ASSESSMENT OF ANTIFUNGAL ACTIVITY AND CYTOTOXICITY OF CN-AMP1-DERIVED PEPTIDES AGAINST *CANDIDA ALBICANS*

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Candida albicans is a major opportunistic fungal pathogen associated with increasing antifungal resistance and limited treatment options. Antimicrobial peptides (AMPs) offer a promising alternative due to their membrane-targeting mechanisms and low likelihood of inducing resistance. In this study, we evaluated the antifungal efficacy and safety profiles of six synthetic peptides derived from the natural peptide Cn-AMP1, including CAP-X, CAP-Y, and four leucine-substituted derivatives. These peptides were synthesized via Fmoc-based solid-phase peptide synthesis and tested in vitro against C. albicans ATCC 10231 using broth microdilution assays to determine minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC). CAP-Y exhibited the strongest antifungal activity, followed by CAP-X, while the leucine-substituted derivatives showed similar or reduced potency. Hemolysis assays revealed that CAP-X and CAP-Y induced minimal hemolysis (< 10% at 512μM), and MTS assays confirmed no significant cytotoxicity on 4T1 murine breast cancer cells at concentrations up to 512μM. These findings suggest that Cn-AMP1-derived peptides, particularly CAP-Y and CAP-X, possess moderate antifungal activity with acceptable safety profiles and may serve as lead candidates for the development of novel antifungal therapeutics.

Keywords: Antimicrobial peptides, Candida albicans, Cytotoxicity, Hemolysis, Antifungal activity, Peptide therapeutics.

I. INTRODUCTION

Fungal infections caused by *Candida albicans* are a major clinical concern, particularly in immunocompromised individuals.[1] The increasing prevalence of antifungal resistance and the limitations of current treatments, such as toxicity and narrow spectrum, highlight the need for new therapeutic options with novel mechanisms of action. Antimicrobial peptides (AMPs) have gained attention as promising alternatives due to their broad-spectrum activity, membrane-targeting mechanism, and reduced potential for resistance development.[2] AMPs are typically short peptides (10 – 50 amino acids) that possess both cationic and amphipathic properties, enabling them to interact with and disrupt microbial membranes.[3] Their secondary structures often include α-helices or β-sheets, with α-helical AMPs being the most common. Structural parameters such as net positive charge, hydrophobicity, and hydrophobic moment play key roles in modulating their selectivity and activity toward microbial versus

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Received: 20/06/2025
Accepted: 07/07/2025

host cells.[4]

Cn-AMP1 is a naturally occurring AMP isolated from coconut water, known for its low cytotoxicity but relatively weak antimicrobial activity.[5,6] In our previous study, we designed and synthesized a series of Cn-AMP1 derivatives by modifying their hydrophobicity and net charge. Among these, several analogs, specifically CAP-X, CAP-Y, and four derivatives of CAP-X in which leucine residues were substituted for glycine, showed notable improvement in antibacterial activity against both Gram-negative and Gram-positive bacterial strains (see Figure 1 for peptide sequences).

While these peptides were originally optimized for antibacterial purposes, their antifungal properties had not been thoroughly investigated. In this study, we explored their antifungal potential against *C. albicans*. These peptides include CAP-X and CAP-Y as well as the four leucine-substituted derivatives of CAP-X. This study aims to perform a preliminary *in vitro* evaluation of the antifungal activity and cytotoxicity of Cn-AMP1-derived peptides, and to assess their potential as lead candidates for antifungal peptide therapeutics.

