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Two novel antimicrobial and anticancer peptides prediction from *Vibrio sp*. strain ES25

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ABSTRACT

Proteins from microbes are a large source of potential raw material for novel pharmaceuticals. Endophytic bacteria associated with algae associated have been shown to be particularly promising candidates. In a previous study, a crude protein extract was isolated from an endophytic *Vibrio sp*. strain ES25. The extract was found to have anti-microbial activity. Despite these promising results, further work was needed to characterize both the protein and the bacteria that produce it. In this study, we sought to increase the activity of the previously discovered protein through pepsin digestion as well as provide sequence data to characterize the bacterial isolate. Here, we show promising evidence that the hydrolysate produced (QA1d) was found to have a promising antimicrobial as well as anticancer properties. Through the DNA sequencing, we also provide better taxonomic information on the isolate. Finally using Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS) and *in silico* prediction, we identify to novel peptides produced from the bacterial protein that were predicted to have multifunctional activity. These results shown here indicate these peptides are promising candidates for anticancer drug development or antibacterial agents for the food industry.

INTRODUCTION

Bioactive peptides are a versatile raw material for producing pharmaceutical drugs. This is due to their specific and interactive nature. Low-molecular weight peptides in particular have been shown to have strong bioactivity (Wang and Zhang, 2016). These bioactive peptides are produced from proteins by breaking the peptide bonds to produce simple chain amino acids (Kadam *et al*., 2015).

Peptides found endophytically within microbial hosts have been shown to be bioactive. For example, in the endophytic fungus, *Aspergillus awamory* found in *Acacia nilotica* produces antidiabetic peptides (Singh and Kaur, 2016). Unguisin F, a novel cyclic peptide, has been isolated from *Mucor irregularis* that is hosted in *Moringa stenopetala*, and has antibacterial and antifungal properties (Akone *et al*., 2016). An endophytic bacteria isolated from red pepper leaf has also been found to produce antimicrobial and antiangiogenic peptides (Jung *et al*., 2015).

A crude protein extract from the endophytic *Vibrio sp*. bacterial strain ES25, isolated from *Eucheuma spinosum*, was previously proved to be intermediately active as an antibacterial and anticancer agent (Sugrani *et al*., 2019b). However, the bioactivity of a protein can be increased by the hydrolysis of proteins into peptides (Wang and Zhang, 2016). In this study, authors have first sought to provide better taxonomic information on the ES25 isolate that was previously identified through morphology by using DNA sequencing. The authors have then hydrolyzed the crude protein extract into peptides and tested its antagonistic activity against pathogenic bacteria and HeLa cell lines as well as predict functionality *in silico.* Finally, we present two novel peptides: QA1d-4 and QA1d-8 identified using Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy (LC-MS/MS). These peptides are promising candidates.

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MATERIALS AND METHODS

Strain and growth conditions

The bacterial strain ES25 was previously reported to originate from the genus *Vibrio* and was isolated from *E. spinosum* from Takalar, South Sulawesi, Indonesia (Sugrani *et al*., 2019b). It was grown for 48 hours on a heated orbital shaker in a nutrient broth (NB, Oxoid) at 37°C and 150 rpm. The antibacterial activity of the isolate was tested against *Staphylococcus aureus* and *Eschericia coli* (collected from Hasanuddin University Medical Research center, Makassar, Indonesia).

Identification of bacteria

Isolated strains were identified by the rRNA sequencing. DNA was isolated using a Cetyl trimethylammonium bromide (CTAB) method (Kakade and Chaphalkar, 2017). RNA template amplification using universal primary numbers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387R (5′-GGGCGGAWGTGTACAAGGC-3′) (Hailu *et al*., 2015). DNA amplification was carried out in a T100™ BioRad thermocycler machine provided applied biosystems with conditions: 95°C (15 minutes); 40 cycles of 94°C (60 seconds), 55.0°C (30 seconds), 72.0°C (60 seconds), final extension 72°C (5 minutes). The product was examined by 1% agarose gel stained with ethidium bromide. The gel was eluted by the SIGMA gel elution kit. The bacterial sequence was identified using the Basic Local Alignment Search Tool (BLAST) algorithm on NCBI GenBank. Phylogenetic trees were built by the Neighbor-Joining method using Clustal Omega online.

Isolation of proteins from bacteria

Fifty grams of the bacterial culture added to 500 ml of HCl tris buffer and was stirred with an electric stirrer for 24 hours at 5°C. The bacterial cells were ultrasonicated (BANDELIN Sonopuls, frequency 20 Hz, 60% amplitude, for 5 minutes) and then freeze-thawed 2–3 times, 500 ml mixture was centrifuged at a speed of 3,200 rpm for 10 minutes. After that, the supernatant was fractionated using ammonium sulfate $(NH_4)_2SO_4$. The dissolved protein at each fractionation was extracted and then pelletized through centrifugation at 13,000 rpm for 10 minutes at 4°C. The protein obtained was dialyzed for 12 hours at a temperature that remained cool at 25°C–35°C. The protein concentration in the extract was then measured using Lowry method, 4 ml sample added with Lowry B [mixture of 100 ml sodium carbonat (Na_2CO_3 2% dalam 0.1 M NaOH), 1 ml potassium sodium tartate ($KNaC_4H_4O_6$ 2%), and 1 ml copper sulfate pentahydrate ($CuSO_4.5H_2O$ 1%)]; shaken and allowed to stand at room temperature for 30 minutes; then added 0.5 ml Lowry A [Folin-Ciocalteu dalam H_2O (1:1 *v/v*)]; allowed to stand again at room temperature for 30 minutes. The absorbance was read at 650 nm, Bovine Serum Albumin as a standard (0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg/ml) (Maehre *et al*., 2018)

