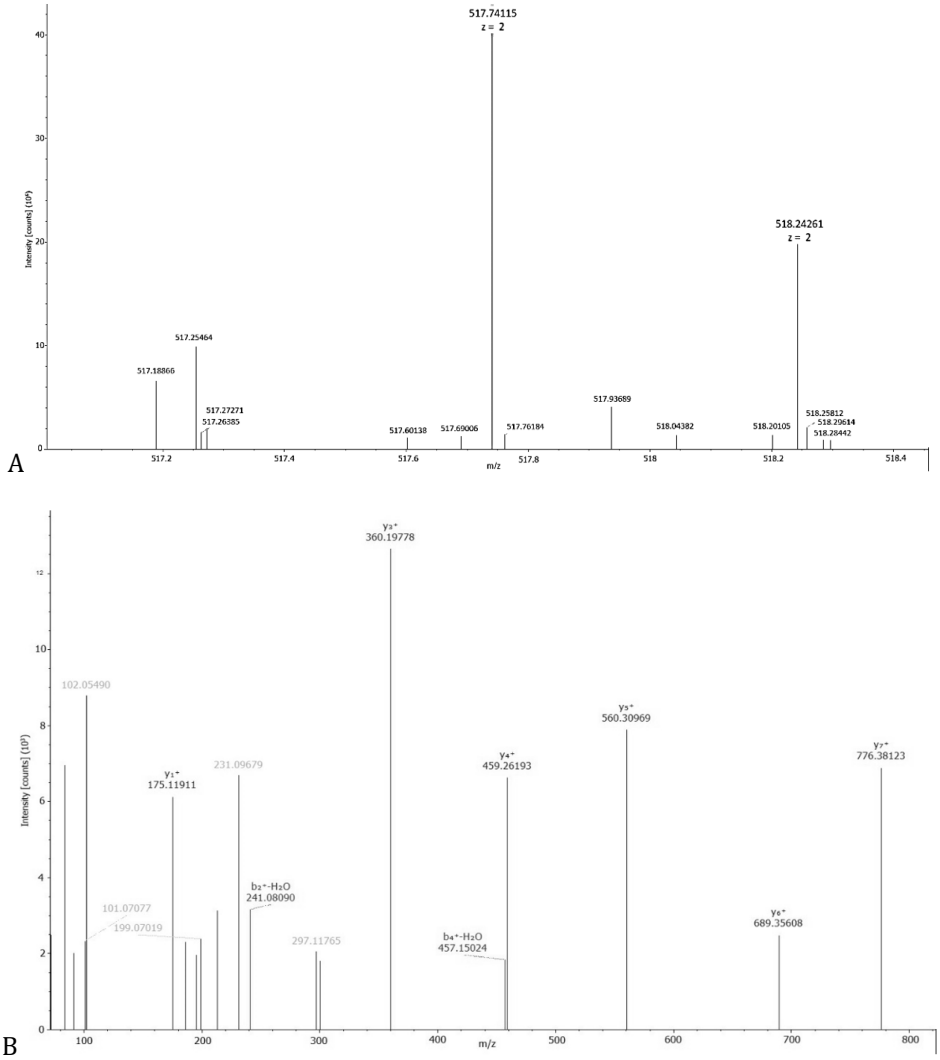

Figure 3. MS spectra of peptide number 1 of the pH 4 fraction of *R. communis* seed protein hydrolysate. MS^1 identifies the isotopic pattern of the peptide ion (a), whereas MS^2 identifies the b^+ and y^+ ions from the peptide fragmentation data (b).

The TIC shows the raw data of all ions detected using both MS^1 and MS^2. MS^2 was performed using data-dependent MS^1 (dd-MS), with 10 ions of MS^1 with the highest intensity subjected to collision ionization of MS^2. Three MS^2 data from TIC of pH 4 fraction were confirmed as peptides of Jatropha protein.

