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Discovery of New Amphipathic Helix Antibacterial Peptides with Proteomic Analysis using LC-HRMS and Virtual Screening

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ABSTRACT

Peptides like antimicrobial peptide (AMPs) and cell penetrating peptides (CPPs) characterized by amphipathic and α-helical structures represent promising therapeutic alternatives to combat escalating antibiotic resistance through membrane disruption or antibiotic delivery. The current study employed a machi ne learning-driven virtual screening approach to discover novel AMPs from the *Chondrus crispus* proteome, generated via LC-HRMS following tryptic digestion. An initial pool of 3,645 candidate peptides was subjected to multi-stage computational filtering based on physicochemical properties, sequence parameters, and predictive scoring utilizing CAMPR4 and dbAMP. The screening pipeline isolated three primary candidates: CC1 (FSTSSRALRFFR), CC2 (RDLQQAISMVKK), and CC3 (IAAKIQLLRSYR). Circular dichroism (CD) spectroscopy confirmed that these peptides adopt random coil conformations in aqueous solutions but effectively transition to functional α-helical structures in membrane-mimetic environments (50% TFE). *In vitro* assays revealed that CC1 and CC3 achieved up to 100% growth inhibition against *Escherichia coli* at 250 µg/mL; however, efficacy was notably reduced (50–60% inhibition) against *Staphylococcus aureus*. While this computational framework successfully identifies structurally viable AMPs, further sequence optimization is required to enhance their targeted antibacterial potency. Nevertheless, the inherent physicochemical profiles of these novel peptides underscore their robust potential for secondary application as cell-penetrating peptides (CPPs) in advanced intracellular drug delivery systems.

Keywords: Antibacterial, Discovery, Peptide, Proteomic, Virtual Screening

INTRODUCTION

Antimicrobial resistance (AMR) has become one of the most serious challenges in treating microbial infections. In 2019, it was estimated that there would be 1.27 million cases of death caused by antibiotic resistance[1]. The World Health Organization (WHO) projected that the causes of death due to AMR will rise to 10 million cases by 2050 (Naghavi, 2022). With the high impact of AMR, the world will lose 3.4 trillion USD in gross domestic product (GDP) by 2030, and it will rise to 3.9 trillion USD by 2050 (O'Neill, 2014). Therefore, there is an urgent need to discover novel antimicrobial agents to fight AMR. Antimicrobial peptides (AMPs) can be an alternative to new antibacterial agents in dealing with antimicrobial resistance. AMPs are bioactive macromolecules produced by a narrow class of organisms as an essential component of their innate immune response (Huan et al., 2020). AMPs are generally defined as peptides with residues of fewer than 100 amino acids and a net charge of +2 to +9. Active

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peptides are considered a potential and promising drug candidate for the future, particularly due to their broad-spectrum activity, low toxicity, and decreased resistance process of the cell target (Hancock & Patrzykat, 2002). Some of the biological activities found in antimicrobial peptides are antibacterial, antifungal, antiviral, anticancer, and antiparasitic (Li et al., 2012).

In addition to their primary mechanism of disrupting bacterial cell membranes, antimicrobial peptides (AMPs) can also function effectively as cell-penetrating peptides (CPPs). In this specialized role, CPPs demonstrate the unique ability to breach the formidable defenses of antibiotic-resistant bacteria. Once inside, they act as efficient delivery vehicles, transporting other antibiotics or synergistic AMPs directly into the cell. Interestingly, while CPPs excel at penetration, they typically exhibit low or completely inactive antibacterial properties on their own (Hadjicharalambous et al., 2022). Structural characteristics, specifically amphipathicity and helical conformation, are crucial parameters that enable these peptides to successfully cut through complex bacterial membranes (Habibie et al., 2025; Putri et al., 2025).

Proteomic analysis plays an important role in the identification and characterization of bioactive peptides (Abril et al., 2022). Two commonly employed approaches in discovering new antimicrobial peptides from organisms are the empirical and bioinformatics approaches (Capriotti et al., 2016). Liquid chromatography tandem high-resolution mass spectrometry (HRMS) perhaps represents the leading method for proteomics, as it is sufficiently sensitive to identify new AMPs in low concentrations (Schrader et al., 2014). *In silico* evaluation using molecular docking to target protein is generally utilized to predict biological activity of the AMPs in the intracellular mechanism of action (Rahman et al., 2020). On the other hand, molecular dynamics study using membrane models is generally used to evaluate activity and mechanism of AMPs in *in silico* approach (Huynh et al., 2021).