Hydrolysis of protein

The protein fraction was diluted into a 3% buffer solution and was hydrolyzed using pepsin (EC 3.4.23.1) using a 6% w/w enzyme to substrate ratio (E/S) at 37°C, pH 2 for 2 hours. The reaction was stopped by heating the mixture to the boiling point of water for 10 minutes. The hydrolysate was then cooled and centrifuged at 10,000 rpm and 4°C for 20 minutes. Finally, the hydrolysate was lyophilized before storage (Wang and Zhang, 2016). A 27.15% degree of hydrolysis was found to have occurred.

Ultrafiltration

The lyophilized hydrolysate was ultrafiltrated by separation membrane molecular-weight-cut-off using a Vivaspin 20 Fisher Scientific. Fractions were collected in the range of: <3 kDa, 3–5 kDa, 5–10 kDa, and >10 kDa and stored at −20°C (Guo *et al*., 2015). These fractions were named as QA1d, QA1c, QA1b, and QA1a, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the collected peptides was determined by SDS-PAGE using a 2–250 kDa marker and a Dual Xtra 161-0377 Bio-Rad. The 3 mg/ml of the peptide and the markers were dissolved in the sample buffer and transferred to the gel for electrophoresis (height 8 cm, width 10 cm, and thickness 0.6 mm). Electrophoresis was conducted at 60 mA for 3–4 hours (Naimah *et al*., 2018).

Toxicological assay

Toxicological thresholds were determined using a brine shrimp lethality assay (Sugrani *et al*., 2019b). *Artemia salina* eggs were hatched using seawater for 48 hours. The protein compounds were then prepared in concentrations of 1, 10, 100 ppm in 5 ml seawater. Ten shrimp larvae were put into a bottle, but before the bottle has been filled with seawater. After 24 hours, the live larvae were counted. Seawater was used as a negative control and Dimethyl sulfoxide (DMSO) as a positive control. Probit analysis was then used to obtain LC_{50} values.

Antibacterial assay

The antibacterial activity of the collected proteins and peptides were tested using an agar diffusion method (Sugrani *et al*., 2019a) on the bacteria *S. aureus* and *E. coli*. Medium Muller Hinton Agar was sterilized using an autoclave set to 121°C a pressure of 15 psi. The 15 ml of media was poured aseptically into a petri dish and then inoculated 200 µl of bacterial suspension. Paper discs (about 6 mm in diameter), containing of the peptides or proteins applied as a 20 ul were then placed on the surface of the agar and incubated for 24 hours at 37°C before measuring the inhibition zone.

Antiproliferation assay

100 µl of HeLa cancer cells at a density of 106 cells per well were placed into 96 wells. The cell suspension was incubated in an incubator at 37°C for 24 hours. 20 µl peptides (concentration series were 200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 µg/ml) was added to the wells. After 24 hours incubation, 20 µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added in each well and further incubated for 4 hours at 37°C, 5% CO_2. The resulting formazan

precipitate was dissolved by adding 100 µl DMSO and incubated again at 37°C for 5 minutes. Absorbance was measured at 540 nm by a microplate reader to calculate cell viability. Medium without peptide and mixed with cisplatin as negative and positive control (Chu *et al*., 2015).

Mass spectrometry (MS) and de novo sequencing

LC and MS/MS analyses were performed using a UPLC-MS [ACQUITY UPLC®H-Class System (Waters, Milford, MA)] with a binary pump. In this system, the liquid chromatography is associated with a Quadrupole Time of Flight (QTOF) mass spectrometer with an Electrospray ionization (ESI) ionization source. Mass spectroscopy was performed with a Xevo G2-X2 QTof using the positive ionization mode. The ESI parameters used were full scan mode from *m/z* 50–3,000 Da, a column temperature of 50°C, and room temperature 25°C. A UPLC CORTECS C8 Column (2.7 µm, 2.1 mm × 100 mm) was used. Bioactive peptides from protein hydrolysates were identified by their amino acid sequences based on *m/z* spectrum values by the *de novo* sequencing, using the MASCOT Program (Wang and Zhang, 2016).

In silico prediction

Anticancer activity was predicted by the using ANTICP (https://webs.iiitd.edu.in/raghava/anticp/) (Tyagi *et al*., 2013). CAMPR3 (http://www.camp.bicnirrh.res.in/predict/) was used for antibacterial prediction (Waghu *et al*., 2016). ProtParam was used to calculate various physical and chemical parameters of the isolated peptides (http://web.expasy.org/protparam) (Gasteiger *et al*., 2005).