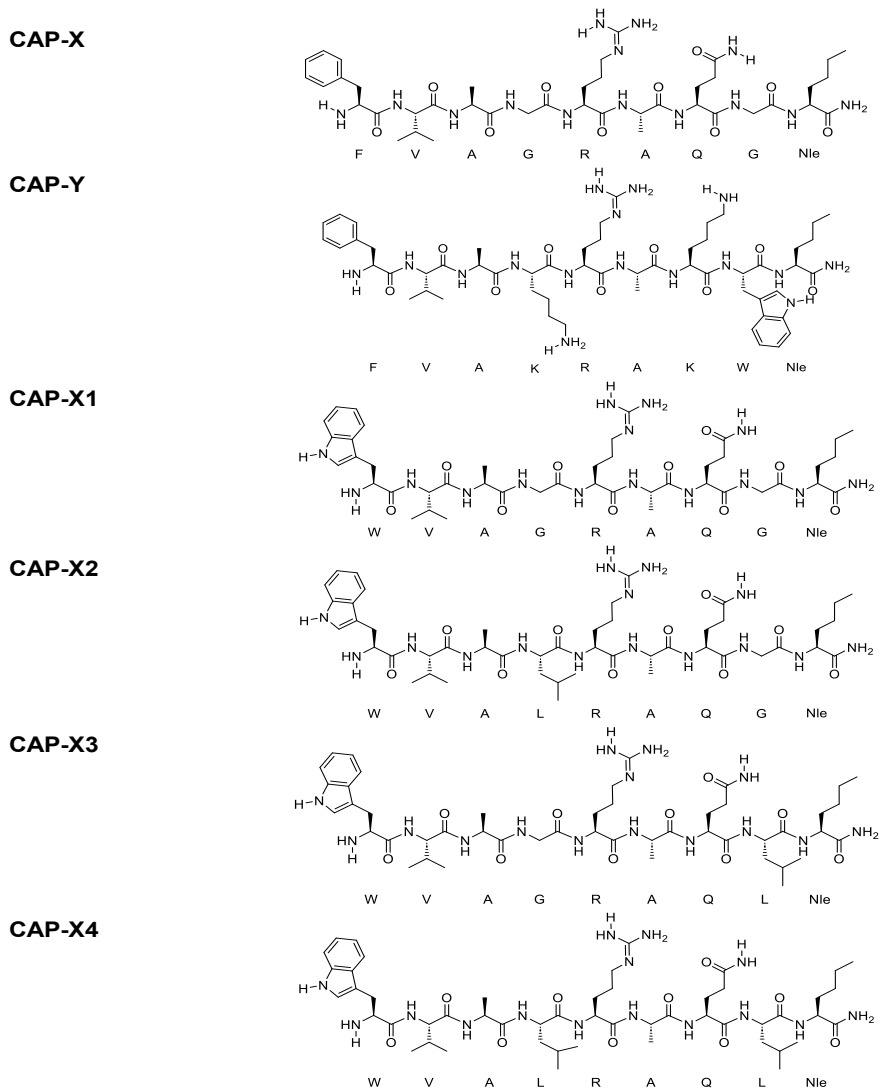

Figure 1. The amino acid sequences of the peptides investigated in this study

II. MATERIALS AND METHODS

1. Subjects

The study focused on a group of synthetic peptides, including CAP-X, CAP-Y, and four derivatives of CAP-X (CAP-X1 to CAP-X4), designed based on the natural peptide Cn-AMP1. These peptides were evaluated *in vitro* for their antifungal activity against *C. albicans*.

Materials

The Fmoc-protected α-amino acids, including Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Nle-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, and Fmoc-Gln(OtBu)-OH, on Rink Amide MBHA resin (100–200 mesh, ≥ 97% purity, Angene, China). Reagents and solvents used included COMU, DIPEA, NMP, DMF, DCM, TFA, and ACN (Daejung, Korea), and TIS (Sigma-Aldrich, Germany). Microbiological media and components were purchased from Himedia (India), Xilong (China), and domestic suppliers (Vietnam). *C. albicans* ATCC 10231 and 4T1 CRL-2539 cells were obtained from the American Type Culture Collection (ATCC). Phenazine methosulfate (PMS) was purchased from ThermoFisher Scientific (catalog number H56718.14).

2. Methods

Peptide preparation

Resin swelling and Fmoc deprotection: The resin was first pre-swelled by sequential treatment with dichloromethane (DCM) for 3 minutes, followed by dimethylformamide (DMF) for 10 minutes to ensure optimal solvent penetration. Fmoc deprotection was carried out using 20% piperidine in DMF. After each deprotection or coupling cycle, the resin was thoroughly washed to remove residual reagents and by-products, using a series of washes with DCM and DMF in the order: DCM, DMF, DCM, DMF, and finally DMF.

Amino acid coupling: Amino acids were coupled sequentially employing COMU as the coupling reagent at a molar ratio of resin to amino acid to COMU of 1:5:5, in the presence of excess N,N-diisopropylethylamine (DIPEA) to facilitate activation and coupling efficiency. This process was repeated until the desired peptide sequence was assembled.

Peptide cleavage from resin: Following the final Fmoc deprotection, the peptides were cleaved from the resin using a cleavage cocktail consisting of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water in a volume ratio of 95:2.5:2.5. The cleavage reaction was conducted at room temperature for 2 hours, after which the solvent was removed and the crude peptides were dried under ambient conditions overnight.

