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JOÃO MOREIRA DE MATOS NETO

PERFIL ANTIMICROBIANO E CITOTÓXICO DE PEPTÍDEOS DO NÉCTAR DE TABACO ORNAMENTAL CONTRA LEVEDURAS DO GÊNERO *Candida*

FORTALEZA
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CDD

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RESUMO

Antibióticos convencionais vêm se tornando cada vez mais ineficazes diante do crescente número de infecções por microrganismos multirresistentes, incapazes de serem tratadas pelos métodos tradicionais. Como resposta, a busca por terapias antimicrobianas inovadoras vem se intensificando no meio científico, onde há certo destaque por parte dos peptídeos antimicrobianos (PAMs), moléculas de amplo espectro que causam prejuízos a múltiplos componentes microbianos e atenuam as chances de desenvolvimento de resistência. Plantas são fontes abundantes destas moléculas, o que direcionou o presente estudo na avaliação da eficácia de seis peptídeos do néctar floral do tabaco ornamental contra leveduras de importância clínica (*Candida albicans*, *Candida krusei*, *Candida parapsilosis* e *Candida tropicalis*), averiguando sua ação sobre a membrana microbiana, acúmulo de espécies reativas de oxigênio no citosol e potencial citotóxico, somada à investigação do perfil estrutural da molécula em diferentes solventes. Dos peptídeos testados, apenas um (Pep6) possuiu atividade contra todas as cepas testadas em concentrações inferiores a 100μM. Ensaios posteriores demonstraram que esse peptídeo, de caráter fungicida, era capaz de causar danos às leveduras mediante permeabilização da membrana e indução de danos oxidativos, sem causar danos significativos nas concentrações testadas a eritrócitos humanos, fibroblastos murinos e embriões de *Danio rerio*. Além disso, dados estruturais previram sua capacidade de formar estruturas α-helicoidais em ambientes miméticos de membrana. Posteriormente, utilizando métodos respaldados na área clínica, os valores de MIC do Pep6 (Nicotianina-I) foram reavaliados e obteve-se uma concentração quase quatro vezes menor (25μM) para *C. tropicalis*, assim como um efeito sinérgico com fluconazol e caspofungina. Por fim, a capacidade de interação a membranas e penetração celular foram confirmadas experimentalmente. Estes resultados destacam o potencial deste peptídeo e podem embasar futuros estudos que visem otimizar sua capacidade enquanto agente antimicrobiano.

Palavras-chave: antifúngicos; mecanismos de ação; peptídeos de defesa; permeabilização de membrana; sinergismo.

ABSTRACT

Conventional antibiotics have become ineffective in the face of the growing number of infections caused by multidrug-resistant microorganisms, leading to untreatable infections. In response, innovative antimicrobial therapies are being extensively explored, emphasizing antimicrobial peptides (AMPs) in this context. These broad-spectrum molecules are capable of damaging multiple microbial components, hindering the chance of resistance development in strains. Plants are abundant sources of highly bioactive biomolecules, including AMPs, which led this study to evaluate the efficacy of six peptides from the floral nectar of ornamental tobacco against six clinically relevant yeasts (*Candida albicans*, *Candida krusei*, *Candida parapsilosis* e *Candida tropicalis*), assessing their effects on membrane integrity, intracellular reactive oxygen species accumulation, and cytotoxic potential, couplet with an investigation of peptide's structural profile in different solvents. Among the tested peptides, only one (Pep6) exhibited activity against all tested strains in concentrations below 100μM. Further assays demonstrated its fungicidal properties and its ability to target yeasts through membrane permeabilization/disruption and oxidative damage induction, without causing any harm at the tested concentrations to human erythrocytes, murine fibroblasts, or *Danio rerio* embryos. Moreover, structural data predicted its ability to adopt an α-helical pattern in membrane-mimetic environments. Subsequent studies, using clinically validated methodologies, reported an almost fourfold reduction in MIC values for Pep6 (Nicotianin-I) against *C. tropicalis*. Additionally, synergistic effects with caspofungin and fluconazole were also confirmed. Finally, the peptide's ability to interact with membranes and penetrate cells were also experimentally confirmed. These findings highlight the potential of Nicotianin-I and may support future studies aimed at optimizing its efficacy as an antimicrobial agent.

Keywords: antifungals; mechanisms of action; defensins; membrane permeabilization; synergism.

SUMÁRIO

1. INTRODUÇÃO GERAL

A área da saúde enfrenta severas problemáticas no que diz respeito à emergência de novas variantes microbianas cada vez menos sensíveis aos tratamentos com antimicrobianos convencionais. O sistema de saúde global vem lidando continuamente com o desenvolvimento de cepas resistentes e multirresistentes que dificultam a adoção de abordagens terapêuticas efetivas e, em casos mais graves, chegam a eliminar completamente as possibilidades de intervenções médicas cabíveis, caracterizando as chamadas infecções intratáveis (Gray e Wenzel, 2020; Murray *et al.*, 2022).

Segundo a Organização Mundial de Saúde (OMS), os microrganismos multirresistentes são considerados uma séria ameaça à saúde pública, sendo estimado pelo Grupo de Coordenação Interinstitucional em Resistência Antimicrobiana que, até 2050, a taxa anual de mortalidade ocasionada por organismos resistentes escalará ao patamar de 10 milhões (Painuli *et al.*, 2023). Em resposta ao surgimento acelerado de novas cepas resistentes, vê-se um grande esforço da comunidade científica e farmacêutica na busca por novas moléculas antibióticas, visando seu potencial isolado ou combinado com drogas já estabelecidas no mercado para o tratamento de superinfecções, focando sempre na redução da possibilidade de desenvolvimento de resistência pela estirpe por meio da busca de drogas multialvo e combinação de moléculas com ação sinérgica (Gray e Wenzel, 2020).

No intuito de guiar a comunidade científica em tal esforço conjunto, a OMS publica listas de agentes patogênicos de prioridade, sendo divididos em três principais categorias: ‘Importância Crítica’, ‘Alta Importância’ e ‘Média Importância’. Sendo organismos de destaque as bactérias e leveduras: *Cryptococcus neoformans*, *Candida albicans e C. auris* (Importância Crítica); *C. parapsilosis*, *C. tropicalis*, *Enterococcus faecium*, *Staphylococcus aureus* e *Pseudomonas aeruginosa* (Alta Importância) (World Health Organization, 2022, 2024). Isto, somado aos dados epidemiológicos previamente citados, põe luz acerca da relevância e enfoque necessário na prospecção de novas drogas e os principais alvos a serem salientados neste âmbito de estudo.

É neste momento que a biotecnologia se mostra como estratégia pertinente à solução de tal problemática. Uma vez que a busca por moléculas antimicrobianas a partir de fontes biológicas já mostrou ser uma ferramenta em potencial no combate a infecções, como no caso de metabólitos secundários de microrganismos (Singh e Dubey, 2020), óleos essenciais de plantas (Coimbra, Ferreira e Duarte, 2022), proteínas e enzimas de classes líticas variadas, como: Proteínas Ligantes a Quitina (*Chitin Binding Proteins* - CBPs) (Silva Neto, da *et al.*,

2022), inibidores de peptidases (Melo *et al.*, 2019), lectinas (Nabeta *et al.*, 2021), quitinases (Loc *et al.*, 2020) e peptídeos antimicrobianos (PAMs) (Aguiar, Neto, *et al.*, 2023; Branco *et al.*, 2022; Neto *et al.*, 2022). Essa última classe de biomoléculas apresenta características interessantes do ponto de vista clínico, tais como: amplo espectro de ação, diversidade de alvos celulares, biocompatibilidade e possibilidade de ação combinada com fármacos já bem estabelecidos no mercado (Chen e Jiang, 2023; Xuan *et al.*, 2023).

Nos estudos de prospecção de antimicrobianos é importante a adoção de um alvo e metodologia justificáveis para uma sondagem rápida e custo-efetiva. Microrganismos destacam-se na área de busca por novos compostos tanto por uma questão histórica relacionada à primeira geração de antimicrobianos descobertos, como no caso da penicilina e nistatina (Fleming, 1941; Michel, 1977), quanto pela menor complexidade metabólica em comparação a organismos pluricelulares. Dentre as diferentes abordagens adotadas por pesquisadores destacam-se a mineração de genomas (Blin *et al.*, 2021) e a prospecção ativa, que avaliam a produção de novos metabólitos secundários com ação antimicrobiana (Eshboev *et al.*, 2024), peptídeos antimicrobianos de ação generalista (Jeon *et al.*, 2023) e *lasso-peptides* de ação específica (Raphel e Halami, 2024), por exemplo. Ainda assim, organismos vegetais exibem potencial equiparado ao microbiano neste aspecto em virtude de sua maior robustez metabólica, o que justifica uma maior diversidade de moléculas bioativas distribuídas nos diferentes órgãos vegetais, o que vem trazendo resultados importantes na área médica através da descoberta de moléculas com potencial antibacteriano (Özcan, 2020), antifúngico (Zhang, W. *et al.*, 2021), antinociceptivo (Bhuiyan *et al.*, 2020), anti-inflamatório (Shovo *et al.*, 2021), entre outros.

O néctar floral - uma fonte subexplorada de moléculas antimicrobianas – um fluido vegetal que apesar de altamente rico em fontes de carbono e nitrogênio, apresenta uma acentuada resistência à ação microbiológica (Ma *et al.*, 2017) decorrente dos efeitos combinados de fatores diversos, tais como: alta concentração de peróxido de hidrogênio (H_2O_2), presença de proteínas vegetais de defesa (PVDs) e peptídeos com ação antimicrobiana (Carter e Thornburg, 2004). Assim, o estudo de PAMs de néctar floral é um campo de pesquisa promissor e inovador, sendo o estudo de Parra *et al.* (2022) o primeiro notificado neste âmbito, onde houve a identificação de 793 peptídeos do néctar de plantas de tabaco ornamental e, mediante estudos *in silico*, a seleção de seis destes peptídeos para posteriores estudos *in vitro* que validaram suas ações antibacteriana e/ou antifúngica.

Portanto, o presente estudo visou avaliar o efeito *in vitro* dos seis peptídeos antimicrobianos do néctar de tabaco ornamental contra leveduras patogênicas do gênero

Candida, elucidando questões relacionadas aos mecanismos da ação destes PAMs, caracterizando suas estruturas secundárias sob diferentes condições (temperatura, pH e solventes). Além disso, o estudo põe luz acerca do potencial tóxico *in vitro* do peptídeo mais promissor, utilizando eritrócitos humanos e fibroblastos murinos, e *in vivo*, utilizando embriões de peixe-zebra (*Danio rerio*). Por fim é avaliada a capacidade do peptídeo mais promissor de agir de maneira sinérgica com drogas antifúngicas de classes metabólicas distintas.