RESULTS AND DISCUSSION

Based on the BLAST analysis of the 16S rRNA sequence (Table 1), ES25 was 97% similar to *Vibrio algivorus* strain SA2 (GenBank ID: NR_151933.1), and therefore is unlikely to be a new species (Mansfield *et al*., 2015). The further assessment of the taxonomic position of the isolate using a phylogenetic tree confirmed a close relationship to. *Vibrio algivorus* strain SA2 (Fig. 1). The fact that ES25 sits on a new branch may indicate a novel linage to those previously identified.

The four protein hydrolysates were tested for their inhibitory activity against bacteria (*E. coli and S. aureus*). The results showed that QA1c and QA1d had a strong activity against *S. aureus*, whereas for *E. coli*, all four hydrolysates had a weak activity (Table 2). In addition, QA1d was found to be very toxic to *Artemia salina* (Table 3). Based on these antibacterial and toxicology results, QA1c and QA1d continued at the next stage. SDS-PAGE electrophoresis results showed that both QA1c and QA1d had a low-molecular weight (Fig. 2).

Based on the relationship of peptide concentrations with % viability of HeLa cells (Figs. 3 and 4), only QA1d peptides can contribute to HeLa cell inhibition but QA1c peptides can't. According IC_{50} to concentration values published by Weerapreeyakul *et al*. (2012), the IC_{50} value for QA1d (248.18 µg/ml) and (>4,491.88 µg/ml) would place them in the moderate and not cytotoxic categories, respectively.

QA1d amino acid composition was identified using LC-MS/MS analysis and *de novo* sequencing. The results of the liquid chromatography analysis showed nine peaks (Fig. 5) indicating nine separate peptides in the QA1d protein hydrolysate (Stan *et al*., 2016). Each chromatography peak was further analyzed by MS/MS using Masslynx V4 (Fig. 6). Results from *de novo* sequencing using the MASCOT program can be seen in Table 4. One peptide was excluded from further analysis as it scored less than 60 (Wang and Zhang, 2016).

The eight peptides were further analyzed using *in silico* antibacterial analysis using CAMPR3 (http://www.camp.bicnirrh.res.in/predict/) and anticancer using AntiCP (https://webs.iiitd.edu.in/raghava/anticp) (Table 5). Although, *in vivo* and *in vitro* assays have been used elsewhere, these are both financially and time costly and require more sampling. Conversely, *in silico* analysis, can reduce costs, speed up quantification time and minimize the use of test samples (Sucher, 2014).

Table 1. 16S rRNA sequence of endophytic bacterial strain ES25.

Species	16S rRNA sequence
Vibrio sp. Strain ES25	GATGGAGGGAGTTTCGGATTCTGGGCGTAAGCGCATGCAGGTGGTTTGTTAAGTCAGATG
	TGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGAGTACTGTAG
	AGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTG
	GCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACA
	GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTG
	AGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGAT
	TAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
	TGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTACCAGAGATGCATTGG
	TGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATG
	TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAATGTCGG
	AAACTCCAGGGAGACTGCCGGTGATAAAACGGAGGAAGGGGGGGACGACACAAGTCGTCA
	TGGGCCCGTACCAAT

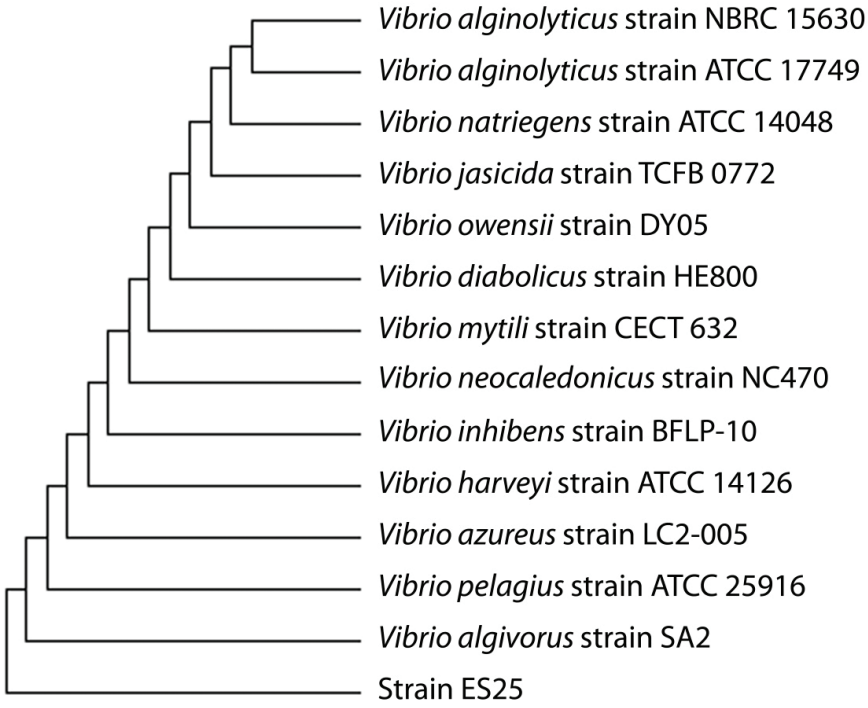


Figure 1. Phylogenetic tree of strain ES25. The phylogenetic tree was constructed by the neighbor-joining (NJ) method.