The first peptide has m/z = 517.74115 (z=2) that indicate several isotopic ions with low intensity (Figure 3a). The molecular mass (MH^+) of the peptide is 1,034.47502, and on the basis of this mass, the peptide is predicted to be composed of 9 to 10 amino acids. The MS^2 data of the peptide confirm the presence of several y^+ ions and two b^+ ions and y_1^+ ion=175.1191 representing the R amino acid of trypsin, confirming the presence of R or K at the carboxyl (C) terminal of the peptide (Figure 3b). The difference between the y_3^+ ion (360.19778) and the y_1^+ ion (175.11910) is 185.07867, which matches the mass of QG supported by the presence of the immonium ion (101.07080) of Q. The difference between the y_4^+ ion (459.26103) and the y_3^+ ion is 99.06415, representing the V amino acid, followed by T (101.04866), E (129.04639), and S (87.02515), as derived from the difference between the y_5^+ ion (560.30969) and the y_4^+ ion, between the y_6^+ ion (689.35608) and the y_5^+ ion, and between the y_7^+ ion (776.38123) and the y_6^+ ion, respectively. The interpretation of the y^+ ion confirmed the presence of the sequence SETVGQR of part of the first peptide, according to Proteome Discoverer analysis. The interpretation of the b^+ ion confirmed the presence of the sequence SE (216.018191), as derived from the difference between the b_4^+ ion (457.15654) and the b_2^+ ion (241.08191). The presence of the strong immonium ion of E (102.05490) indicated the presence of E at the amine end of the peptide [32, 33]. The analysis of the MS/MS raw data using the database search engine Proteome Discoverer confirmed the sequence EESETVGQE of the peptide with the molecular mass (MH^+) = 1,034.47502. The peptide is part of 2S albumin of *R. communis* located at amino acid numbers 129–137.

The MS^1 result of the second peptide with m/z = 574.76862 (z = 2) or molecular ion (MH^+) = 1,148.52995 (Figure 4a). MS^2 nine y^+ ions and four b^+ ions were detected (Figure 4b). The y_1^+ ion = 175.11896 confirms the presence of the R amino acid at the C terminal of the peptide. The mass difference between the y_2^+ ion (304.16147) and the y_1^+ ion confirms the presence of E (129.04251), leading to the sequence ER at the C terminal. Consecutively, the mass difference between the y_3^+ ion (432.21982) and the y_2^+ ion is 128.05858 (Q), that between the y_4^+ ion (560.28015) and the y_3^+ ion is 128.05858 (Q), that between the y_5^+ ion (617.30017) and the y_4^+ ion is 57.02147 (G), that between the y_6^+ ion (718.34924) and the y_5^+ ion is 101.04768 (T), that between the y_7^+ ion (775.36969) and the y_6^+ ion is 57.02147 (G), that between the y_8^+ ion (876.41632) and the y_7^+ ion is 101.04768 (T), and that between the y_9^+ ion (963.44830) and the y_8^+ ion is 87.03203 (S), which confirm the amino acid sequence STGTGQQER. Meanwhile, additional information was obtained from the interpretation of the b^+ ion mass. The lowest mass of the b_2^+ ion, i.e., 186.08737, indicates that the amine end of the peptide could be QG or GQ. The mass difference between the b_3^+ ion (273.11890) and the b_2^+ ion is smaller than that between b_4^+-H_2O (356.15588) and b_2^+ and that between b_6^+-H_2O (514.22394) and b_4^+-H_2O, confirming the sequence identified using the interpretation of the y^+ ion mass. De novo sequencing of the MS/MS raw data using the Proteome Discoverer database confirmed the presence of the sequence GQSTGTGQQER of the peptide with the molecular mass (MH^+) = 1,148.52995. The peptide is part of legumin B of *R. communis* located at amino acid numbers 125–135.