2. HIPÓTESE

Peptídeos antimicrobianos (PAMs) do néctar floral de *Nicotiana* possuem ação antifúngica contra diferentes espécies de *Candida*, perturbando o *status quo* da membrana celular microbiana, mediante a formação de estruturas α-helicoidais, promovendo o acúmulo de espécies reativas de oxigênio (EROs), ao passo que apresentam baixa citotoxicidade em células não-alvo. Além disso, somada à permeabilização da membrana, esses PAMs conseguem penetrar a membrana das leveduras e aumentar a sensibilidade das leveduras a tratamentos antifúngicos convencionais.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar e caracterizar o potencial antimicrobiano de seis peptídeos do néctar floral de tabaco ornamental, avaliando-os em relação ao seu mecanismo de ação contra leveduras de interesse clínico.

3.2 Objetivo Específicos

- Determinar o potencial de inibição de seis peptídeos quanto ao crescimento de diferentes espécies de *Candida*, selecionando o peptídeo mais promissor para os testes seguintes;
- Avaliar permeabilização da membrana, formação de poros e o perfil ultraestrutural das células fúngicas tratadas com o peptídeo;
- Identificar o possível acúmulo de espécies reativas de oxigênio (EROs) pelo tratamento com o peptídeo;
- Estudar as características físico-químicas do peptídeo sob diferentes temperaturas, solventes e pHs;
- Avaliar, por simulação *in sílico*, qual perfil de ação sob a membrana justifica a ação do(s) peptídeo(s);
- Verificar a citotoxicidade *in vitro* e *in vivo* do peptídeo;
- Averiguar a presença de sinergismo entre o peptídeo e drogas antifúngicas de diferentes classes sobre leveduras;
- Avaliar alterações no potencial superficial da membrana das leveduras tratadas com o peptídeo;
- Inferir se o peptídeo possui a capacidade de penetrar a célula e o núcleo das leveduras-alvo.

4. REFERENCIAL TEÓRICO

4.1 Doenças Infecciosas

Uma doença infecciosa é descrita como uma enfermidade provocada pela proliferação de microrganismos, como bactérias, fungos filamentosos, leveduras, protozoários e/ou seus subprodutos nocivos (Seventer, van e Hochberg, 2016), sendo tais quadros responsáveis por um massivo número de hospitalizações e mortes a nível mundial. Apenas em 2019, um total de 13,7 milhões de mortes por síndromes infecciosas foram mundialmente contabilizadas (Ikuta *et al.*, 2022), número que vem ganhando força pela contínua emergência de novos patógenos multirresistentes sobretudo na era pós-COVID, devido ao aumento do uso de antibióticos prescritos pela necessidade de evitar o desenvolvimento de infecções microbianas oportunistas em pacientes afetados pelo vírus, somado ao aumento de pacientes sob tratamento intensivo que geraram uma maior proliferação de cepas nosocomiais e um consequente incremento no número de relatos de isolados resistentes (Catalano *et al.*, 2023).

Dentre os agentes causadores de doenças infecciosas, há um certo destaque nas áreas clínica e médica para fungos e leveduras, microrganismos eucarióticos que podem ser patógenos oportunistas (Ciurea *et al.*, 2020). Dentre os patógenos fúngicos, o gênero *Candida* mostra grande destaque no âmbito clínico pelo elevado percentual de infecções ocasionadas por estas leveduras, majoritariamente em decorrência da sua existência como microrganismo comensal em diversos tecidos humano, bem como sua habilidade de comutar certos aspectos morfofisiológicos que implicam no estabelecimento de características patogênicas sob determinados quadros clínicos, como imunossupressão ou desregulação da microbiota do hospedeiro (Lopes e Lionakis, 2022). Dentre as espécies mais comumente isoladas de hospedeiros humanos, tanto como comensal quanto como patógeno, destacam-se as espécies: *Candida albicans* – a mais bem descrita na literatura – e outras, chamadas de não-*albicans*, que possuem importância pareada ou até mesmo superior a esta, como a *Candida auris, Candida glabrata, Candida krusei e Candida parapsilosis* (Ciurea *et al.*, 2020; Lopes e Lionakis, 2022).

Cryptococcus neoformans, o agente infeccioso responsável pelo desenvolvimento da meningoencefalite criptocócica (Rathore *et al.*, 2022), e fungos dermatófitos pertencentes aos gêneros *Tinea* e *Trichophyton* (Dahdah e Scher, 2008) também se destacam neste âmbito.

4.2 Infecção por *Candida spp.*

4.2.1 Candidíases

A infecção mediada por espécies de *Candida* podem ser divididas em dois subtipos: (1) Candidíase, classificada pelo desenvolvimento de hifas em tecidos mucosos, como a mucosa oral, vaginal e gastrointestinal; e (2) Candidemia, um tipo de candidíase invasiva de proporção sistêmica – a forma mais preocupante de infecção por *Candida* – que atinge múltiplos órgãos e está associada a uma alta taxa de mortalidade, se desenvolvendo principalmente em pacientes acometidos por fatores de risco, tais como: tratamento intensivo (sobretudo em condições de intubação, uso de sondas ou cateteres), pós-transplante, sob tratamento por antibióticos de amplo espectro, imunocomprometidos ou diabéticos (McCarty, White e Pappas, 2021).

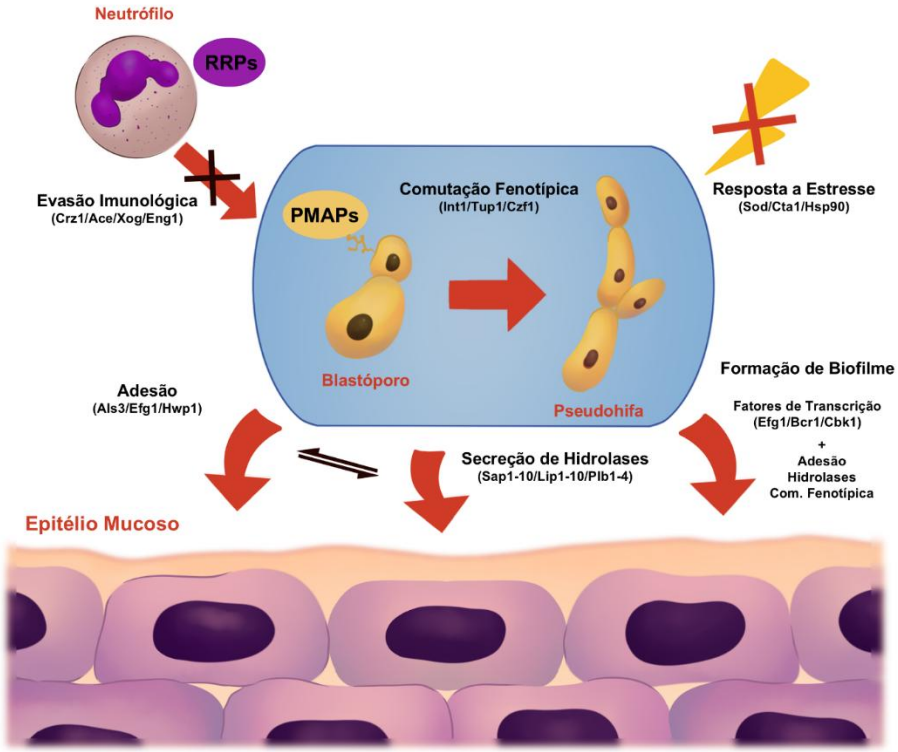
Candidemias estão entre as causas mais comuns de infecções associadas ao sistema de saúde (Magill *et al.*, 2018), sendo *C. albicans* o patógeno mais comumente isolado (~50%) em casos de candidíase, tanto mucocutâneas quanto sistêmicas. Apesar disso, este percentual vem sofrendo uma redução gradual, sobretudo em virtude de um maior predomínio de resistência em espécies não-*albicans* (Pfaller *et al.*, 2019) e à aparição da *C. auris*, um novo patógeno emergente, que possui uma alta capacidade de resistir a antifúngicos e desinfetantes de superfície (amônio quaternário), além de uma alta capacidade formadora de biofilmes e virulência associadas (Lionakis e Chowdhary, 2024).

4.2.2 Mecanismos Moleculares Envolvidos no Processo Infeccioso

Previamente ao desenvolvimento dos quadros de candidíase, a levedura necessita que haja um processo de sinalização molecular que contribua para o desenvolvimento da patogênese mediante a expressão dos chamados genes de virulência (Figura 1). Dentre os elementos fundamentais envolvidos neste processo, destacam-se: Genes de Adesão (GA), relacionados com a aderência dos microrganismos às superfícies biológicas ou inorgânicas – no caso de catéteres ou outros aparatos médicos – mediante a expressão de proteínas (adesinas ou invasinas, como a Als3 e Hwp1) de parede que sejam responsáveis pelo reconhecimento e/ou ligação a proteínas do tecido epitelial ou a polímeros abióticos, sendo a expressiva variedade de adesinas resultante da pluraridade de superfícies que estes microrganismos podem vir a colonizar (Liu e Filler, 2011; Martin, Kavanagh e Velasco-Torrijos, 2021; Talapko *et al.*, 2021);

Genes de Hidrolases (GH), envolvidos na síntese de proteases aspárticas (Sap1-10), lipases (Lip1-10) e fosfolipases (Plb1-4), cuja expressões permitem tanto adaptabilidade metabólica quanto a indução de danos às células epiteliais mucosas (Lopes e Lionakis, 2022); Genes de Comutação Fenotípica (GCF), cuja expressão está associada à invasão dos tecidos epiteliais cutâneos por meio da alternação morfológica das leveduras entre os estados de blastósporos, pseudohifas e hifas (Int1, Tup1 e Czf1), apesar de não ser um processo comum a todas as espécies (Lopes e Lionakis, 2022); e Genes de Formação de Biofilme (GFB) – cruciais para a perpetuação do processo infeccioso – como Efg1, Bcr1, Tye7, Ire1 e Cbk1 (Ganguly e Mitchell, 2011; Talapko *et al.*, 2021), também associado aos próprios processos de adesão, invasão e colonização do tecido para promover a formação do biofilme, que atua como uma barreira física capaz de dificultar o acesso de antimicrobianos e células de defesa do hospedeiro aos patógenos (Pereira *et al.*, 2021).