Table 2. Antibacterial activity of peptides against *S. aureus* and *E. coli.*

Fraction[a]	Inhibition area (mm)[b]			
	S. aureus	Category	*E. coli*	Category
QA1a	1.70 ± 0.57	Weak	1.40 ± 0.14	Weak
QA1b	3.25 ± 0.07	Weak	1.35 ± 0.07	Weak
QA1c	10.95 ± 0.35	Strong	2.00 ± 0.28	Weak
QA1d	13.26 ± 0.65	Strong	3.10 ± 0.57	Weak
K(+)	18.40 ± 2.40	Strong	9.30 ± 5.66	Moderate
K(−)	0.00	Not active	0.00	Not active

[a]Fraction: QA1a: >10 kDa; QA1b: 5–10 kDa; QA1c: 3–5 kDa; QA1d: <3 kDa; K(+): Positive control; K(−): Negative control.
[b]Average of two measurements.

Table 3. LC_{50} values of protein hydrolysate

Fraction[a]	LC_{50} (µg/ml)	Toxicity level
QA1a	>1,000	Not toxic
QA1b	320.01	Moderate
QA1c	4.93	Very toxic
QA1d	1.03	Very toxic

[a]Fraction: Q = Crude extract; QA = Protein 20%; QA1 = Protein hydrolysate; QA1a: >10 kDa; QA1b: 5–10 kDa; QA1c: 3–5 kDa; QA1d: < 3 kDa.

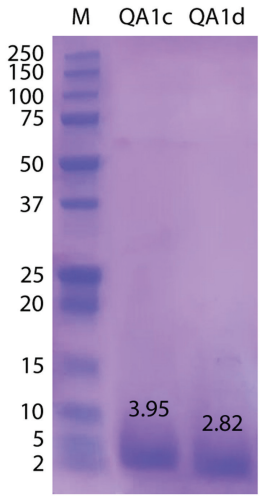


Figure 2. SDS PAGE chromatogram of protein hidrolysate QA1c (MW 3–5 kDa) dan QA1d (MW < 3 kDa) and M = Marker.

Based on the *in silico* analysis, there are two peptides have the potential to be responsible for the antibacterial and anticancer activity of QA1d, QA1d-4 (HAILRGLLCLSLTLAFQPAF), and QA1d-8 (ALHLPLKLMLRPALPLRLKLTL). The exact mechanism of antibacterial and anticancer peptides is not identified in this study and would require further work (Cullen *et al*., 2015; Tyagi *et al*., 2015). There are several potential mechanisms that could be responsible for the activities observed, including the barrel-stave model, the toroidal model, and the carpet model (Li *et al*., 2018; Wang *et al*., 2017). This model mechanism occurs due to electrostatic interactions between cationic peptides (positively charged) with anionic components (negatively charged) from the cancer cell membranes (such as phosphatidylserine) or Gram-positive bacteria (such as peptidoglycan). In general, an interactive relationship peptide and membrane molecules requires direct