Peptide purification by HPLC: Crude peptides were purified by preparative high-performance liquid chromatography (HPLC) using a Zorbax C18 column (Agilent, 5µm, 9.4 × 250mm). The purification was performed with a gradient elution program starting from 2 – 30% solvent B over 11 minutes, ramping to 100% B in 1 minute, maintained at 100% B for 5 minutes, then returning to 2% B in 2 minutes, and finally held at 2% B for an additional 2 minutes. Solvent A was 0.1% TFA in water, while solvent B was 0.1% TFA in acetonitrile (ACN), with a flow rate of 3 mL/min.

Analytical HPLC and LC-MS/MS characterization: Peptide purity was subsequently assessed by analytical HPLC under a gradient of 5 – 100% B over 8 minutes, held at 100% B for 1 minute, then decreased to 5% B over 2 minutes and maintained at 5% B for 1 minute, at a flow rate of 1 mL/min. Finally, peptides were identified and characterized by LC-MS/MS using a gradient from 30 – 80% B

over 2 minutes followed by 80 – 30% B over 2 minutes, with solvent systems identical to those used in HPLC. The flow rate was set to 1 mL/min, with mass detection performed in the range of 400 to 2000Da. All peptide samples were monitored at a wavelength of 220nm.[7]

Antifungal assay

Peptides were serially diluted in PBS to concentrations ranging from 1024μM to 8μM. *C. albicans* was cultured in YM medium (10 g/L glucose, 5 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract). Fungal suspensions (10^5 CFU/mL) were mixed 1:1 with peptide solutions, yielding final peptide concentrations of 512 to 4μM. After 24 hours of incubation at 37°C, MIC was defined as the lowest peptide concentration that inhibited visible growth. MFC was determined as the lowest concentration that resulted in no visible colonies on Mueller-Hinton agar (MHA) plates.[8]

Hemolysis assay

Rat red blood cells (10% v/v) were incubated with peptide solutions at 256μM and 512μM for 60 minutes at 37 °C. Diclofenac sodium was used as a negative control, and 0.1% Triton X-100 as a positive control. After centrifugation (3000rpm, 10 min), the supernatant was transferred to a 96-well plate and absorbance was read at 405nm.[9] Hemolysis percentage was calculated as follows:

$$\text{Hemolysis (\%)} = \frac{[\text{OD (sample)} - \text{OD (negative control)}]*100}{\text{OD (positive control)} - \text{OD (negative control)}}$$

Cytotoxicity assay

4T1 murine breast cancer cells (ATCC CRL-2539) were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were seeded in 96-well plates (5×10^3 cells/well) and incubated overnight. Peptides (128 to 512μM) were added in serum-free medium for 12 hours. MTS assay was then performed by adding 20μL of MTS/PMS solution. The plates were incubated for 2 hours at 37°C in a humidified atmosphere with 5% CO_2. Experiments were performed in five replicates. The blank wells contained only medium, but during the assay, the MTS solution was also added to the blank wells, just like to all other wells. Absorbance at 490nm was measured using a microplate reader.[10] Cell viability was expressed as a percentage compared to untreated control wells using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD (sample)} - \text{OD (blank)}*100}{\text{OD (control)} - \text{OD (blank)}}$$

Statistical analysis and data visualization

All quantitative experiments were performed in replicate. For the cytotoxicity assay, experiments were conducted with five independent replicates (n = 5). Data are expressed as mean ± standard deviation (SD) and were analyzed using a two-way analysis of variance (ANOVA) to assess the main effects of peptide type and concentration, as well as their interaction. Tukey's HSD (Honestly Significant Difference) test was used for post-hoc pairwise comparisons.

For the hemolysis assay, experiments were performed in triplicate (n = 3), and data were analyzed using a one-way ANOVA. Dunnett's post-hoc test was used to compare each treatment group to the negative control. A p-value < 0.05 was considered statistically significant.

The MIC and MFC values were determined from three independent experiments. As identical results were obtained across replicates due to the discrete nature of the two-fold serial

dilution method, the reported values represent the consistent modal result.

All statistical analyses and graphs were generated using Microsoft Excel 2016.

III. RESULTS

Peptide preparation

The synthetic scheme of the peptides used in this study is illustrated in Figure 2.