Several machine learnings, such as dbAMP and CAMR4, are available and exist to address the challenges in new AMPs discovery. The dbAMP webserver is a database consisting of more than 35,000 AMPs, with 30,000 different structures registered. This web server provides two-stage predictions to determine peptide potency as an AMP (Yao et al., 2025). Similar to dbAMPs, the Collection of Anti-Microbial Peptides (CAMPR4) was created to provide curated information on natural and synthetic AMPs in new AMP discovery. The web server provides prediction tools based on two different datasets, natural and synthetic AMPs, utilizing Random Forest (RF), Support Vector Machine (SVM), and Artificial Neural Network (ANN) models (Gawde et al., 2022).

To screen antimicrobial peptides that target bacterial membrane, physicochemical analysis can also be employed using Heliquest and ProtParam. Peng et al. (2024) identified a new AMP from *Heteropoda venatoria* venom using SignalP 6 program (Peng et al., 2024; Teufel et al., 2022). In this study, they used Heliquest and ProtParam to evaluate new candidate AMPs of HvAMP1 and HvAMP5. The HvAMP5, with better physicochemical properties, like AMP and helical secondary structure, exhibited more promising activity against Gram-negative and Gram-positive bacteria. The peptide showed a membrane disruption mechanism against *S. aureus* (Peng et al., 2024). The results indicate that physicochemical properties screening is important in the virtual screening process of bacteria.

In another study, Heymich et al. (2021) conducted a virtual screening to discover new peptides from chickpea storage protein. The proteomic study was performed by digesting chickpea storage protein using chymotrypsin. The hydrolysate of chickpea storage protein was analyzed using micro-liquid chromatography-electrospray ionization quadrupole time-of-flight tandem mass spectrometry (micro-LC–ESI–QTOF-MS/MS) to identify new peptides. The identified peptides from the proteomic analysis were selected using virtual screening by correlating structural elements related to antibacterial activity. All identified peptides were searched for antimicrobial properties using Antimicrobial Peptide Database (APD) version 3. Following the database search, physicochemical properties of the peptides, such as helicity, hydrophobicity, total charge, and amphipathicity, were predicted. Peptides with a negative charge, <20% hydrophobicity, and a lack of helical structure were excluded. In this study, two peptides of Leg1 (RIKTVTSFDLPALRFLKL) and Leg2 (RIKTVTSFDLPALRWLKL) were obtained, with MIC of 62.5 and 15.6 µmol/L against *E. coli* and *B. subtilis*, respectively. The results showed that virtual screening approaches are potential methods for discovering new antimicrobial peptides (Heymich, 2021).

In a previous study, Caprani et al. (2021) found 39 potential sequences of AMPs from *Chondrus crispus* macroalgae. Thirty-one AMPs were identified from some fraction in our previous study, which were obtained through empirical separation using strong cationic exchange methods (Habibie et al., 2023). Based on the potential of *Chondrus crispus*, this study aims to identify new potential antimicrobial peptides through virtual screening using proteomic and bioinformatics approaches. The candidate peptides were obtained by performing a proteomic study using LC-HRMS on *Chondrus crispus* protein. The identified peptides were then filtered using AMP similarity characteristic analysis and machine learning with dbAMP and CAMPR4. The secondary structure of candidate peptides was analyzed using CD spectroscopy. The potency of the antibacterial activity of the identified peptide was assessed *in vitro*.

MATERIALS AND METHODS

Materials used in the Proteomic study were trypsin enzymes (G-bioscience), protein concentrator 3kDa cutoff (Thermo Scientific), acetonitrile (hypergrade for LC-MS, LiChrosolv), water (MS grade), and trifluoroacetic acid (TFA, Merck). For antibacterial assay, the utilized materials were the Mueller-Hinton Broth, agarose (Fisher Scientific), *Escherichia coli* ATCC 23522, and *Staphylococcus aureus* ATCC 23523. For the circular dichroism (CD) study, sodium phosphate monobasic ($NaH_2PO_4.H_2O$), sodium phosphate dibasic ($Na_2HPO_4.2H_2O$), and sodium citrate ($C_6H_5Na_3O_7$) were purchased from Fisher Scientific.