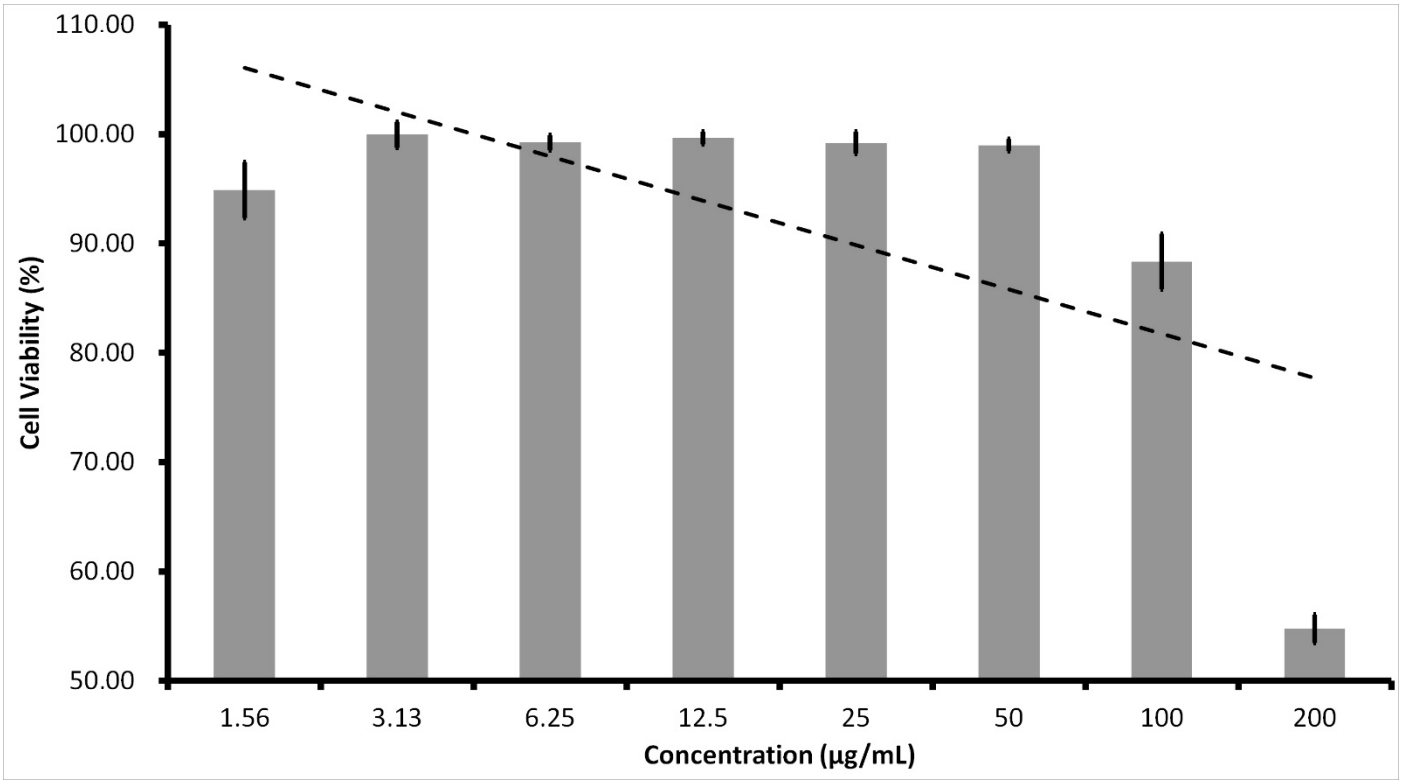


Figure 3. Relationship of QA1d peptide concentrations to the percentage of HeLa cell viability.

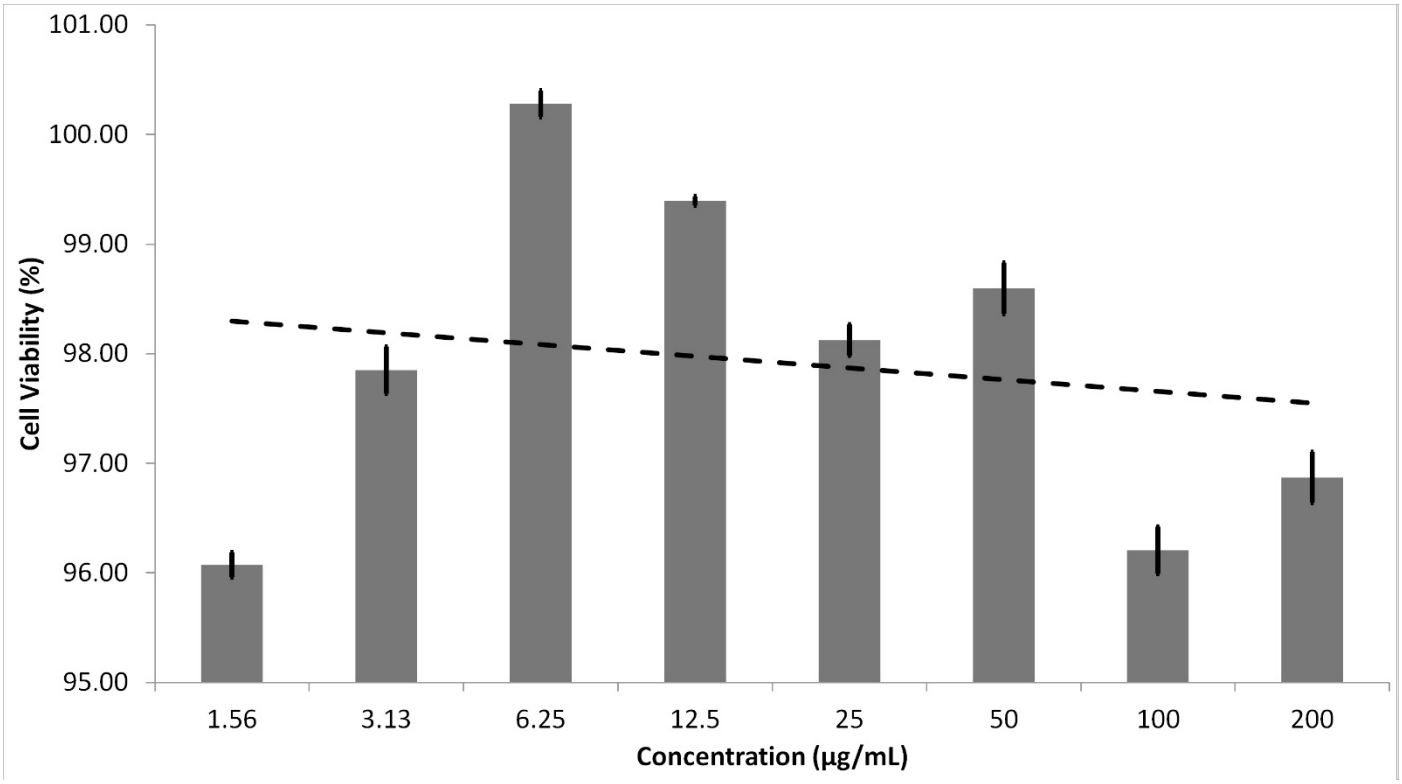


Figure 4. Relationship of QA1c peptide concentrations to the percentage of HeLa cell viability.