Figura 1. Esquematização de mecanismos moleculares envolvidos no processo infecciosos de leveduras do gênero *Candida*.



Fonte: Autor, 2025.

Apesar de quadros infecciosos estarem intimamente associados a períodos de depressão imunológica, o sistema imunológico humano já possui mecanismos inatos de

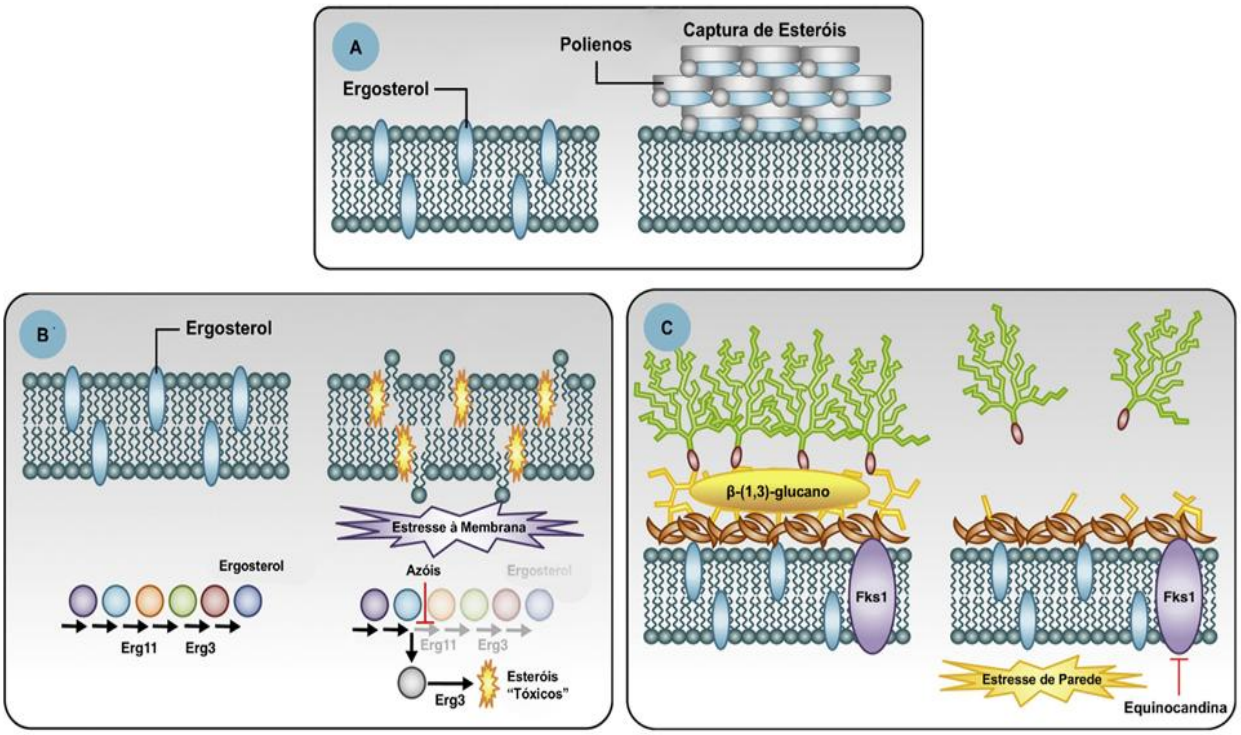
resposta a infecção por *Candida,* que se manifestam em maior ou menor nível a depender do grau de imunossupressão (Oliva *et al.*, 2023). Padrões moleculares associados a patógenos (PMAPs) – padrões de glicosilação existentes na parede fúngica – serão identificados por meio de receptores de resposta a padrões (RRPs) existentes na membrana de neutrófilos, macrófagos e células dendríticas e intermediarão processos de indução à resposta inflamatória e oxidativa, estimulando o recrutamento de células de defesa que participarão do processo de fagocitose dos alvos (Bojang *et al.*, 2021).

O biofilme, por si, já é um grande entrave ao processo de resposta imune. Porém, somado aos GFB, muitos outros fatores contribuem para a adaptação/evasão destas leveduras aos mecanismos de defesa do hospedeiro, sobretudo: Genes de Evasão Imunológica (GEI), que dificultam o reconhecimento de PMAPs das leveduras mediante modificação estrutural de componentes da parede celular fúngica (manoproteínas, quitina e β-1,3-glucanos) a partir de fatores que modifiquem a proporção (Crz1 e Ace) ou estrutura (Xog1 e Eng1) de β-glucanos da parede celular fúngica, no último caso promovido pela secreção de glucanases (Lopes e Lionakis, 2022; Ma *et al.*, 2024); ou Genes de Resistência a Estresse (GRE), que garantem a adaptabilidade do patógeno às condições adversas impostas pelo sistema imunológico do hospedeiro, como resistência a estresses oxidativo, térmico e osmótico (Hog1, Sod e Cta1) (Lopes e Lionakis, 2022), onde a modulação da expressão de genes de resposta a estresse é muitas vezes intermediado pela ação de *Heat Shock Proteins* (HSP), chaperonas que participam do enovelamento proteico pós-transcricional, com especial destaque para a Hsp90, uma HSP predominante mesmo entre diferentes espécies do gênero e que possui papel chave no processamento proteico de inúmeros fatores de virulência (Robbins e Cowen, 2023).

4.3 Agentes Antifúngicos e Mecanismos de Resistência

No intuito de se controlar uma infecção fúngica, a administração de substâncias antifúngicas se faz necessária. Sendo os Azóis, as Equinocandinas e os Polienos as principais classes de antifúngicos atualmente utilizados. Cada uma destas moléculas é responsável por abalar o *status quo* do ciclo de vida microbiano à sua maneira (Figura 2), gerando alterações em processos fisiológicos essenciais à sobrevivência do microrganismo e implicando em morte celular sob mecanismos similares dentro de cada classe (Kaur e Nobile, 2023; Seventer, van e Hochberg, 2016b).

Figura 2. Esquema geral dos mecanismos de ação de (a) polienos, (b) azóis e (c) equinocandinas em leveduras.



Fonte: Adaptado de Revie *et al.* (2018).

4.3.1 Polienos

Em 1949 tomou-se conhecimento da primeira droga antibiótica fungo-específica: a Nistatina (NIS), purificada a partir da cultura de actinobactérias do gênero *Streptomyces* e que apresentava ação antifúngica de amplo espectro (Hazen e Brown, 1951). Anos depois (1953), uma nova droga com ação similar – considerado até hoje como um dos antifúngicos mais potentes existentes – foi isolado a partir da fermentação de *Streptomyces nodosus*: a Anfotericina B (ANF)(Dutcher, 1968).

NIS e ANF, em conjunto com mais de 200 moléculas, compõem os Polienos, moléculas cuja estrutura química é formada por duas cadeias de carbono interligadas: sendo uma cadeia de hidrocarbonetos (hidrofóbica) e uma rica em hidroxilas (hidrofílica), o que confere caráter anfipático a essas moléculas. Isso garante sua incorporação à membrana plasmática microbiana mediante interação com ergosteróis presentes na bicamada. Essa interação promove a formação de poros de proporções molecular, causando um desequilíbrio osmótico somado ao acúmulo de espécies reativas de oxigênio no citosol (Houšť, Spížek e Havlíček, 2020; Ngece *et al.*, 2024).

Mecanismos de resistência associados à ação de polienos, dentre as diferentes classes de antifúngicos, estão entre as mais raras (Carolus *et al.*, 2020; Vincent *et al.*, 2013) pois estes fármacos possuem ação direcionada aos próprios componentes estruturais dos fungos e não seus precursores. Os principais mecanismos que conferem resistência a polienos estão associados a: (1) modificações na composição de ergosteróis da membrana fúngica a partir de mutações, inativações ou perdas de função de genes da via biossintética de ergosterol (Erg2, Erg3, Erg5 e Erg11), levando a redução dos níveis de ergosterol na membrana síntese e incorporação de moléculas análogas ao ergosterol na bicamada (Ahmady *et al.*, 2024; Carolus *et al.*, 2020); (2) modulação da expressão gênica de fatores relacionados com a mitigação de danos oxidativos enzimas do sistema redox (Cta1 e Sod) ou resposta a estresse (HSPs) sendo capazes de reduzir significativamente a eficácia dos danos acarretados pela formação de poros na membrana celular (Mello *et al.*, 2022); e (3) alterações de características estruturais da parede celular fúngica, incluindo a proporção de componentes e aumento de espessura, dificultando o acesso dos polienos aos ergosteróis de membrana (Mesa-Arango *et al.*, 2016).

4.3.2 Azóis

Em 1959 foi descoberto o primeiro composto azólico a ser introduzido no mercado farmacêutico como agente de controle de infecções fúngicas: o Cloromidazol (CMZ), o que culminou no incentivo a buscas por moléculas com características similares por parte da indústria (Fromtling, 1988). Os azóis compreendem uma gama de moléculas orgânicas cuja estrutura é composta por múltiplos anéis heterocíclicos nitrogenados, sendo subdivididos em duas subclasses: os imidazóis (Miconazol e Cetoconazol) que possuem dois átomos de nitrogênio compondo sua estrutura heterocíclica (anel imidazólico), e triazóis (Itraconazol, Fluconazol e Voriconazol) compreendendo três átomos de nitrogênio nos anéis azólicos (Assress *et al.*, 2021). Além disso, ambas as subclasses são altamente utilizadas no manejo de infecções fúngicas em decorrência do baixo custo relativo de produção, somados a uma boa efetividade clínica e efeitos adversos moderadamente leves (Shrestha, Garzan e Garneau-Tsodikova, 2017).

Estas moléculas atuam na inibição da lanosterol-14-α-demetilase, uma enzima dependente do citocromo P450 e que possui papel chave na via biossintética de ergosterol. Ao passo que a desmetilação dos precursores esteroidais é inibida, há um impedimento no processo de maturação da molécula de ergosterol e uma consequente depleção nos níveis de ergosterol

na membrana e o acúmulo de outros esteróis, que não cumprem adequadamente sua função estrutural (Assress *et al.*, 2021; Houšť, Spížek e Havlíček, 2020). Como consequência, há um prejuízo da função da membrana: seja por perda de características como fluidez e integridade ou impedimento de processos secundários associados à ancoragem de proteínas transmembrana envolvidas em processos de síntese da parede celular (Maertens, 2004).