Methods

Proteomic study

The crude protein of *Chondrus crispus* macroalgae was extracted following our previous method (Habibie et al., 2023). The fine *Chondrus crispus* was diluted in water at a ratio of 1:25 (g/mL). The mixture was sonicated with a 42 kHz frequency at room temperature for 1 hour. Supernatant was collected by centrifuging the mixture for 10 minutes at 200 rpm. The protein was extracted using 80% ammonium sulphate (w/v). The salting out was performed with a dialysis membrane overnight, and the obtained extract was used for a proteomic study.

Proteomic analysis was carried out by liquid chromatography-tandem quadrupole time-of-flight mass spectroscopy (LC-QTOP-MS/MS). The crude protein sample was digested using trypsin enzyme at a 1:10 enzyme-to-substrate ratio and incubated for 20 h at 37 °C. The hydrolysis reaction was stopped by heating the mixture to 85 °C for 15 min. The peptide hydrolysate was filtered through a 3 kDa ultrafilter before injection. The mobile phases consisted of 0.05% TFA in purified water as solvent A and 0.1% TFA in water/acetonitrile (20:80) as solvent B. The gradient elution program for LC was 96–90% solvent A in 0–3 min, 90–80 % solvent A in 3–30 min, and 80–5% solvent A in 30–70 minutes. The flow rate applied in this analysis was 0.3 µL/min. MS/MS analyses were carried out using the electron spray ionization (ESI) method, with positive ion mode at 250–1.800 m/z range and full-MS/dd-MS2. The resolving power was adjusted to 140.000 FWHM for full-MS and 17.500 FWHM for dd-MS2. Raw data were analyzed using Proteome Discoverer 2.1 with the Sequest HT algorithms against the *Chondrus crispus* from UniProt database (www.uniprot.org/).

Virtual screening

All identified sequences were filtered with three parameters: +2 minimum charge, 12–50 residual length, as well as Pro, Gly, and Asp as rejected amino acids. The second filtering employed CAMPR4 and dbAMP 3.0 to predict AMP potential (Gawde et al., 2022; Yao et al., 2025). The prediction in dbAMP 3.0 was presented by a single AMP score probability, while the synthetic and natural AMPs dataset with RF, SVM, and ANN models were employed in CAMPR4 prediction tools. The final score from this machine learning filtering process was presented as the total score of every dataset and model (Table Appendix 1). The third filter was performed through manual analysis based on AMP-like characteristics (hydrophobicity >20%, amphipathicity, and helical structure) to obtain final candidates.

The physicochemical properties of the peptide, such as charge and molecular weight, were analyzed by utilizing PepCalc.om (https://pepcalc.com/). The Heliquest web server (https://heliquest.ipmc.cnrs.fr/index.html) was employed to determine the hydrophobicity (H) and mean vector of the hydrophobic moment (µH) of peptides (Gautier et al., 2008). The toxicity was predicted through ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/)(Gupta et al., 2013). The final peptide candidates were synthesized by DG-Peptide Co. Ltd (Sanghai, China) with 95% purity.

CD spectroscopy

The peptides' secondary structure and stability analysis were conducted by circular dichroism spectroscopy on a Jasco-815 spectrophotometer, using a 1 mm path length cell. The secondary structure of the peptides was analyzed in 0% and 50% TFE in 10 mM sodium phosphate buffer (pH 7). The peptide's concentration used in the current study was 125 ppm. All CD spectra were converted from millidegrees (m°) to molar relative ellipticity ([θ]), utilizing equation 2. All analyses were carried out in 5 scans with wavelength regions of 195–250 nm.

Antibacterial assays

Antibacterial assay of the selected peptides was performed by the micro broth dilution method against *Escherichia coli* ATCC 23522 and *Staphylococcus aureus* ATCC 23523 to obtain inhibition of bacterial growth. The bacterial inoculant was prepared by growing the bacteria in the Mueller-Hinton broth (MHB) for 24 h and diluted to ~105 CFU/mL for the assay. Streptomycin and 10 mM sodium phosphate (pH 7.0) were used as positive and negative controls, respectively. The peptide/Streptomycin stocks of 500 µg/mL were prepared by dissolving solid peptides in 10 mM sodium phosphate buffer (pH 7.0) and subsequently diluted to a final concentration of 0.49–250 µg/mL in a sterile polystyrene 96-well plate. 50 µL of bacterial inoculants were transferred into the well plate and incubated at 37 °C for 22 hours. The bacterial growth was analyzed by a microplate reader at 650 or 600 nm wavelength.