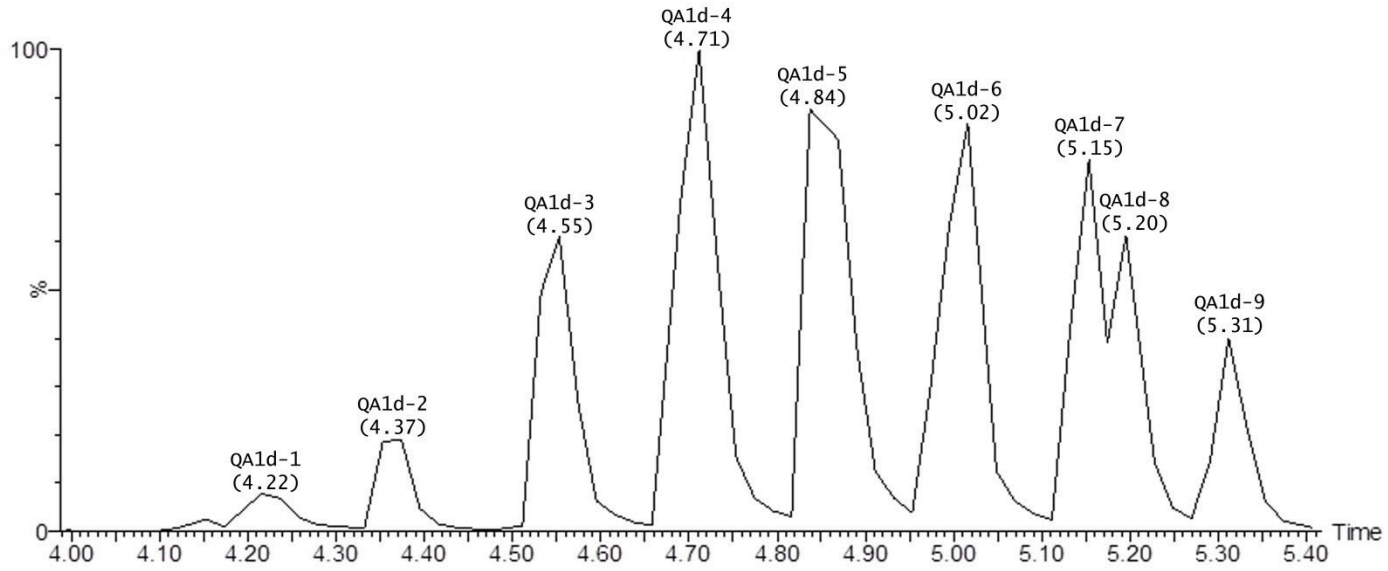
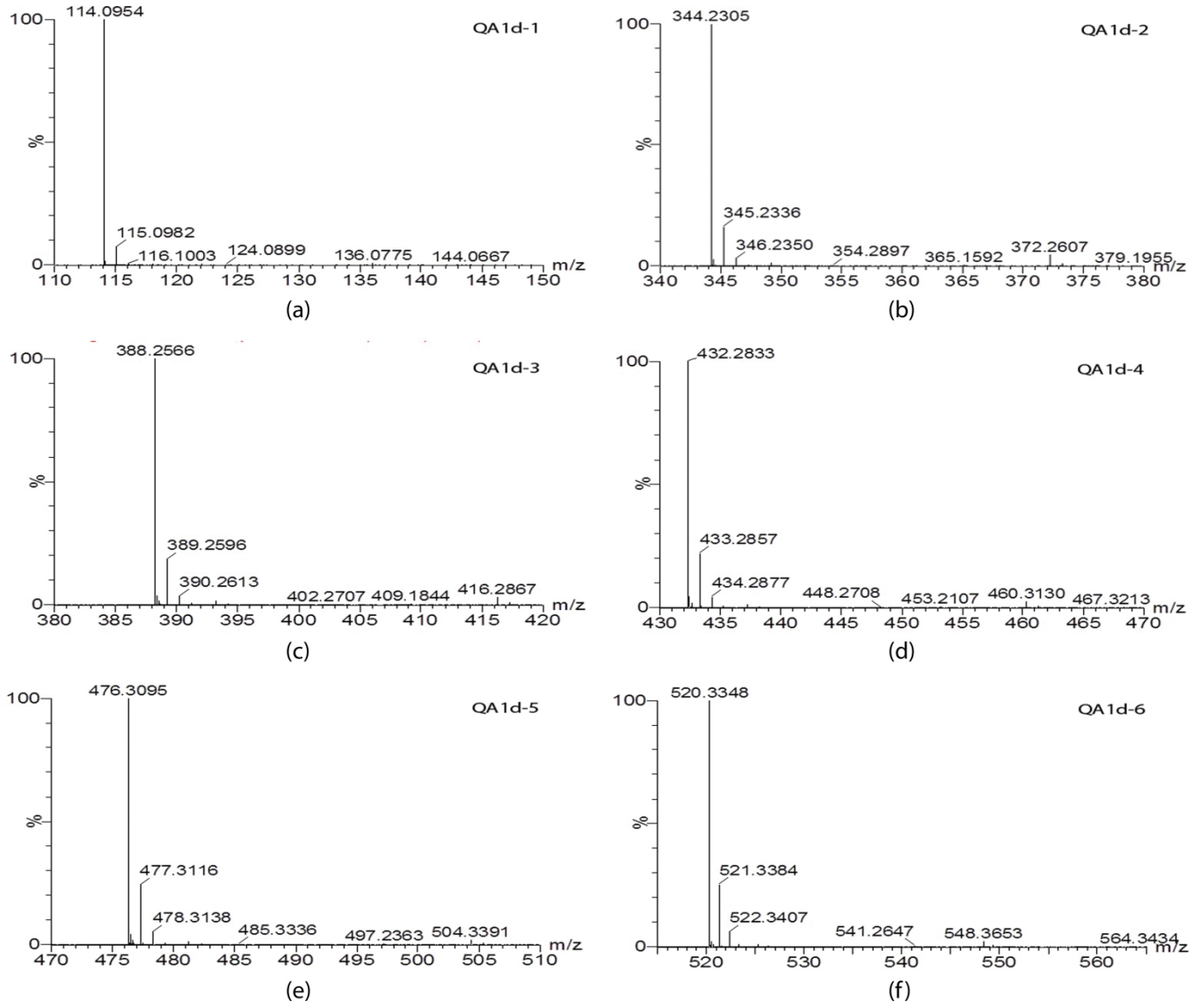