A resistência a azóis se tornou um tópico de importância apenas poucas décadas após a liberação destes compostos no mercado, com um rápido aumento no número de relatos associados, sobretudo, ao uso indiscriminado do composto no tratamento de pacientes sob tratamento intensivo e/ou imunossuprimidos (Sanglard e Odds, 2002; Sobel *et al.*, 2001), sendo atualmente um dos tipos de resistência mais comumente desenvolvidas por fungos. Como o alvo destas substâncias possui natureza proteica e localização intracelular, inúmeros mecanismos podem estar diretamente relacionados com a resistência a estas drogas. Dentre os principais, destacam-se: *bypasses* metabólicos que alteram as rotas biossintéticas de esteróis de membranas – como a repressão de Erg3 – gerando acúmulo de 14-α-metilfecosterol na membrana e consequentemente na efetividade tanto de azóis quanto de polienos (Revie *et al.*, 2018); a redução na carga intracelular da droga mediante superexpressão de transportadores de membrana responsáveis pelo efluxo de drogas sejam eles dependentes de ATP (Cdr1 e Cdr2) ou facilitadores inespecíficos (Mdr1 e Mdr2), o que evita que a droga atinja concentrações capazes de inibir completamente a síntese do ergosterol (Xie *et al.*, 2014); e, por fim, a superexpressão ou mutações nos genes Erg11, Cyp51A ou Cyp51B, reduzindo a efetividade destes fármacos sob concentrações usuais ou reduzindo consideravelmente a afinidade de interação à enzima alvo, sendo os mecanismos de resistência mais comum (Assress *et al.*, 2021).

4.3.3 Equinocandinas

Em 1974 após ter sido identificada como um metabólito de um fungo filamentoso do gênero *Aspergillus* a Equinocandina B foi descoberta, sendo a primeira integrante do que viria a se tornar uma das três grandes classes de moléculas antifúngicas: as Equinocandinas. Apesar disso, apenas no início do século 21 que a Caspofungina (CAS), um derivado da pneumocandina B_0, foi aprovada para uso médico (Szymański *et al.*, 2022).

Equinocandinas são moléculas lipopeptídicas cíclicas, com cadeias laterais compostas por ácidos graxos acetilados a um hexapeptídeo cíclico contendo grupos hidroxila em seus núcleos estruturais (Perrine-Walker, 2022; Szymański *et al.*, 2022). A grande maioria

das equinocandinas atualmente comercializadas são de natureza semissintética e são usualmente desenvolvidas a partir de alterações nas cadeias laterais de equinocandinas naturais, visando maximizar suas funções antimicóticas e minimizar seus efeitos adversos (Hüttel, 2021). A ação destas moléculas está relacionada à perda de função da parede celular microbiana, onde a cadeia lateral de ácidos graxos permite sua ancoragem na membrana microbiana e a interação com a subunidade catalítica de um complexo enzimático transmembranar (β-(1,3)-glucano sintase) que é responsável pela síntese dos glucanos da parede celular fúngica. Essa interação de caráter inibitório gera danos estruturais que sensibilizam as células a fatores externos como: pressão osmótica, temperatura e fatores imunológicos (inflamação, estresse oxidativo e fagocitose), produzindo efeitos fungistáticos ou fungicidas (Gamaletsou, Walsh e Sipsas, 2018; Wheeler *et al.*, 2008). O tratamento mediado por equinocandinas também possuem indicativos de resultados mais satisfatórios numa perspectiva clínica – menores taxas de mortalidade quando associados ao tratamento de candidemias (Kato *et al.*, 2021).

Apesar de serem fármacos relativamente novos, não durou muito até que aparecessem os primeiros relatos de resistência a equinocandinas, seja ela intrínseca – como no caso de *C. neoformans* (Aguiar *et al.*, 2024; Walker, Gow e Munro, 2010) – ou adquirida mediante seleção artificial pelo uso indiscriminado de antimicóticos (Arendrup e Perlin, 2014).

Dentre os principais mecanismos moleculares envolvidos no processo de resistência a equinocandinas, destacam-se: a ineficácia de ação pela perda da interação droga-alvo mediante mutações nos genes envolvidos na síntese da subunidade catalítica (Fks1, Fks2 e Fks3) do complexo enzimático da β-(1,3)-glucano sintase (Suwunnakorn *et al.*, 2018), onde mudanças de um único aminoácido em regiões altamente conservadas (*Hotspots*) destes genes podem aumentar de 500 a 3000 vezes as concentrações mínimas inibitórias (MICs) para estas drogas (Cowen *et al.*, 2015); ou mecanismos adaptativos que atuam suprindo a carência de glucanos mediante síntese de outros componentes compensatórios da parede celular, um sinal mediado pela GTPase Rho1 – subunidade da β-(1,3)-glucano sintase – que coordena a função da proteína cinase C (PKC), sinalizando para a síntese de fatores de transcrição associados ao processo de síntese de quitina.

Outras biomoléculas também participam de processos regulatórios similares relacionados à síntese de parede celular, como Proteínas Cinase Ativadas por Mitogênio (MAPKs), Proteínas do Glicerol de Alta Osmolaridade (HOGs, que promovem a transdução de sinal mediante detecção de alta osmolaridade extracelular) e Calcineurina, que coordena vias regulatórias dependentes do influxo de íons Ca^{2+} para o citosol (desregulação osmótica ou

danos a membrana) induzindo a expressão de múltiplos genes associados com processos metabólicos essenciais e resposta a estresse (Levin, 2005; Szymański *et al.*, 2022).

4.4 Biotecnologia e Prospecção de Novos Antifúngicos

Como consequência da escassez de classes antifúngicas variadas, somado ao abuso e mau uso de tratamentos antifúngicos, podemos rastrear a justificativa do crescente número de isolados clínicos resistentes e multirresistentes (Berman e Krysan, 2020). Para apresentar características de resistência, as células devem desenvolver um ou mais, no caso de multirresistentes, mecanismos para contornar a ação do fármaco ou inativá-lo, como uma alta atividade de bombas de efluxo (Keereedach, Hrimpeng e Boonbumrung, 2020); evasão da interação droga-alvo por modificações metabólicas, via mutação nos genes de expressão do alvo proteico, tornando a ligação e a ação farmacológica ineficiente; superexpressão do alvo, saturando o fármaco e tornando a dose necessária para uma ação efetiva muito superior à usual (Robinson *et al.*, 2017).

A biotecnologia enquanto ciência multi e transdisciplinar se mostra como a maior aliada no processo de sobrepujar a crescente ineficiência de tratamentos envolvendo antifúngicos e antibióticos, unindo estudos de avaliação das estratégias bioquímicas e fisiológicas envolvidos no processo de resistência a fármacos para guiar: a prospecção de novas moléculas antibióticas (Liu *et al.*, 2023), modificação de fármacos pré-existentes (Gonzalez Gomez e Hosseinidoust, 2020) e predição de novos (Lima e Lima, de, 2023) otimizando o desenvolvimento de novas abordagens alternativas eficazes.

4.5 Peptídeos Antimicrobianos (PAMs)

A contínua prospecção de novos metabólitos com mecanismos promissores que podem de alguma forma contornar as táticas de resistência previamente citadas conduz a comunidade científica a diversos candidatos apropriados, como é o caso dos Peptídeos Antimicrobianos (*Antimicrobial Peptides* - AMPs), biomoléculas naturais que são ubiquamente distribuídas dentre todos os reinos taxonômicos (Gallardo-Becerra *et al.*, 2024). Estas moléculas participam do sistema de defesa inato de organismos contra uma grande variedade de patógenos e apresentam determinadas características físico-químicas (α-hélices anfifílicas, afinidade a proteínas moderadamente baixa, carga líquida positiva e outras) que possibilitam

interações de amplo espectro a membranas microbianas, negativamente carregadas em sua face externa, provocando danos celulares através da alteração da permeabilidade da membrana e levando ao desbalanço osmótico, extravasamento intracelular, superprodução de espécies reativas de oxigênio (EROs), além de alterações metabólicas ocasionadas pela interferência em processo de enovelamento proteico e processos enzimáticos diretos (atuando como inibidores) (Bechinger e Gorr, 2017; Erdem Büyükkiraz e Kesmen, 2022; Yao *et al.*, 2023).

Apesar dos aspectos citados e do elevado número de estudos exploratórios buscando o desenvolvimento e a implementação de PAMs como abordagens terapêuticas, ainda existe uma grande lacuna entre os resultados obtidos *in vitro* e a sua usabilidade de fato. Majoritariamente devido às características encontradas *in vivo* – como pH e força iônica, digestibilidade ou fatores multivariados difíceis de prever e simular – que implicam em alterações estruturais dos peptídeos e, consequentemente, na perda da sua ação enquanto agente antimicrobiano (Zainal Baharin *et al.*, 2021).

Outro gargalo na implementação de tratamentos terapêuticos envolvendo PAMs diz respeito às suas características intrínsecas, que culminam numa baixa estabilidade e biodisponibilidade, com uma possível ação citotóxica contra células de mamíferos e, consequentemente, células humanas (Svendsen *et al.*, 2019). Fatores estes intimamente associados à inespecificidade de ação dos peptídeos, que acabam por reconhecer e interferir em processos metabólicos tanto de células microbianas quanto de células humanas, restringindo ainda mais a progressão no uso de peptídeos na área terapêutica. Esta característica acaba sendo o principal limitador do potencial pleno dos PAMs, uma vez que um ajuste fino entre a citotoxicidade e potencial antimicrobiano deve ser mensurado e levado em consideração em todas as fases de estudos envolvendo estas moléculas (Greco *et al*., 2020).

A averiguação da ação citotóxica promovida por PAMs pode ser mensurada por diversas técnicas, sendo um dos métodos mais empregados neste intuito a avaliação da atividade hemolítica por meio da incubação dos peptídeos com suspensões de eritrócitos diluídas, seguida da quantificação da hemoglobina liberada no meio em comparação com o controle positivo de hemólise, geralmente um surfactante (Greco *et al.*, 2020). Apesar disso, um método definitivo para a verificação da presença de citotoxicidade ainda é algo irreal, devido ao quão complexo é um organismo em sua totalidade, fazendo com que os dados obtidos *in vitro* em muitos casos não gerem os mesmos efeitos ao serem avaliados em modelos *in vivo*, variando inclusive entre diferentes modelos (Oddo e Hansen, 2017).