The MS^1 result of the third peptide with m/z = 521.76379 (Figure 5a). MS^2 detected eight y^+ ions and two b^+ ions (Figure 5b). The y_1^+ ion = 175.11896 confirms the presence of the R amino acid at the C terminal of the peptide. The mass difference between the y_2^+ ion (289.16189) and the y_1^+ ion confirms the presence of N (114.04293), leading to the sequence NR at the C terminal. Consecutively, the mass difference between the y_3^+ ion (404.18884) and y_2^+ ion is 115.02695 (D), that between the y_4^+ ion (501.24161) and the y_3^+ ion is 97.05277 (P), that between the y_5^+ ion (630.28421) and the y_4^+ ion is 129.04260 (E), that between the y_6^+ ion (743.36828) and the y_5^+ ion is 113.08407 (L), that between the y_7^+ ion (814.4054) and the y_6^+ ion is 71.03712 (A), and that between the y_8^+ ion (929.43235) and the y_7^+ ion is 115.02695 (D), which confirm the amino acid sequence DALEPDNR. Meanwhile, additional information was obtained from the interpretation of the b^+ ion mass. The mass of the b_2^+ ion, i.e., 229.11830, indicates the presence of D (115.02695). Therefore, the mass difference between the b_2^+ ion and the b_1^+ ion (D) is equal to 114.09135. The presence of two amino acids, i.e., I and L, indicate two possible sequences at the N terminal.