Figure 5. LC profile of protein hydrolysate QA1d.



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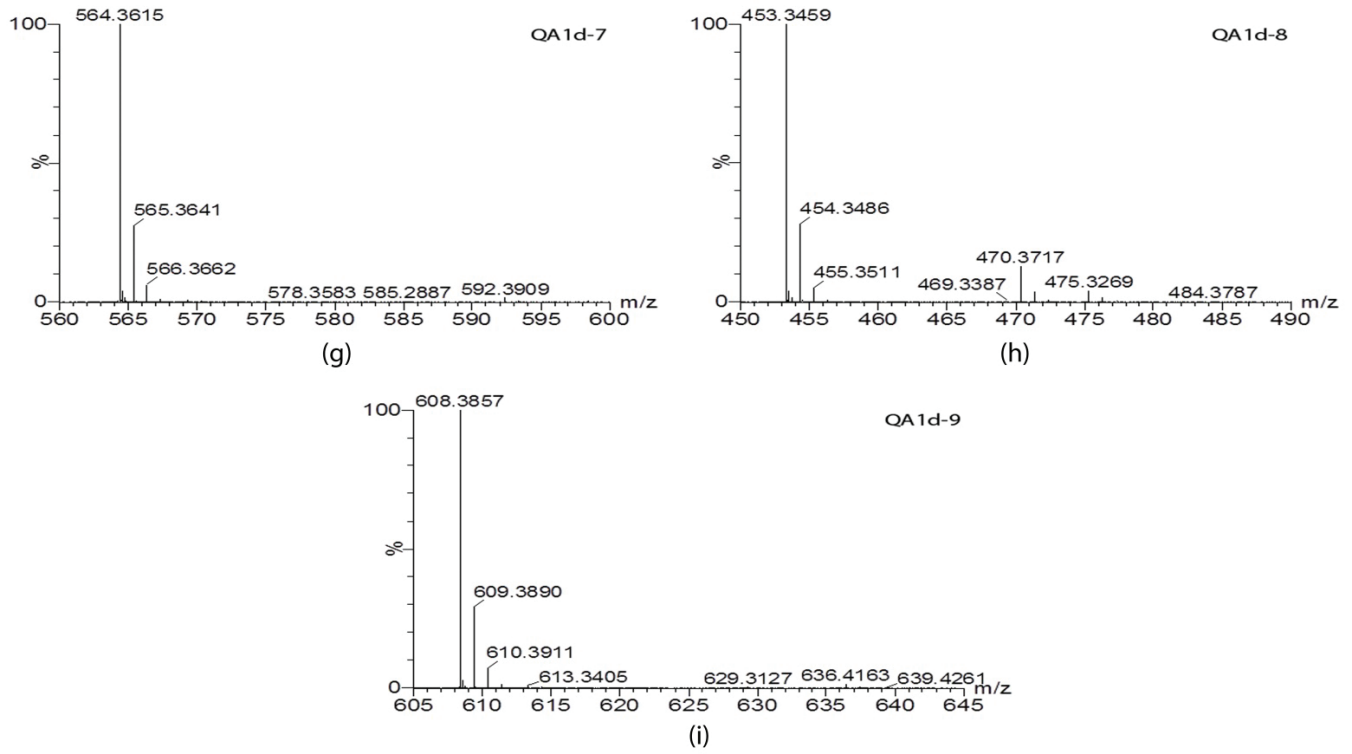

Figure 6. MS/MS spectra QA1d. (a) MS/MS spectra QA1d-1, (b) MS/MS spectra QA1d-2, (c) MS/MS spectra QA1d-3, (d) MS/MS spectra QA1d-4, (e) MS/MS spectra QA1d-5, (f) MS/MS spectra QA1d-6, (g) MS/MS spectra QA1d-7, (h) MS/MS spectra QA1d-8, and (i) MS/MS spectra QA1d-9.