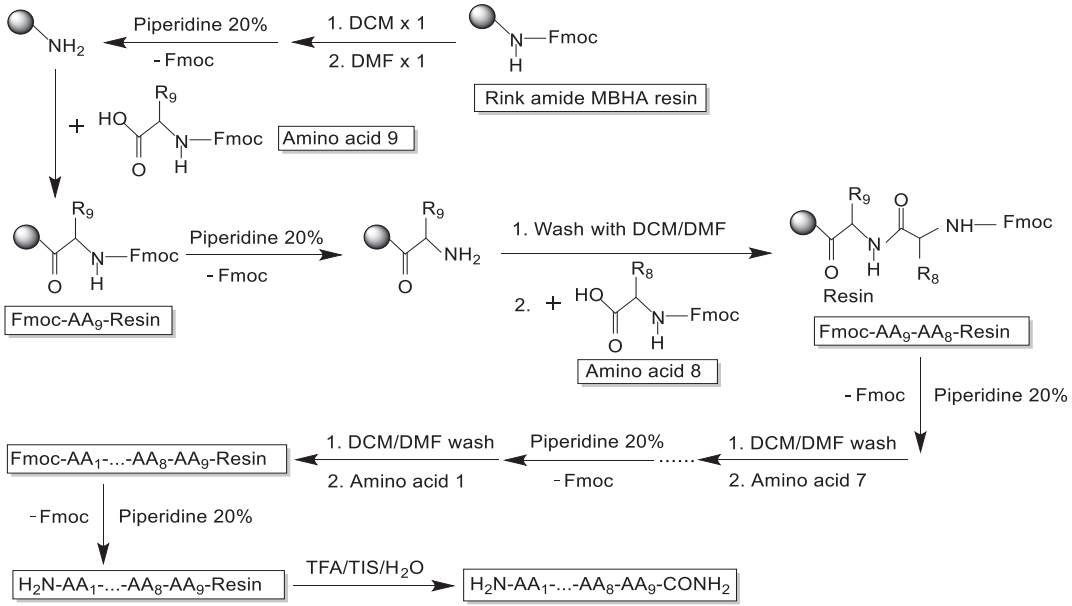

Figure 2. Schematic diagram of peptide synthesis in this study

The obtained peptide products were in the form of white, fluffy, odorless powders. Purity was assessed using HPLC, where it was defined as the percentage area of the product peak in the total chromatogram area (excluding system-related peaks). As shown in Table 1, the HPLC purity of all peptides was above 95%. In addition, molecular mass was determined by LC-MS for selected purified peptides. The experimental m/z values closely matched the theoretical values, confirming the identity of the products.

Table 1. Peptide characterization by HPLC and LC-MS

Peptide	Chemical formula	HPLC data		LC-MS data	
		Retention time*	Purity (%)	m/z calculated**	m/z found
CAP-X	$C_{41}H_{68}N_{14}O_{10}$	4.882	100.0	459.27	459.40
CAP-Y	$C_{55}H_{88}N_{16}O_9$	5.432	100.0	559.35	559.50
CAP-X1	$C_{43}H_{69}N_{14}O_{10}$	5.238	99.2	478.78	478.90
CAP-X2	$C_{47}H_{77}N_{14}O_{10}$	5.568	100.0	506.81	507.00
CAP-X3	$C_{47}H_{77}N_{14}O_{10}$	5.668	100.0	506.81	506.90
CAP-X4	$C_{51}H_{85}N_{14}O_{10}$	5.995	100.0	534.84	535.00

**: minutes; **: [M+2H]/2.*

Antifungal activity toward *C. albicans*

The antifungal activity of the peptides was evaluated against *C. albicans*. Among them, CAP-Y showed the strongest antifungal activity with MIC and MFC values of 128 µM and 256 µM, respectively (see Table 2). CAP-X showed a slightly weaker MIC (256 µM), although the MFC remained equivalent to that of CAP-Y. Notably, the leucine-substituted derivatives of CAP-X displayed equal or reduced antifungal activity compared to CAP-X.