4.5.1 Mecanismos de ação dos PAMs

Apesar de apresentarem diversos perfis físico-químicos indicadores de atividade antimicrobiana, sobretudo em etapas de predição *in silico*, os peptídeos antimicrobianos acabam por não possuir um modo de ação específico, mas sim uma pluralidade de mecanismos associados que podem se manifestar individual ou conjuntamente de acordo com o PAM e a cepa testada. Destacam-se, dentre dos mecanismos isolados de ação antimicrobiana a permeabilização da membrana plasmática microbiana, o estímulo direto ou indireto à superprodução de espécies reativas de oxigênio (EROs), indução de apoptose e o prejuízo de processos metabólicos essenciais mediante inibição enzimática ou interferência em processos de enovelamento proteico (Aguiar, Mesquita, *et al.*, 2023; Bechinger e Gorr, 2017; Luo e Song, 2021).

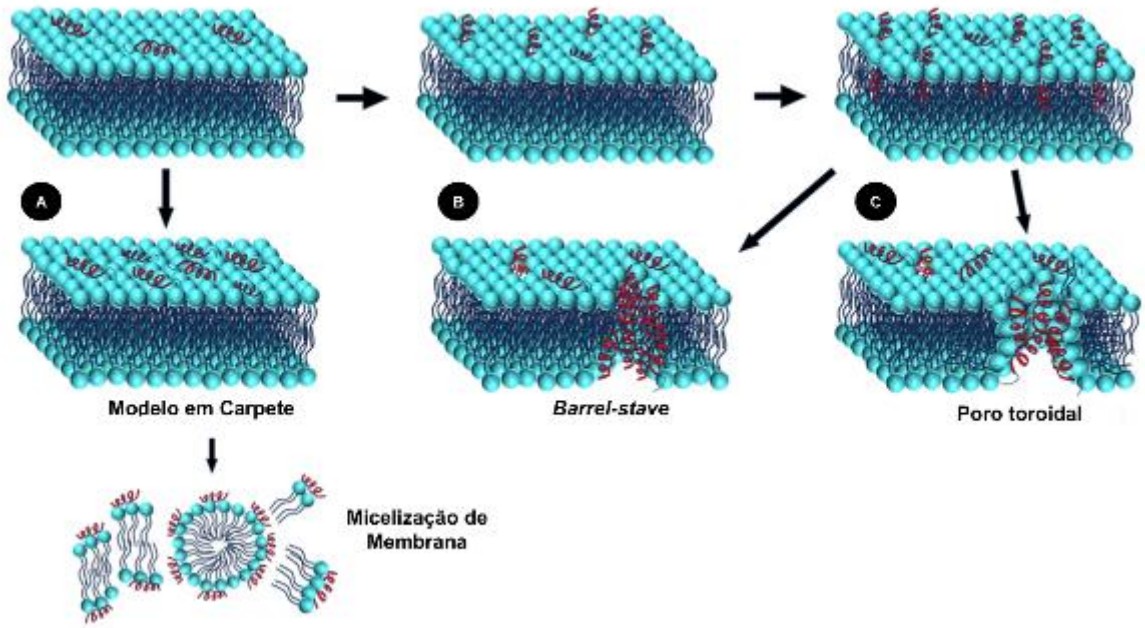
4.5.1.1 Permeabilização e Penetração de Membrana

Dentre a multiplicidade de mecanismos associados à ação antimicrobiana de peptídeos, grande destaque é dado a modelos de ação antimicrobiana baseado na interação à membrana plasmática, sobretudo à porção extracelular de microrganismo que possuem carga líquida negativa, o que dificulta a adoção de mecanismos de defesa pelo microrganismo afetado, uma característica altamente conservada de um ponto de vista evolutivo, somado ao desencadeamento de danos celulares secundários promovidos pela permeabilização membrana, como acúmulo de EROs e indução à morte celular programada (Zhang, Q. Y. *et al*., 2021). Devido à maior vantagem deste modo de ação, um dos principais fatores desejados ao se avaliar o potencial antimicrobiano são: sua carga líquida positiva, o índice de hidrofobicidade, a presença de α-hélices anfipáticas e um momento hidrofóbico que proporcione mudanças conformacionais que facilitem a interação e/ou inserção do peptídeo na membrana celular microbiana (Lima *et al.*, 2021).

A promoção da hiperpermeabilização, formação de poros ou lise membranar promovidos por peptídeos é discutido principalmente a partir de três mecanismos distintos esquematizados na Figura 3. Os diferentes modelos teóricos de ação destes englobam: (1) *Barrel-stave* ou Poros em Barris, que é explicada a partir da formação de poros transitórios instáveis nas membranas celulares microbiana, através da própria estrutura molecular de peptídeos anfotéricos e/ou de oligômeros (Tornesello *et al.*, 2020); (2) *Toroidal pores* ou Poros

Toroidais, onde os peptídeos combinam-se com os lipídeos, formando poros anulares em decorrência desta interação, podendo ser liberados para o meio intracelular no processo, mediante desestabilização das interações e consequentemente do poro (Sani e Separovic, 2016); (3) *Carpet* ou Carpete, onde os peptídeos com alta afinidade à membrana se aglomeram em sua superfície, formando uma diferença de potencial entre as bicamadas que geram alterações transitórias entre as camadas interna e externa. O reequilíbrio destes parâmetros promove a desestabilização transitória da membrana, internalizando parte dos peptídeos no processo ou promovendo sua micelização. Sob concentrações críticas esse processo de formação de micelas pode culminar em uma desestruturação completa que leva à lise celular dos microrganismos (Ciumac *et al.*, 2019).

Figura 3. Modelos de permeabilização e disrupção de membrana mediado por PAMs. (a) Carpete (b) Poros em barril e (c) Poros toroidais.



Fonte: Adaptado de Karmakar, Das e Banerjee (2024).

4.5.1.2 Alvos Intracelulares

Em complementaridade à perturbação da membrana, é comum que o peptídeo possua mecanismos complementares associados a inibição ou degradação de proteínas e ácidos nucleicos. Por se tratar de mecanismos intracelulares, a internalização prévia dos PAMs para o meio citosólico é um passo essencial desse processo. A ação de penetração à célula pode ser

realizada por mecanismos distintos e variam de acordo com a molécula e a célula em questão. Há dois métodos principais envolvidos nesse processo: a endocitose, promovida pela interação dos PAM com receptores de membrana, seguida da invaginação da bicamada lipídica e a entrada destas moléculas a um custo energético (ATP) (Ulmschneider, 2017); ou, durante os processos de permeabilização já previamente citados parte dos peptídeos podem ser internalizados, conferindo ação antimicrobiana e penetrante ao AMP que apresenta tal característica.

Quando já localizados no meio intracelular, as características dos peptídeos ditam seu alvo e consequentemente sua ação. Diferentes tipos de peptídeos com mecanismos distintos de ação intracelular já foram reportados na literatura, sendo os mais comuns: (1) Ação Relativa a DNA/RNA - degradação de gDNA e RNA (He *et al.*, 2018), inibição da síntese de ácidos nucleicos (Subbalakshmi e Sitaram, 1998) e inibição da atividade da RNA polimerase (Braffman *et al.*, 2019); (2) Interferência na Síntese Proteica - inibição do enovelamento proteico (Cardoso *et al.*, 2019) e inibição do processo de tradução (Mangano *et al.*, 2020); (3) Ação Metabólica Generalista – inibição do ciclo celular e, consequentemente, da divisão celular (Chileveru *et al.*, 2015; Subbalakshmi e Sitaram, 1998), destruição de organelas (Zhou *et al.*, 2023) e inibição de enzimas essenciais ao metabolismo energético (Maria-Neto *et al.*, 2012).

Conforme previamente comentado, os mecanismos aqui individualmente descritos não só podem como normalmente ocorrem conjuntamente. Desta forma, um mesmo peptídeo pode apresentar mais de um alvo intracelular, além da ação usual sobre a membrana. A capacidade de uma simples molécula de interagir com múltiplos alvos, causando um estresse generalizado à célula são limitadores do processo de aquisição/desenvolvimento de resistência pelas células microbianas (Ho *et al.*, 2016).

4.6 Plantas como fontes de PAMs

Em se tratando de PAMs originado de plantas é importante salientar seu potencial como candidatos promissores ao tratamento de infecções humanas, sobretudo pela carência de fatores coevolutivos envolvendo patógeno-hospedeiro, que levariam a uma maior probabilidade da pré-existência de estratégias resistivas à ação antimicrobiana por agentes não fitopatogênicos (Baindara, Ghosh e Mandal, 2020). PAMs são componentes essenciais ao sistema de defesa inato das plantas e podem ser encontrados em diversos órgãos vegetais, como frutos (Ochoa-Zarzosa *et al.*, 2021), flores (Parra *et al.*, 2022), sementes (Diz *et al.*, 2006) e o néctar (Parra *et al.* 2022) que, apesar de ser uma solução rica em fontes de carbono, é altamente resistente à

proliferação de microrganismos (Ma *et al.*, 2017), graças às propriedades inerentes ao néctar: acúmulo de peróxido de hidrogênio, presença de proteínas de defesa do néctar (nectarinas) e PAMs de néctar (Carter e Thornburg, 2004).