RESULTS AND DISCUSSION

Proteomic study using LC-HRMS

Proteomic analysis was carried out by LC-HRMS to identify peptide sequences from the macroalgae *Chondrus crispus* protein. Tryptic digestion with trypsin was carried out to cleave *Chondrus crispus* proteins to get cleavage in lysine and arginine residues in peptides' C-terminal (Zhang et al., 2020). Two different digestion methods were carried out, and a total of 3645 peptide sequences were identified from both methods.

854 peptide sequences exhibited two missing cleavage clusters, suggesting that peptide sequences in this cluster consisted of three arginine and/or lysine residues (Figure 1A). The clusters with one and zero missed cleavages consisted of 1477 and 1314 peptide sequences. In these clusters, peptide sequences consisted of two and one basic residues (arginine and/or lysine), respectively. Most of the identified peptide sequences exhibited more than 12 kDa molecular weight, with the highest number of peptide sequences was observed in the 1.2–1.4 kDa range (Figure 1B). Most sequences exhibited a residue length of 10–13 amino acids (Figure 1C). The majority had retention times of more than 30 minutes and contained 50% hydrophobic residues (Figure 1D).

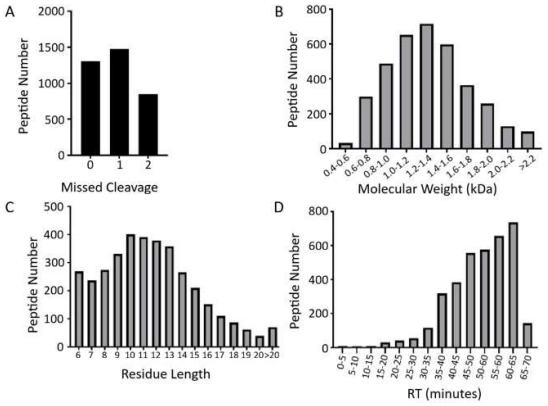


Figure 1. Proteomic study results: (a) missed cleavage peptide, (b) molecular weight of identified peptides, (c) residue length of identified peptide, and (d) retention time of identified peptide.

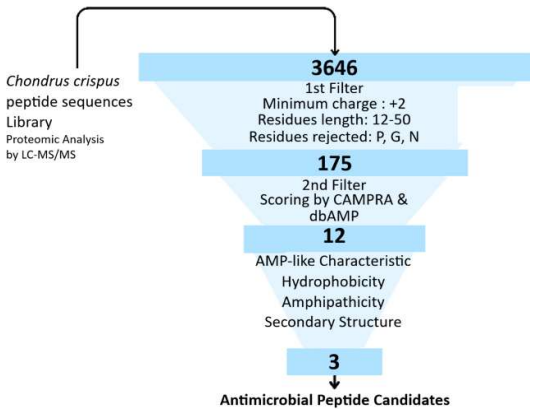


Figure 2. Virtual screening workflow

Peptide selection

All identified peptides from the proteomic study were filtered to obtain peptide sequences with a minimum charge of +2, a residue length of 12–50 amino acids, and exclusion of peptides that contain glycine, proline, and asparagine (Figure 2).

Table I. Physicochemical properties of the identified peptide by virtual screening

Peptide	Sequence	Charge	MW [a] (Da)	<H> [b]	<μH> [b]	Toxicity [c]
CC1	**FSTSSRALRFFR**	+3	1474.67	0.374	0.404	Non-toxic
CC2	**RDLQQAISMVKK**	+2	1416.69	0.168	0.507	Non-toxic
CC3	**IAAKIQLLRSYR**	+3	1431.73	0.443	0.271	Non-toxic

[a] MW= molecular weight in Dalton (Da); [b] <H> Hydrophobicity and <μH>= mean vector hydrophobicity, determined by Heliquest web server (Gautier et al., 2008); [c] Toxicity was predicted by ToxinPred web server (Gupta et al., 2013)