Table 2. Antifungal activity of peptides against *C. albicans*

Peptides	MIC (µM)	MFC (µM)
CAP-X	256	256
CAP-Y	128	256
CAP-X1	128	512
CAP-X2	256	512
CAP-X3	256	512
CAP-X4	512	>512

Hemolytic activity

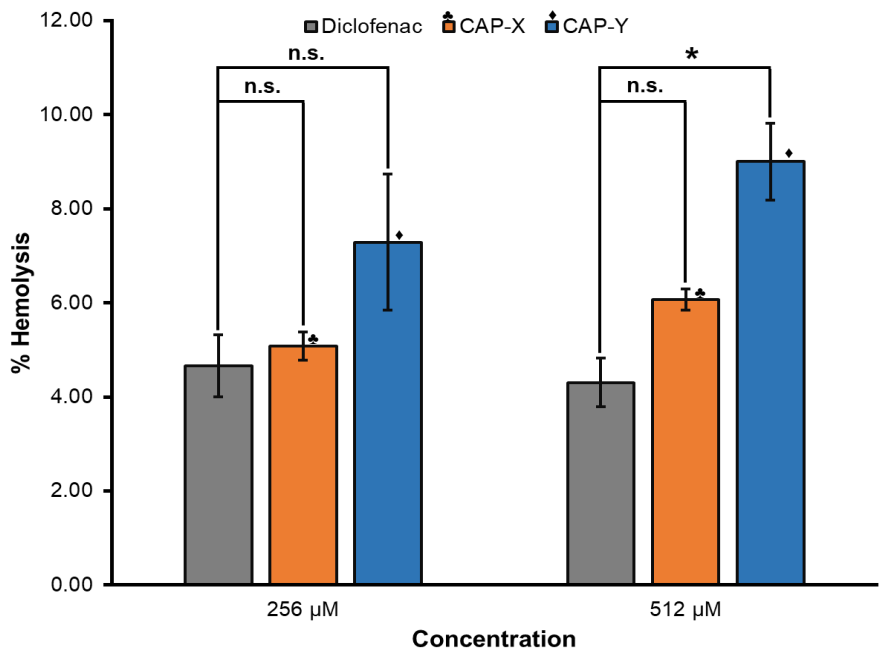

Chart 1. Hemolytic activity of CAP-X and CAP-Y on rat erythrocytes

Peptides were incubated with rat red blood cells at concentrations of 256 µM and 512 µM. Data are presented as the mean ± standard deviation (SD) from three independent experiments (n = 3). Statistical significance was determined by a one-way ANOVA followed by Dunnett's post-hoc test, comparing each peptide treatment to the negative control (Diclofenac sodium) at the same concentration. (, $p < 0.05$; n.s., not significant)*

CAP-X and CAP-Y, which exhibited the best antifungal profiles, were selected for hemolysis evaluation. Hemolysis is a common side effect associated with AMPs. Results are presented in Chart 1. Diclofenac sodium, known for its lack of hemolytic activity, was used as a negative control and showed hemolysis rates below 5% at both 256μM and 512μM. At both concentrations, the hemolysis induced by CAP-X was below 6% and not significantly different from the negative control. Only at 512μM, CAP-Y showed a small but statistically significant increase hemolytic activity (9%) compared to the negative control, while their difference at 256μM was not significant.

Cytotoxicity on 4T1 Cells

The cytotoxicity of CAP-X and CAP-Y was also evaluated *in vitro* on 4T1 murine breast cancer cells. Both peptides showed minimal effects on cell viability. At all tested concentrations (128 – 512μM), cell viability remained above 95%, indicating virtually no cytotoxicity (see Table 3). CAP-Y consistently exhibited significantly less cytotoxicity, even slightly promoted cell growth, than CAP-X at all the tested concentrations.

Table 3. Cytotoxicity of peptides on 4T1 cells

Peptide	Cell viability (%) ± SD		
	128μM	256μM	512μM
CAP-X	98.29 ± 2.87[a]	97.70 ± 4.70[a]	96.92 ± 3.39[a]
CAP-Y	101.39 ± 5.08[b]	99.33 ± 4.03[b]	105.43 ± 4.05[b]

Cell viability is expressed as mean ± SD from five independent experiments (n = 5). Statistical analysis was performed using a two-way ANOVA followed by Tukey's HSD post-hoc test. Means within the table that do not share a common superscript letter (a, b) are significantly different from each other (p < 0.05).