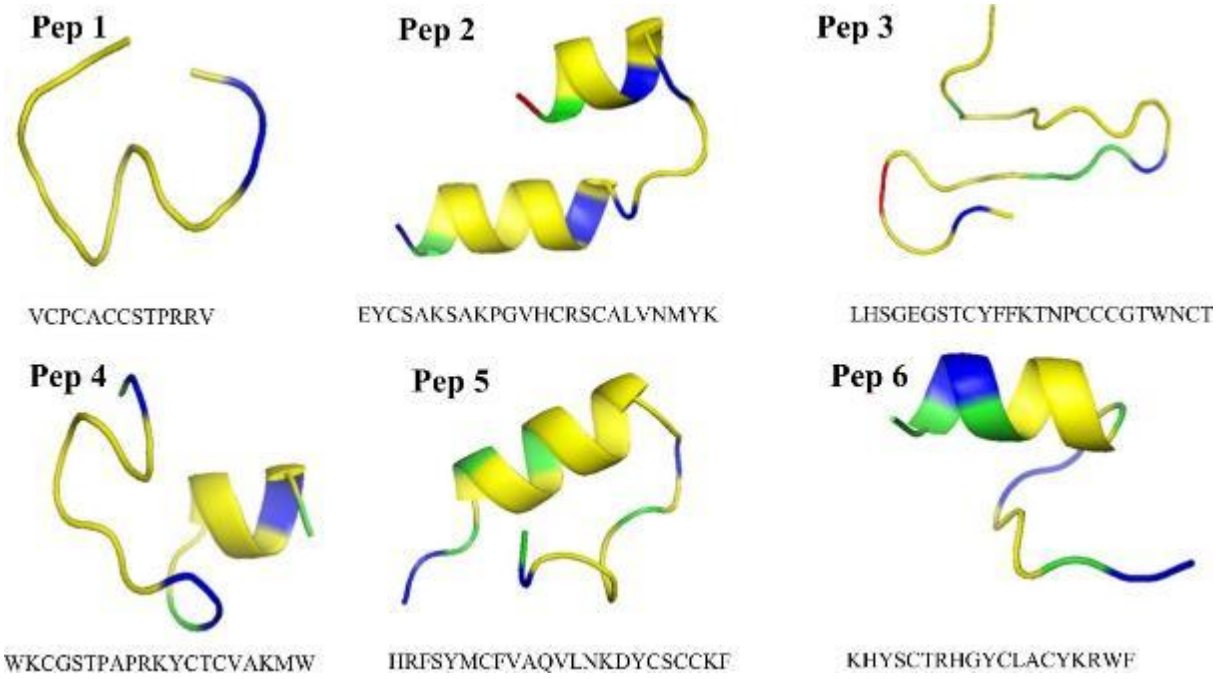
4.7 *Nicotiana* spp.

O tabaco, nome popular pelo qual são conhecidas as plantas do gênero *Nicotiana* (*Solanaceae,*) são majoritariamente distribuídos nos continentes Americano e Australiano (Ivanov, Sierro e Peitsch, 2020). A espécie *Nicotiana tabacum,* uma das mais importantes do gênero *Solanaceae*, é um vegetal amplamente estudado em decorrência de determinadas características que possibilitam suas aplicações em campos medicinais, agroeconômicos e científicos, seja pela sua utilização na obtenção de compostos de caráter fitoterapêutico, na sua utilização como plantas ornamentais ou pela sua aplicação como organismo modelo em estudos de engenharia genética. São plantas altamente exploradas para produção heteróloga de compostos de alto valor agregado (Ahrazem *et al.*, 2022), como modelos de estudos fisiológicos envolvendo estresses bióticos e abióticos (Berruezo *et al.*, 2021; Xie *et al.*, 2022) ou na bioprospecção de moléculas naturais do tabaco com diferentes aplicações biotecnológicas (Al-Lahham *et al.*, 2020).

4.7.1 PAMs de Nicotiana – Perspectivas no Tratamento de Doenças Infecciosas

Estudos emergentes envolvendo o néctar floral como uma fonte rica em PAMs promovido por Parra *et al*., (2022) identificaram, por meio de técnicas de LC-MS/MS, cerca de 800 peptídeos provenientes do néctar de plantas de tabaco ornamental. Após a obtenção dos dados referentes às sequências de aminoácidos, foi realizada a avaliação *in silico* de carga líquida, índice de *Boman*, hidrofobicidade, comparação com bancos de dados de peptídeos antimicrobianos, dentre outros. Os peptídeos que obtiveram o melhor perfil de predição da atividade antimicrobiana *in silico* foram selecionados para as etapas seguintes, sendo nomeados de: Pep1, Pep2, Pep3, Pep4, Pep5 e Pep6 (Figura 4), com estudos *in vitro* para a comprovação da ação antimicrobiana dos peptídeos contra fitopatógenos (*Aspergillus niger*, *Penicillium digitatum*, *Colletotrichum spp.*, *Pseudomonas syringae*, *Xanthomonas perforans* e *Erwinia amylovora*), detalhados na Tabela 1.

Figura 4. Estrutura tridimensional dos peptídeos antimicrobianos de néctar floral (Pep 1-6), preditos a partir do software PEP-Fold 4.0.



Fonte: Parra *et al.* 2022.

Tabela 1. Resumo dos resultados obtidos pela avaliação da ação antimicrobiana dos seis peptídeos selecionados pelo estudo de Parra *et al*., (2022).

Cepa	Pep 1	Pep 2	Pep 3	Pep 4	Pep 5	Pep 6
P. syringae pv. syringae	-	+	+	-	-	++
P. syringae pv. Tomato	-	-	-	+	-	++
X. perforans	-	-	-	+	-	++
E. amylovora	-	-	-	-	-	+
A. niger	++	+	+	+	+	++
P. digitatum	+	+	+	++	+	++
Colletotrichum spp.	+	++	+	++	+	++

\+ = Inibição de crescimento/germinação presente
++ = Alta inibição de crescimento/germinação
\- = Inibição de crescimento/germinação ausente

Fonte: Autor, 2024.

5. CAPÍTULO II - ARTIGO CIENTÍFICO I

Artigo Científico Publicado na Revista: ACS Omega - Qualis A4 / IF 3.7

Nicotianin-I: a tobacco floral nectar peptide with anticandidal activity

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Abstract: Multidrug-resistant microorganisms are major threats to society, leading to the necessity of alternative molecules to fight them back. Antimicrobial peptides (AMPs), especially those derived from plants, have become relevant for multiple reasons. Therefore, this study evaluated six peptides identified in the floral nectar of ornamental tobacco for their effectiveness against four clinically relevant yeast species (Candida albicans, C. krusei, C. parapsilosis, and C. tropicalis). Pep6 (KHYSCTRHGYCLACYKRWF) was the only one that showed activity against all yeast species, with IC50 values ranging from 24 ± 1.9 μM to 80 ± 4.6 μM. Pep6 (named Nicotianin-I) was able to alter permeability or generate pores in the cell membranes of all microorganisms, in addition to inducing an overproduction of reactive oxygen species (ROS). Nicotianin-I adopted a structure similar to polyproline II (PPII) helix in buffer and SDS and α-helical pattern when subjected to a membrane mimetic environment (50% TFE). It demonstrated negligible hemolytic activity and exhibited no cytotoxic effects on murine fibroblast cells. Only at the highest concentration assessed (100 μg/mL or 42 μM), Nicotianin-I caused a 40% mortality rate in zebrafish embryos and delayed their hatching. This study demonstrates the potential of a novel peptide with anticandidal activity and opens the door to the rational design of new analogous peptides with improved properties.

Keywords: Antimicrobial peptide; Antifungal; Candidiasis; Circular dichroism; Cytotoxicity; Membrane Damage.

5.1 Introduction

Pathogenic microorganisms, including bacteria, filamentous fungi, and yeasts, are responsible for a significant number of hospitalizations and fatalities worldwide. In 2019, infectious syndromes accounted for more than 13 million deaths[1]. This scenario has intensified largely due to the emergence of new pathogenic strains that exhibit treatment resistance[2]. Yeasts, particularly those of the *Candida* genus, have garnered significant attention as pathogens implicated in infectious diseases[3].

Candida species-mediated infection can be classified into two subtypes: (1) Candidiasis, which is characterized primarily by yeast proliferation on mucosal tissues, such as the oral mucosa, gastrointestinal tract, and vaginal mucosa; and (2) Candidemia, which is a systemic type of candidiasis that affects multiple organs and is associated with a high mortality rate[3,4]. Furthermore, the emergence of clinical isolates resistant to available antifungal drugs can exacerbate these threats[5].

Consequently, some surveys have been developed to discover novel therapeutic options that can surpass and outperform the existing ineffectiveness of antifungal antibiotics[6]. Antimicrobial peptides (AMPs) have distinguished themselves in this aspect since they are recognized for their amphiphilic α-helices and overall positive charge. These characteristics have been associated with their primary mechanisms of action, including damage to the cell membrane and inducing oxidative stress by overproducing Reactive Oxygen Species (ROS)[7].

Plant antimicrobial peptides possess biochemical properties that make them highly favorable candidates for the treatment of human infections[8]. These AMPs are crucial elements of the innate immune system and are present in various plant tissues and organs, such as leaves[9], seeds[10] and others. Floral nectar has recently been identified as an abundant biological source of AMPs. Parra and colleagues[11] conducted the sequencing and characterization of almost 800 peptides derived from the floral nectar of ornamental tobacco. Afterwards, they selected six peptides and proved their ability to kill some phytopathogenic fungi and bacteria. Due to their high biotechnological potential, the current study focuses on the use of these six tobacco floral nectar peptides as anticandidal agents. Therefore, we describe the *in vitro* antifungal potential of these six peptides against *C. albicans, C. krusei, C. parapsilosis,* and *C. tropicalis*, with a focus on their mechanisms of action. Moreover, we characterized by circular dichroism and performed *in vitro* and *in vivo* toxicity tests with the most promising peptide.

5.2 Results and Discussion

5.2.1 Anticandidal activity

The present study aimed to assess the anticandidal properties of six peptides derived from ornamental tobacco floral nectar (a cross of *Nicotiana sanderae* and *N. langsdorffii*). The study specifically targeted *C. albicans, C. krusei, C. parapsilosis,* and *C. tropicalis*, assessing both the inhibitory capabilities and mechanisms of action. With respect to the *in vitro* antifungal activity, peptides Pep1, Pep2, Pep3, Pep4, and Pep5 exhibited negligeable or no significant inhibition against all strains tested (Figure 1). On the other hand, peptide Pep6 not only suppressed the growth of all tested organisms but also showed the lowest concentrations of inhibition among other peptides that were anticandidal (Figure 1). The respective IC_{50} and MIC values for peptide Pep6 against the *Candida* species were as follows: 80 ± 4.6 µM (194 µg/mL) and 120 ± 9.3 µM (292 µg/mL) for *C. albicans*, 57 ± 3.7 µM (140 µg/mL) and 133 ± 8.6 µM (323 µg/mL) for *C. krusei*, 46 ± 2.2 µM (111 µg/mL) and 141 ± 7.1 µM (342 µg/mL) for *C. parapsilosis*, and 24 ± 1.9 µM (59 µg/mL) and 86 ± 3.6 µM (210 µg/mL) for *C. tropicalis*.