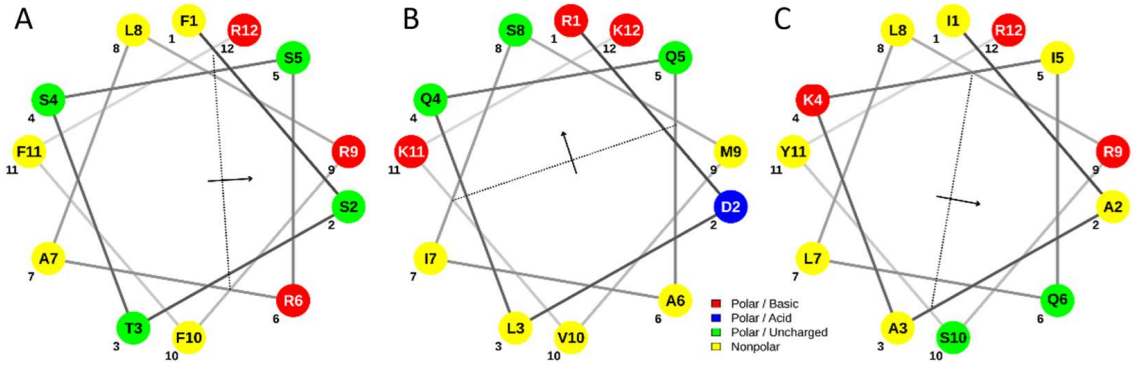


Figure 3. The helical wheel and projection of (A) CC1, (B) CC2, and (C) CC3. The helical wheel of peptides NetWheels: Peptides Helical Wheel and Net projections maker (http://www.lbqp.unb.br/NetWheels/). The arrow is directed to the peptide hydrophilic face and the dashed line divides the hydrophobic and hydrophilic faces. The polar basic residues with a positive charge and polar acid residues with a negative charge are indicated as red and blue, respectively. The polar uncharged and non-polar residues are marked as green and yellow, respectively.

As shown in Appendix 1, 175 peptide sequences were identified from the first filter. To carry out the second filter, all peptide sequences were tested and scored using CAMPR4 and dbAMP 3.0 (Table Appendix 1) to predict their antimicrobial peptide probability and activity against bacteria (Gawde et al., 2022; Yao et al., 2025).

The physicochemical properties of twelve peptide sequences obtained from the second filtering were analyzed to select the best peptide with antimicrobial peptide-like characteristics (Appendix 1). In this step, hydrophobicity, mean residue hydrophobicity, total charge, and secondary structure of peptides were used to select peptides. The three peptides with the best antimicrobial peptide-like characteristics were FSTSSRALRFFR (64), RDLQQAISMVKK (133), and IAAKIQLLRSYR (175). In the next step, the three peptides will be referred to as CC1, CC2, and CC3, respectively.

The majority of antimicrobial peptides originated from animals, with 78.29%, followed by those from bacteria and plants with a percentage of 12.17% and 7.95%, respectively (Saubenova et al., 2025). Several AMPs from animals exhibited activity against broad-spectrum bacteria. For example, Lc-NK-lysin-1 demonstrated activity against Gram-positive and Gram-negative bacteria (Zhou et al., 2016). In contrast, AMPs from marine plants are still rare. In 2020, only 0.15% of AMPs were registered from the *Protista* kingdom (Wang et al., 2022). In our previous study, several peptides were found in active fractions of the macroalgae *Chondrus crispus* hydrolysate protein (Habibie et al., 2023). Several peptides from the macroalgae *Porphyra yezoensis* also exhibited antibacterial activity against *S. aureus* (Jiao et al., 2019). The opportunities to discover new antimicrobial peptides from macroalgae are still wide open.

Physicochemical properties of candidate peptides

Physicochemical properties of three peptides were analyzed by applying bioinformatic approaches. The first identified candidate peptide, CC1, exhibited a high positive charge of +3 with a molecular weight of 1474.67 Da (Table I).