IV. DISCUSSION

The escalating threat of antifungal resistance necessitates the development of novel therapeutic agents like AMPs. This study aimed to provide a preliminary evaluation of the antifungal potential and safety profile of six synthetic peptides derived from Cn-AMP1. Our findings demonstrate that rational sequence modifications can yield derivatives with moderate but promising activity against *Candida albicans*, coupled with an excellent safety profile, identifying CAP-Y and CAP-X as viable lead candidates for further development. While CAP-Y showed the strongest activity with MIC and MFC values of 128μM and 256μM, respectively, CAP-X, despite a slightly higher MIC, exhibited the same fungicidal threshold. This enhancement of antimicrobial activity can be attributed to fundamental principles of AMP design. CAP-Y incorporates additional tryptophan and cationic residues compared to CAP-X (as inferred from their structures and properties), which likely optimizes its amphipathic character and increases its net positive charge. This combination is known to be critical for the initial electrostatic attraction to the negatively charged fungal cell surface and subsequent membrane disruption. This aligns with numerous studies demonstrating that a well-balanced amphipathicity is more crucial than hydrophobicity alone for potent activity.[11,12]

Perhaps the most insightful finding from a drug design perspective was the failure of

leucine substitutions to improve antifungal activity. The progressive decline in potency from CAP-X1 to the inactive CAP-X4 strongly suggests that there is an optimal hydrophobic threshold for this peptide scaffold. Exceeding this threshold, as was done with increasing leucine content, likely leads to detrimental effects such as peptide self-aggregation in the aqueous YM broth, reducing the concentration of active, monomeric peptides. This phenomenon, where excessive hydrophobicity leads to a loss of activity, is a well-documented principle in AMP design and underscores that a simple, linear increase in hydrophobicity is not a guaranteed strategy for improving efficacy.[3]

When contextualizing the potency of our lead peptide, CAP-Y, its MIC of 128 µM against *C. albicans* indicates moderate activity. For comparison, the widely studied human peptide LL-37 exhibits a wide range of MIC (4.5–55 µM) against *C. albicans* in different reports, while the clinical antifungal drug fluconazole inhibits most *Candida* spp. at ~26µM.[13-15] However, the activity of CAP-Y is comparable to, or better than, many other unoptimized, naturally-derived peptides in early-stage discovery. Therefore, CAP-Y should be viewed not as a final drug candidate, but as a promising and validated scaffold for further optimizations. The hemolytic activity of CAP-X and CAP-Y remained low, with less than 10% hemolysis observed even at 512µM, suggesting a favorable therapeutic window. Additionally, the MTS assay confirmed that these peptides exhibited minimal cytotoxicity on 4T1 murine cells, further supporting their potential as safe antifungal agents. A critical parameter for any potential therapeutic is its selectivity for microbial versus host cells, often expressed as a Selectivity Index (SI). Calculating the SI as the ratio of the highest non-toxic concentration to the MIC value, CAP-Y demonstrates an SI of at least 4, while CAP-X has an SI of 2. While CAP-Y's higher SI suggests a better therapeutic window, its safety profile is complex. Our cytotoxicity data shows CAP-Y is significantly less toxic to 4T1 cancer cells than CAP-X. Conversely, it exhibits slightly, but significantly, higher hemolytic activity. This differential toxicity highlights the distinct biophysical interactions of the peptides with different mammalian membrane types and warrants further investigation.

We acknowledge the limitations inherent in this preliminary study. Our investigation was limited to a single reference strain of *C. albicans*. Future work should broaden this scope to include clinical isolates and other pathogenic *Candida* species. Secondly, while our data suggest a membrane-disruptive mechanism, subsequent studies employing techniques like membrane permeabilization assays or electron microscopy are required to elucidate the precise mechanism of action. Finally, peptide stability and aggregation evaluation studies would be critical to develop peptide therapeutics.

Our findings contribute new insights into their antifungal potential, particularly against *C. albicans*, a clinically important opportunistic pathogen. While the activity observed is moderate compared to conventional antifungals, these peptides could serve as lead compounds for further structural optimization or combinatorial therapy.

V. CONCLUSION

This study evaluated the antifungal activity of a series of Cn-AMP1-derived peptides against *Candida albicans*. CAP-Y and CAP-X demonstrated the most promising profiles, with moderate antifungal efficacy and low cytotoxicity. Leucine-substituted derivatives did not enhance the activity, highlighting the

importance of balanced physicochemical properties in AMP design. These findings provide preliminary evidence supporting the potential of Cn-AMP1 derivatives as scaffolds for developing novel peptide-based antifungal agents.

Acknowledgements

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 108.05-2021.06. The authors acknowledge the facility support from Phenikaa University for the chemical synthesis, chemical analysis, hemolysis, cytotoxicity assay, and Nguyen Tat Thanh University for the antifungal assay.

Conflict of interest:We have no conflict of interest to declare.

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