Table 4. Identification of peptides by the MASCOT program.

Code	Peptide sequence	MW (Da)	Amino acid amount	score	Protein coverage (%)	ID protein (NCBIprot)	Original protein	Original organism
QA1d-1	FLP	375.47	3	40	12	KKT52525.1	Hypothetical protein UW44_C0001G0077	*Candidatus Collierbacteria bacterium GW2011_ GWB2_44_22*
QA1d-2	MGFVLLQLLCFTVF	1,631.07	14	84	4	XP_021659746.1	Non-classical arabinogalactan protein 31-like	*Hevea brasiliensis*
QA1d-3	MFLATLLSIGLMTFRIL	1,940.48	17	84	24	WP_088762647.1	hypothetical protein	unclassified *Sphingobacteriales* (miscellaneous)
QA1d-4	HAILRGLLCLSLTLAFQPAF	2,184.67	20	103	4	WP_136162937.1	M48 family metalloprotease	*Sphingomonas sp. ZLT-5*
QA1d-5	AFTFPLYPAFVVFLPGTVL	2,099.54	19	78	8	XP_024692302.1	DUF775-domain-containing protein	*Aspergillus campestris IBT 28561*
QA1d-6	MNYLLLIKFKYTLSFQF	2,169.65	17	80	3	PKP27261.1	Cytochrome C	*Bacteroidetes bacterium HGW-Bacteroidetes-22*
QA1d-7	DEASFFERLGLRRLFFMSSL	2,421.80	20	81	4	WP_144233976.1	Hypothetical protein	*Desulfovibrio marinus*
QA1d-8	ALHLPLKLMLRPALPLRLKLTL	2,521.28	22	86	4	RXN20527.1	Tyrosine- phosphatase 10D	*Labeo rohita*
QA1d-9	LTHELDQSFLSHNFMSIQFLQHVL	2,885.29	24	86	7	XP_015878958.1	Protein LOC107415185	*Ziziphus jujube*

contact and the active peptides to be positively charged. Based on the physicochemical analysis, authors found that peptides (QA1d-4 and QA1d-8) were positively charged, QA1d-4 peptides were δ + 1.25 and QA1d-8 were δ + 4.25.

BLAST searches on NCBIprot and Uniprot show that both QA1d-4 and QA1d-8 peptides have never been reported, thus indicating that both peptides are newly discovered and are therefore promising antibacterial and anticancer agents in the future.

Table 5. The antibacterial and anticancer activity prediction of peptide.

Code	Peptide sequence	Antibacterial prediction	Anticancer prediction
QA1d-2	MGFVLLQLLCFTVF	Non-AMP	Non-Anticp
QA1d-3	MFLATLLSIGLMTFRIL	Non-AMP	Non-Anticp
QA1d-4	HAILRGLLCLSLTLAFQPAF	AMP	Anticp
QA1d-5	AFTFPLYPAFVVFLPGTVL	Non-AMP	Non-Anticp
QA1d-6	MNYLLLIKFKYTLSFQF	Non-AMP	Non-Anticp
QA1d-7	DEASFFERLGLRRLFFMSSL	Non-AMP	Non-Anticp
QA1d-8	ALHLPLKLMLRPALPLRLKLTL	AMP	Anticp
QA1d-9	LTHELDQSFLSHNFMSIQFLQHVL	Non-AMP	Non-Anticp

CONCLUSION

In this study, authors obtained a protein hydrolysate QA1d (<3 kDa) from *Vibrio sp*. ES25 which actively inhibited the growth of *S. aureus* at 13.26 ± 0.65 mm, was very toxic to *Artemia salina* with LC_{50} values 1.03 µg/ml and proliferation of HeLa cancer cells with IC_{50} values 248.18 µg/ml, considered to moderate. Then two novel peptides were then identified from QA1d and predicted to be the source of this bioactivity: QA1d-4 (HAILRGLLCLSLTLAFQPAF) and QA1d-8 (ALHLPLKLMLRPALPLRLKLTL). These could prove to be good candidates for novel drug production in the future.

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AUTHOR'S CONTRIBUTION

Data gathering and idea owner of this study: Sugrani A., Ahmad A., Djide M. N., Natsir H.

Study design: Sugrani A., Ahmad A., Djide M. N.

Data collection: Sugrani A., Natsir H.

Manuscript writing: Sugrani A., Ahmad A.

Editing of final manuscript: Sugrani A., Ahmad A., Djide M. N., Natsir H.

CONFLICT OF INTEREST

Authors declared that there is no conflicts of interest.

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