The peptide revealed 0.374 hydrophobicity and 0.404 mean residue hydrophobicity. The second identified peptide, CC2, showed lower total charge of +2 with 1416.69 Da molecular weight. The peptide exhibited a lower hydrophobicity of 0.168 with a higher mean residue hydrophobicity of 0.507. Meanwhile, the CC3 had the same total charge as CC1 (+3) with a molecular weight of 1431.73 Da. This peptide exhibited the highest hydrophobicity (0.443) but a lower mean residue hydrophobicity (0.271). All identified peptides were predicted to be non-toxic according to ToxinPred results.

CC1 displayed an imperfect amphipathic structure, with two polar residues in the hydrophobic side of the peptide helical wheel (Figure 3). In contrast, CC2 exhibited a more amphipathic structure, though with less distinct polar and non-polar residues. CC3, on the other hand, showed less amphipathic structure, with more separate residues in polar and non-polar peptide faces. As shown in a previous study, amphipathicity is one of several parameters that influence the activity of a peptide.

Secondary structure of identified peptides

The secondary structure of peptides was analyzed by circular dichroism spectroscopy to obtain the secondary structure composition of peptides. Trifluoroethanol (TFE) was employed to mimic the bacterial membrane environment, as short peptides undergo secondary structure self-assembly when interacting with bacterial membranes (Myers et al., 1998).

CC1 exhibited a typical random coil signal at 0% TFE (v/v) in phosphate mM buffer (pH 7) (Figure 4). A random coil signal pattern demonstrated a minima peak at 200 nm. Interestingly, this peptide had a slight peak shifting in 200 nm, indicating the presence of other secondary structures. However, a helix pattern was observed with a double minima peak at 222 and 208 nm, and maxima at 195 nm (Miles et al., 2021). Secondary structure composition was determined by the BestSel web server (Micsonai et al., 2022).

Similar to CC1, CC2 had a random coil pattern observed at 0% TFE, with a higher minima peak intensity at 200 nm (Figure 4). At 50% TFE, the helix pattern was observed with higher intensity than CC1. The last identified peptide, CC3, displayed similar behavior to CC2 and CC1, with a random coil pattern at 0% TFE. CC3 recorded a helix pattern at 50% TFE, with a lower signal than CC2 but higher than CC1.

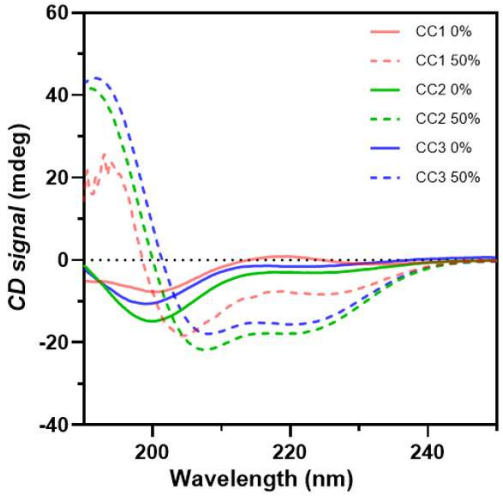


Figure 4. Secondary structure analysis performed using Circular Dichroism spectroscopy. Measurements were performed at 0% (solid line) and 50% (dash line) TFE (v/v) in 10 mM sodium phosphate buffer (pH 7).

Secondary structure	CC1		CC2		CC3	
	0	50	0	50	0	50
Helix	0	15.6	2.5	48.6	0.0	41.4
Antiparallel	31.2	2.6	27.9	3.7	34.5	6.9
Parallel	0	0.0	0.0	0.0	0.0	0.0
Turn	17.6	12.8	17.5	9.5	18.0	10.9
Others	51.2	69.0	52.1	38.2	47.5	40.7

Table II. Secondary structure of identified peptide at 0% and 50% TFE (v/v) in 10 nM sodium phosphate buffer (pH 7), analyzed by BetSel (https://bestsel.elte.hu) (Micsonai et al., 2022). The data are presented as the percentage of secondary structure composition (%).

CC1 exhibited 0% helicity, with antiparallel (31.2%) as the major secondary structure at 0% TFE (Table II). The helicity increased to 15.6% at 50% TFE, followed by an antiparallel decrease to 2.6%. The second identified peptide, CC2, exhibited 2.5% helicity at 0% TFE, with 27.9% antiparallel and 17.5% turn at 0% TFE. The helicity of CC2 increased to 48.6% at 50% TFE, with 3.7% antiparallel and 9.5% turn. On the other hand, CC3 revealed a similar composition to CC1 at 0% TFE, with 34.5% antiparallel and 18.0% turn. At 50%, helicity of CC3 was 41.4%, with 6.9% antiparallel and 10.9% turn. The results indicate that the order of helicity in this study is: CC2> CC3 > CC1.