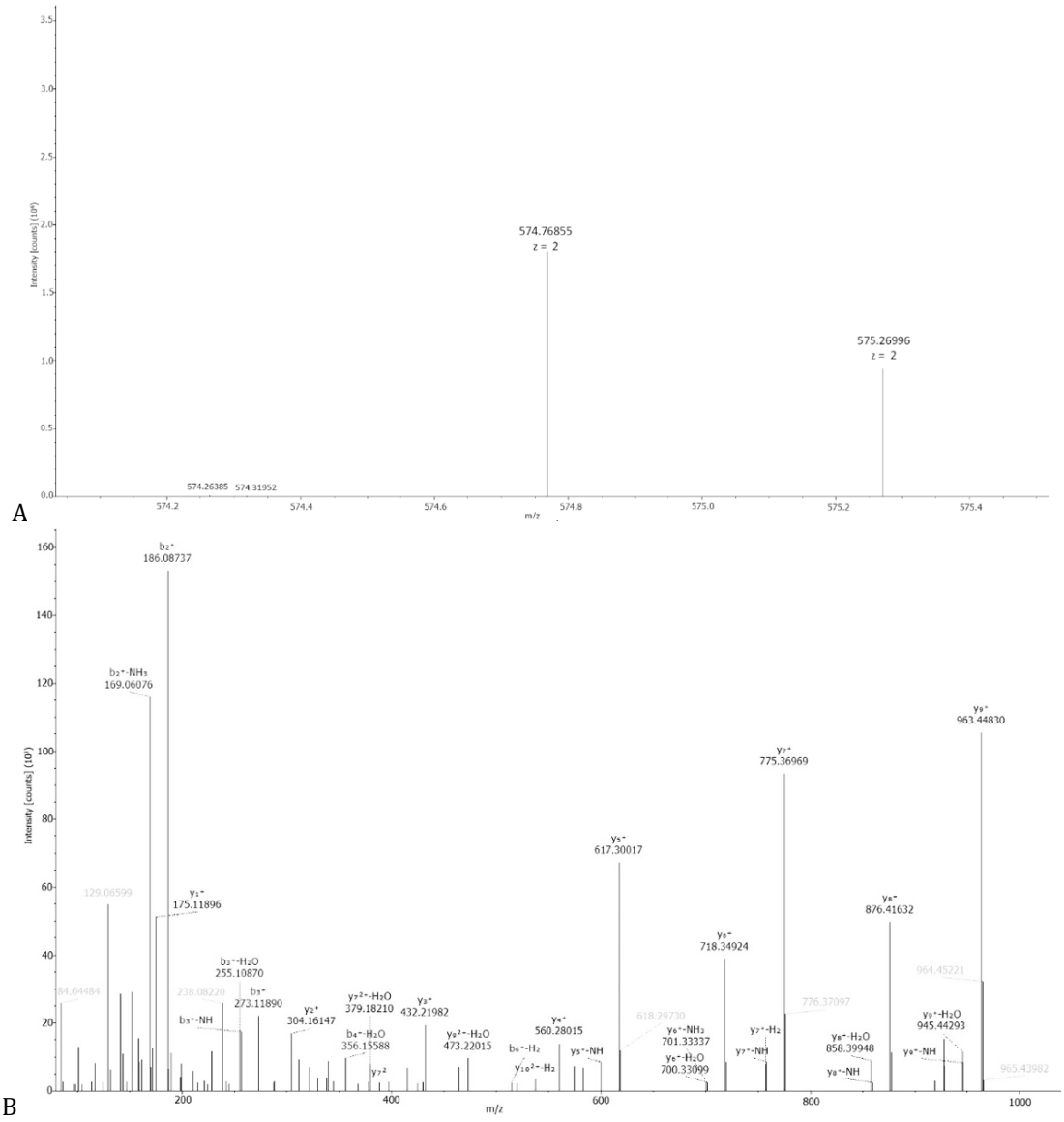

Figure 4. MS spectra of peptide number 2 of the pH 4 fraction of *R. communis* seed protein hydrolysate. MS^1 identifies the isotopic pattern of the peptide ion (a), whereas MS^2 identifies the b^+ and y^+ ions from the peptide fragmentation data (b).

The analysis of the MS/MS raw data using the Proteome Discoverer database confirmed the presence of the sequence LDALEPDNR of the peptide with the molecular mass (MH^+) = 1,042.52031. The third peptide is part of legumin A of *R. communis* located at amino acid numbers 33–41.

The main mechanism of antibacterial compounds is cell lysis due to the inhibition of cell wall biosynthesis through the penicillin mechanism or the disruption of the bacterial cell membrane. Ionic compounds usually follow the cell membrane disruption mechanism. Several peptide antibiotics, such as gramicidin, bacitracin, and polymyxin, have been successfully developed through this mechanism (Xue *et al*., 2018; Zhao *et al*., 2018). Identified peptides from the pH 4 fraction of the Jatropha seed protein hydrolysate (Table II). The amino acid composition shows that all three peptides have ionic properties as only a few amino acids have a nonpolar side chain.