The Antimicrobial Peptide Database (APD) presently comprises 5,099 peptides (https://aps.unmc.edu/, accessed on 13 January 2025), which includes 3,306 natural antimicrobial peptides (AMPs) sourced from the six kingdoms (410 from bacteria, 5 from archaea, 8 from protists, 29 from fungi, 268 from plants, and 2,580 from animals), 1,299 synthetic peptides, and 231 predicted AMPs with different functions or activities. APD contains 926 anticandidal peptides (https://aps.unmc.edu/, accessed on 13 January 2025). Antimicrobial peptides exhibit a wide range of biochemical properties and mechanisms of action, resulting in significant diversity of biological activity. Consequently, comparing MIC and IC_{50} values, especially among different microorganisms, is exceedingly challenging. For example, ToAP2 and NDBP-5.7, which are two peptides discovered through the analysis of scorpion venom cDNA libraries, exhibited MIC values of 12 µM and 25 µM against *C. albicans*[12], respectively. In contrast, it was found that the leaf peptides of *Capsicum annuum* did not exhibit any inhibitory effect on *C. parapsilosis* and *C. tropicalis*[13].

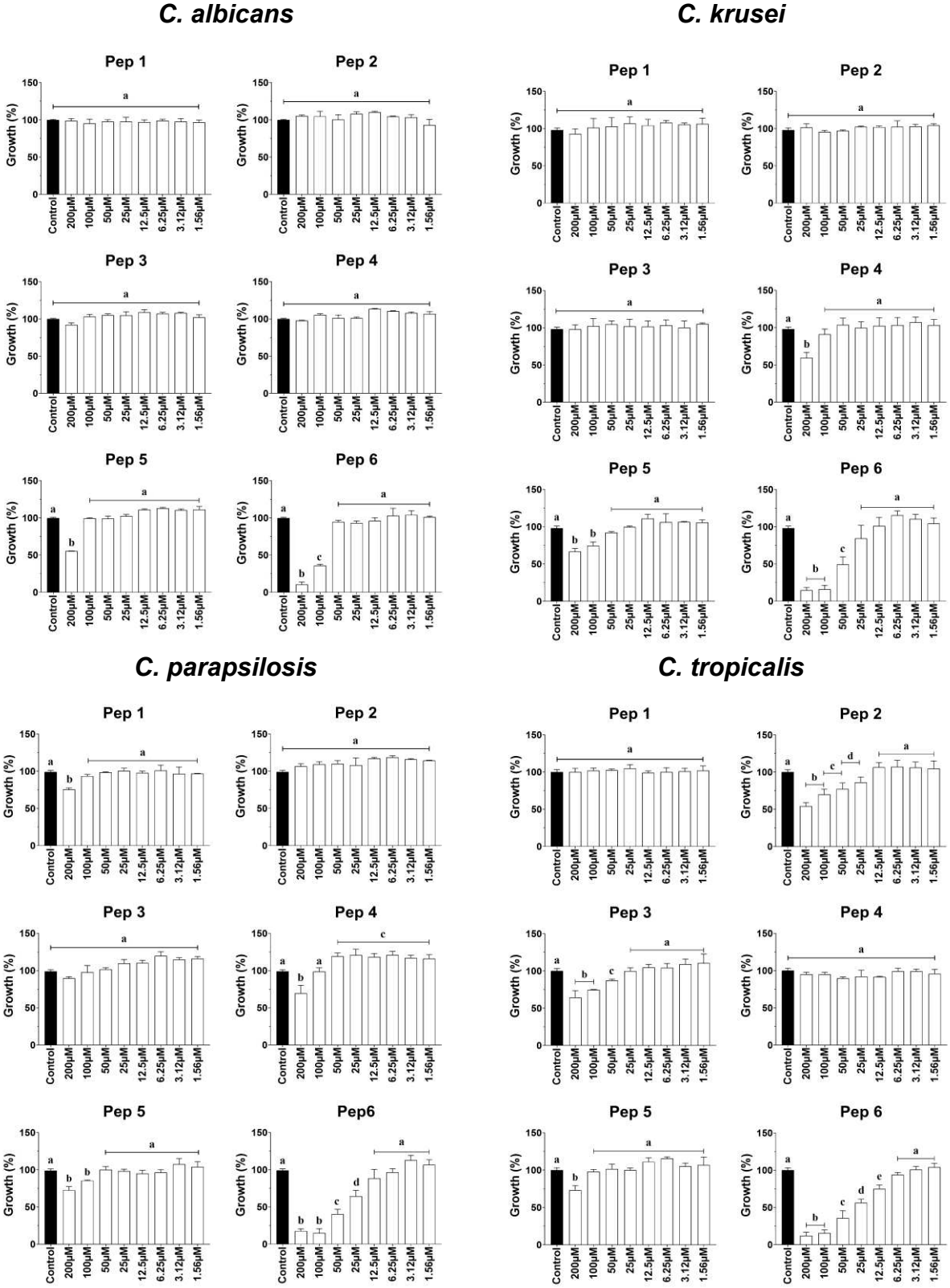

Figure 1. Anticandidal activity of floral nectar peptides. Pep 1 (VCPCACCSTPRRV), Pep 2 (EYCSAKSAKPGVHCRSCALVNMYK), Pep 3 (LHSGEGSTCYFFKTNPCCCGTWNCT),

Pep 4 (WKCGSTPAPRKYCTCVAKMW), Pep 5 (HRFSYMCFVAQVLNKDYCSCCKF) and Pep 6 (KHYSCTRHGYCLACYKRWF), at varying concentrations, were evaluated for their inhibitory effects on *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. The results are presented as mean ± standard deviation. Distinct letters denote significantly different groups ($p < 0.05$) from the control (0.15 M NaCl containing 5% DMSO).

Perez-Rodriguez and colleagues[14] examined the main biochemical characteristics and modes of action of 20 anticandidal peptides derived from diverse biological sources. The peptides ranged from 8 to 101 amino acids and exhibited MIC values from 0.18 to 800 μM[14]. Noteworthy among the identified peptides is NaD1, which was isolated from the flowers of *Nicotiana alata*. It exhibits significant antifungal efficacy against many agronomically relevant filamentous fungi and both susceptible and resistant strains of *C. albicans*[15,16]. NaD1 interacts with the fungal cell surface, leading to membrane permeabilization and the induction of excessive reactive oxygen species (ROS) production[17,18].

Due to its higher inhibitory capacity, peptide Pep6 was selected for all subsequent assays involving characterization, mechanisms of action, and toxicity. Additionally, peptide Pep6 was named Nicotianin-I, referencing the first antimicrobial peptide identified in the floral nectar of a Nicotiana species. A novel experiment was conducted to assess the fungicidal or fungistatic properties of Nicotianin-I. Cell viability tests, using MTT reagent, demonstrated that Nicotianin-I exhibited fungicidal activity against *C. albicans, C. krusei, C. parapsilosis,* and *C. tropicalis*, with efficacy of 83%, 79%, 81%, and 84%, respectively, at 200 μM (Figure S1). Other antimicrobial peptides possessing fungicidal properties against *Candida* strains have been documented, as Kyotorphin-derived conjugated peptides, which showed minimum fungicidal concentration (MFC) of 1000 μM (IbKTP-NH2) against *C. albicans* and *C. krusei*[19] and Temporin B derived peptides, exhibiting fungicidal activity at 1.8 μM (IC_{90})[20].

Our findings also indicate that Nicotianin-I diminished its efficacy at reduced doses (Figure S1). Interestingly, Nicotianin-I treated cells, at 12.5 μM, exhibited improved cell viability compared to control cells of *C. albicans, C. parapsilosis,* and *C. tropicalis* (Figure S1). MTT, a formazan salt, is reduced inside cells by oxidoreductases, dehydrogenases, or reduced coenzymes (NADH and NADPH)[21]. It is used to check if cells are alive or how metabolically active they are. Under stress conditions, certain cells can augment the expression of stress-response proteins, demanding higher metabolic and energy investments[22]. This may partially explain the augmented metabolism of Candida cells with reduced doses of Nicotianin-I. Some

studies examining cell viability in fungi[23], bacteria[24], and malignant (leukemia) cells[25] also observed similar findings.

5.2.2 Mechanism of action

Among the six-tobacco floral nectar peptides[11], Nicotianin-I exhibits the lowest predicted DNA-binding activity (18.7%), a feature that could greatly impede its function, as eDNA, a quorum-sensing molecule from the extracellular matrix of Candida cells, could imprison and inactivate it[26]. On the other hand, Chen and Jiang[27] described that the primary mechanism by which many antimicrobial peptides function is by interacting with the membrane of the target organism. Therefore, our hypothesis is that the anticandidal activity of Nicotianin-I is linked to its interaction with negatively charged microbial membranes, as it has positive net charge (+6). This interaction could lead to disruption of the membrane, either by creating pores or through membrane micellization. Subsequent investigations were conducted to elucidate the mechanism of action by which Nicotianin-I acts on fungal cells.

Candida cells exposed to Nicotianin-I and propidium iodide (PI) exhibited red fluorescence (Figure 2a), while the control cells did not display any fluorescence. PI is a fluorophore that produces red fluorescence upon binding to DNA. Furthermore, it is impermeable to the healthy plasma membrane. Hence, the presence of red fluorescence indicates membrane damage[28], potentially involving the formation of pores. In addition, we conducted an experiment to investigate the formation of pores induced by Nicotianin-I using Fluorescein Isothiocyanate conjugated with a 4 kDa dextran polymer (FITC-D4). In this case, the presence of green fluorescence within the cells indicates the formation of pores, with diameters capable of crossing molecules with minimum molecular masses of 4 kDa. Only the species *C. albicans* and *C. tropicalis*, when treated with Nicotianin-I, exhibited fluorescence raising. Compared to the control groups, the fluorescence increased by 45% for *C. albicans* and 76% for *C. tropicalis*. There was no increment in green florescence inside *C. krusei* and *C. parapsilosis* (Figure 2b).