Antibacterial activity of the identified peptides

The antibacterial activity was carried by microbroth dilution against *E. coli* (ATCC 23522) and *S. aureus* (ATCC 23523). CC1 and CC3 exhibited small activities against *S. aureus* with more than 50% inhibition at a high concentration of 250 µg/mL (Figure 5A). Similar to the antibacterial activity against *S. aureus*, both peptides CC1 and CC3 inhibited *E. coli* growth close to 100% in 250 µg/mL concentration (Figure 5B). In a lower concentration of 125 µg/mL, CC1 revealed ~50% inhibition, while CC3 inhibited bacterial growth up to 35%. On the other hand, CC2 had no activities against both bacteria.

Discussion

The growing antimicrobial resistance poses a significant concern due to the overuse of antibiotics in medicine, as well as in agriculture and animal farming, especially in developing nations (Ayukekbong et al., 2017). AMR resulted in challenging clinical outcomes and economic burdens in different countries (Founou et al., 2017). More than 150 new antibiotics have been developed, but their extensive and often excessive use has contributed to the emergence of heightened resistance, including the formation of multi-resistant strains known as "superbugs." This resistance has elevated mortality rates, as clinical effectiveness wanes because of the overuse or misuse of antibiotics (Hutchings et al., 2019).

AMPs have emerged to be good candidates for a new type of antimicrobial agent. Their physicochemical attributes, including amphipathicity, α-helix structure, and hydrophobicity, contribute to antimicrobial activity covering a wide spectrum of bacteria (Edwards et al., 2016; Narayana et al., 2020). Notably, these peptides exhibit a low probability of inducing bacterial resistance (Spohn et al., 2019). Hence, the quest for discovering new AMPs as effective agents against microorganisms remains imperative.

A proteomic and bioinformatic prediction approach has been actively used to find and study new antimicrobial peptides (Abril et al., 2022). In a previous study, six promising peptides were observed from *Crassostrea gigas* mucus by performing a proteomic study and bioinformatic prediction for the identified peptides (Song et al., 2024). In another previous study, the iAMPCN model was applied to predict the antibacterial activity of the peptides (Xu et al., 2023). Two peptides exhibited significant activity against *E. coli,* with the most active peptide showing a MIC of 1 µM.

In our current study, the bioinformatics prediction was also applied to peptide filtration to obtain the best peptide. CC1 and CC3 obtained 7 score predictions from CAMPR4 and dbAMP 3.0 (Gawde et al., 2022; Yao et al., 2025), while CC2 only resulted in 4 score predictions (Appendix 2),. The results were proportional to their antibacterial activity, whereas the CC1 and CC3 inhibited *E. coli* growth (Fig.5B).

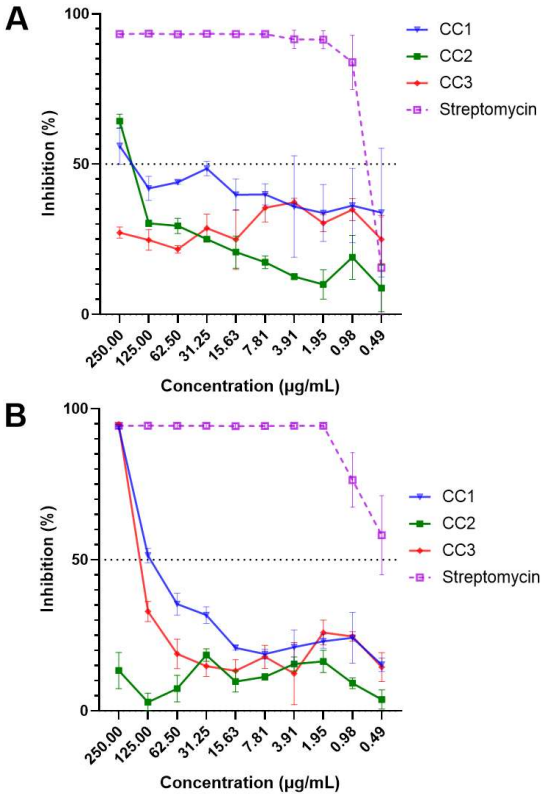


Figure 5. antibacterial activity of identified peptides at 0.49 – 250 µg/mL concentrations against (A) *S. aureus* ATCC 23523 and (B) E. coli ATCC 23522