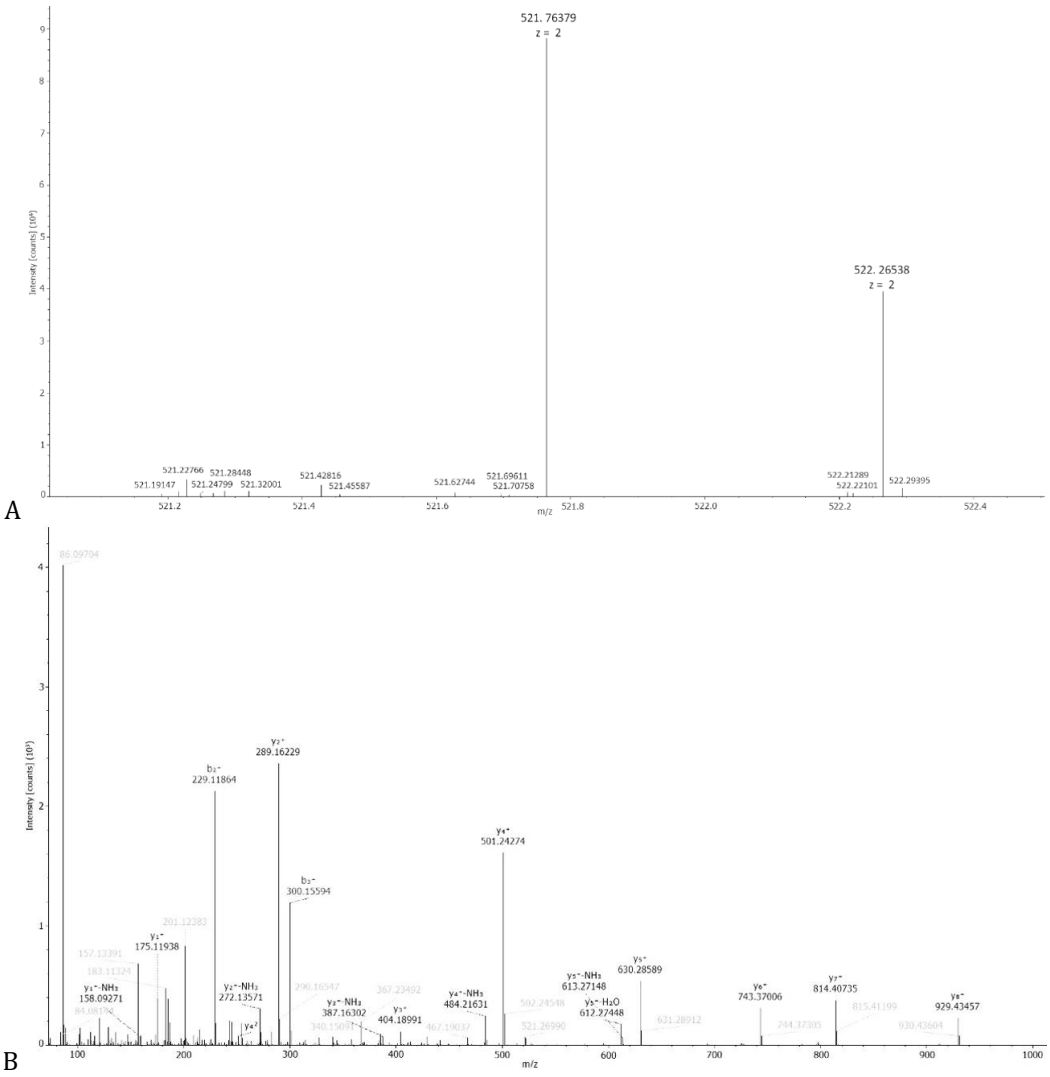

Figure 5. MS spectra of peptide number 3 of the pH 4 fraction of *R. communis* seed protein hydrolysate. MS^1 identifies the isotopic pattern of the peptide ion (a), whereas MS^2 identifies the b^+ and y^+ ions from the peptide fragmentation data (b).

Table II. Characteristics of the peptides of the pH 4 fraction of the jatropha seed protein hydrolysate

No.	Peptide sequences	Predicted pI	MH^+	Protein of *R. communis*	Amino acid position in the protein
1	EESETVGQR	4.25	1,034.47502	2S albumin	129–137
2	GQSTGTGQQER	6.00	1,148.52995	Legumin B	125–135
3	LDALEPDNR	4.03	1,042.52031	Legumin A	33–41

According to the predicted pI, the first and third peptides will present strongly as ions at physiological pH, whereas the second peptide will be slightly ionized. The ionic structure of peptides is considered to be responsible for the disruption of the bacterial cell membrane because it could interact with the charged head of the phospholipid bilayer of the cell membrane. The interaction could disrupt the intermolecular hydrogen bonding between phospholipids, leading to the collapse of the cell membrane.

The secondary structure of the peptide also determines antibacterial activity, where the random coil structure could lead to the disruption of the membrane structure when the peptide interacts with the membrane (Sila *et al*., 2014a). However, the sequence of the characterized peptides is too short to be modeled to identify their secondary structure. Despite that, on the basis of the secondary structure of the entire protein, the secondary structure of the peptide can be considered as a part of the protein. Modeling of 2S albumin, the first peptide identified using JPred 4 (a protein secondary structure prediction server), revealed the sequences of EESETVGQR and EESERVAQR located at the β-turn between α-helix 1 and α-helix 2. Modeling of legumin B, the protein where the second peptide originated, also showed that the sequence GQSTGTGQQER is part of the turn between sixth and seventh β-sheets of the protein. Structural modeling of legumin A, the protein of *R. communis* where the third peptide originated, showed that the sequence LDALEPDNR manifests as a random coil in front of the first β-sheet structure. All data from the three peptides, which manifests in the ionic form with non-helix and sheet secondary structures according to modeling, support the antimicrobial activity of the peptide fraction containing these peptides.

CONCLUSION

The protein of jatropha (*R. communis*) seed can be a source of antibacterial peptides. Tryptic digestion of protein, followed by cation exchange fractionation, showed that the pH 4 fraction of the hydrolysate has activity against both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria. Three peptides having the sequences of EESETVGQR, GQSTGTGQQER, and LDALEPDNR identified in the pH 4 hydrolysate fraction could be responsible for the activity. The antibacterial activity of these peptides, which is due to their ionic properties and secondary structure, supports the disruption of the bacterial cell membrane.

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