All identified peptides in the current study were inactive against *S. aureus*. The previous study showed that the majority of AMPs exhibited less activity against Gram-positive bacteria than Gram-negative bacteria. The AMPs are allegedly less active against Gram-positive bacteria due to interaction between the peptides and lipoteichoic acid (LTA). Malanovic et al. (2015) revealed that LTA slightly hindered the penetration of OP-145, a

LL-37's derivative, to the bacterial membrane (Malanovic et al., 2015; Malanovic & Lohner, 2016). Another study showed that AMP needs a high concentration to disturb the bacterial membrane and kill the bacteria (Roversi et al., 2014).

CC1 exhibited the most activity against *E. coli* (93.93% inhibition in 250 µg/mL and 51.32 % in 125 µg/mL) (Figure 5B). The three peptides recorded the same number of positive residues, but CC2 exhibited a lower total charge of +2 with one aspartic acid. A previous study revealed that total charge, amphipathicity, helicity, and hydrophobicity are important for antibacterial activity of peptides (Chen et al., 2007; Hollmann et al., 2016; Huang et al., 2014; Lunkad et al., 2021). In the current study, CC2 did not exhibit any activity, likely due to the hydrophobicity that is used for membrane permeabilization (Le Huy et al., 2024).

The lower activity of identified peptides in the current study is due to the unoptimized physicochemical properties. Zhang et al. (2025) designed several peptides with rational antibacterial design. The results showed that peptides with 12 residual length exhibited promising activity, and the mean residue hydrophobicity/hydrophobic moment of the peptide (µH) was more than 0.7, with at least +5 total charge (Zhang et al., 2025). In another study, Aurein 1.2 analog also had similar results. KLA-2, which showed the best activity, had 0.674 hydrophobic moment with a total charge of +2, and a hydrophobicity of 0.530 (Liao et al., 2023). In the current study, all identified peptides had a lower value of hydrophobicity (0.168–0.443) and hydrophobic moment (0.271–0.507). To increase peptides' activity, several modifications and mutations might be applied to optimize peptide physicochemical properties (Putri et al., 2025, 2026).

Although the initial biological activities of these sequences were observed to be relatively low, they retain significant therapeutic potential, particularly in their capacity to function as cell-penetrating peptides (CPPs) for drug delivery. Their inherent propensity to adopt an α-helical secondary structure, as demonstrated in Figure 3, provides a highly favorable foundational framework. This pre-existing helicity suggests that these peptides can be strategically modified—such as through targeted amino acid substitutions or peptide stapling—to substantially increase their structural rigidity, half-life, and overall proteolytic stability in physiological environments (Copolovici et al., 2014).

Furthermore, these sequences closely align with the established physicochemical profiles required for effective intracellular delivery. Successful CPPs are predominantly characterized by a high net positive charge, typically rich in basic residues like arginine or lysine, which is essential for mediating initial electrostatic interactions with negatively charged phospholipid headgroups on the cell surface. Coupled with a distinct amphipathic architecture, the peptides can efficiently partition their hydrophilic and hydrophobic faces to drive deeper insertion into the lipid bilayer. Whether adopting an α-helix or β-sheet conformation upon membrane contact, these structural motifs are critical for translocation. Finally, possessing a compact sequence length of 5 to 30 amino acid residues ensures these peptides remain agile enough to traverse the membrane efficiently while simultaneously minimizing unwanted immunogenic responses (Patel et al., 2019).

CONCLUSION

Virtual screening was successfully applied to identify potential peptides from the *Chondrus crispus* algae proteomic data bank. Although the three selected peptides exhibited promising physicochemical properties with helical amphipathic structures, only CC1 and CC3 showed activity against *S. aureus* and E. coli. The results revealed that hydrophobicity is important for peptides' activity, as CC2 with no activity exhibited low hydrophobicity. Although CC1 and CC3 showed antibacterial activities, further development of peptides is needed to increase their antibacterial activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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