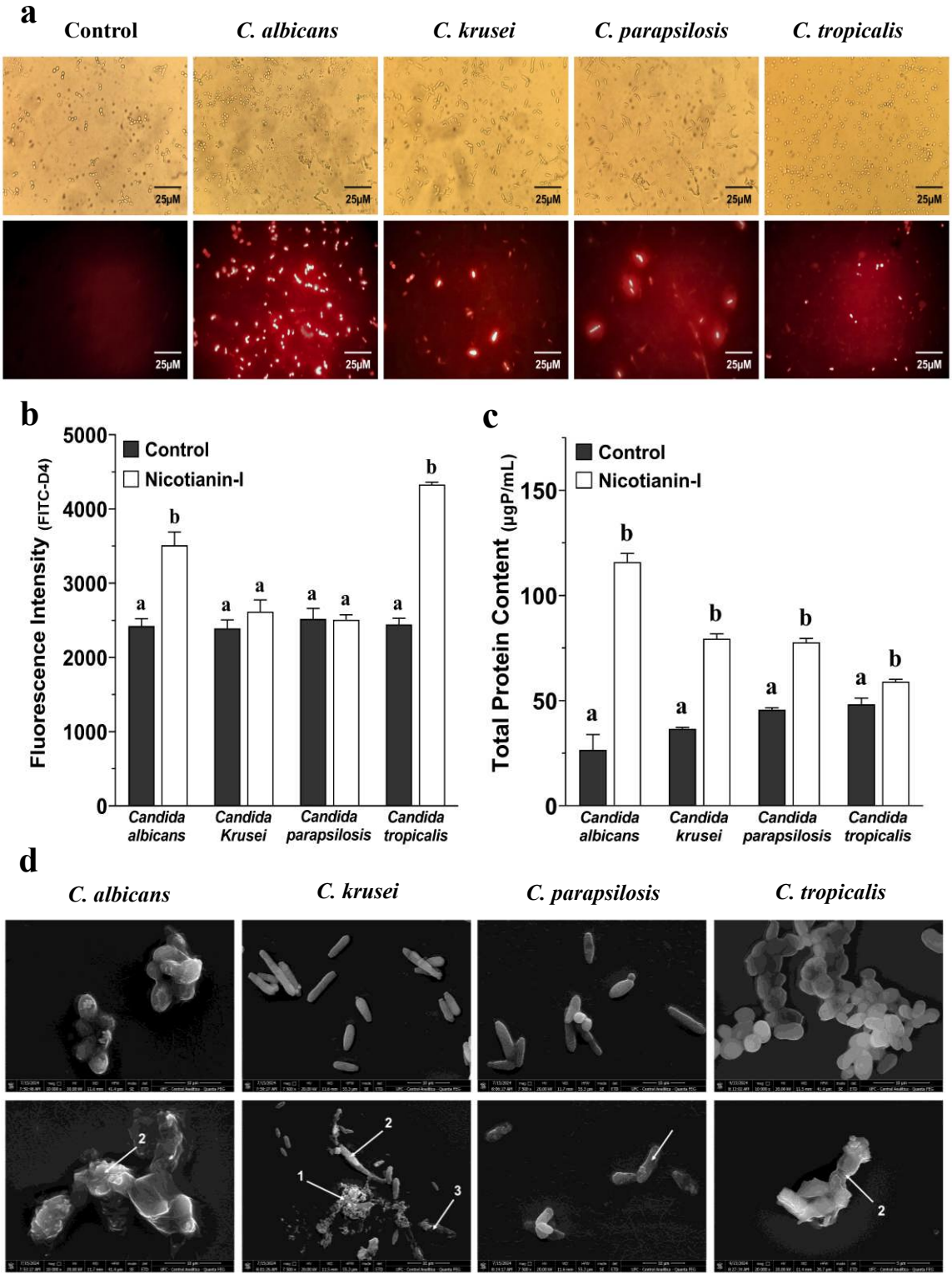

Fig. 2. Analysis of membrane damage and pore formation. **(a)** Propidium iodide fluorescence observed after 24 h of incubation with Nicotianin-I (excitation/emission: 488/525 nm). Superior panel: light microscopy. Bottom panel: fluorescence microscopy. Control: *C. albicans* cells

subjected to 0.15 M NaCl containing 5% DMSO. **(b)** FITC-Dextran (4 kDa) fluorescence was determined after 24 h of incubation with Nicotianin-I. Fluorescence intensity was quantified using emission and excitation wavelengths of 490 and 520 nm, respectively. The results are presented as mean ± standard deviation. Distinct letters denote significantly different groups ($p < 0.05$) relative to the control (0.15 M NaCl containing 5% DMSO). **(c)** Protein content of cell-free supernatant treated with Nicotianin-I (IC_{50}). **(d)** SEM images presenting (1) leakage of intracellular compounds, (2) rugous cells with indicative of membrane damage and (3) cellular debris, all caused by Nicotianin-I after 24 h. Upper panel: control cells (0.15 M NaCl containing 5% DMSO). Bottom panel: Cells treated with peptide Nicotianin-I (IC_{50}).

We also investigated the protein release by *Candida* cells following treatment with Nicotianin-I to assess membrane damage. Extracellular protein levels were significantly increased in all strains treated with Nicotianin-I (Figure 2c). The extracellular protein levels increased by 335% for *C. albicans*, 117% for *C. krusei*, 70% for *C. parapsilosis*, and 22% for *C. tropicalis*. The protein outflow results for *C. krusei* and *C. parapsilosis* appear contradictory when contrasted with the experimental evidence of pore development (Figure 2b). However, the inability to maintain 4 kDa dextran probes within the cells indicates that Nicotianin-I operates through detergent-like mechanisms rather than generating pores. This statement is corroborated by our ultrastructural morphological analyses conducted via scanning electron microscopy (Figure 2d), which revealed that *C. krusei* and *C. parapsilosis* treated with Nicotianin-I exhibited significant membrane damage, characterized by ruptures, intracellular content leakage, and cellular debris. Conversely, the SEM pictures of *C. albicans* and *C. tropicalis* exhibited increased rugosity relative to control cells, potentially indicating pore development induced by the peptide treatment. Consequently, these findings indicate that Nicotianin-I operates via pore-forming mechanism in *C. albicans* and *C. tropicalis* and through a detergent-like lysis mechanism in *C. krusei* and *C. parapsilosis*. The diverse mechanisms of action across different species can be explained by the distinct phospholipid membrane compositions of each microorganism[29], which may result in different oligomerization patterns of Nicotianin-I. Nonetheless, additional research is necessary for accurately determining the membrane-binding characteristics of Nicotianin-I on various cell membrane types.

Coupled with membrane damage, the mechanism of action of AMPs also is commonly associated with ROS overproduction[30]. ROS are subproducts of metabolism and their accumulation are commonly associated with enhanced metabolic response of cells under

stress conditions, signaling for metabolic adjustments and/or programmed cell death[31,32]. Therefore, DCFH-DA assays were performed to evaluate the ROS overproduction induced by Nicotianin-I. This fluorescence probe is deacetylated by cell metabolism (forming DCFH) and oxidizes (Fluorescent DCF-) in the presence of most ROS, presenting an optimal coverage for oxidative stress appraisal, indicating superoxide anions, hydrogen peroxide, peroxyl, and hydroxyl radicals[31].

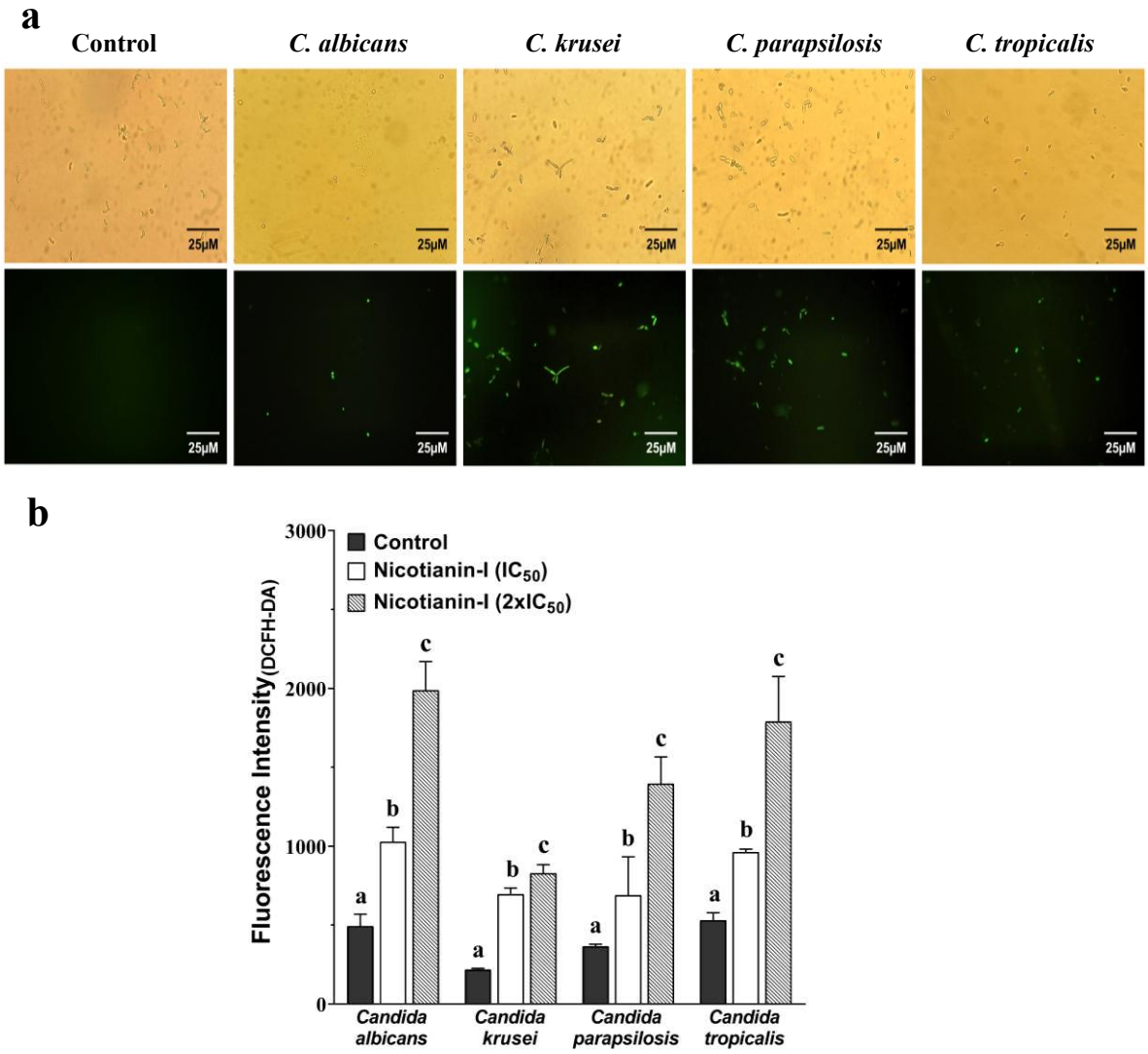

Figure 3. Assessing ROS overproduction induced by Nicotianin-I. **(a)** DCFH-DA fluorescence of Nicotianin-I (IC_{50}) treated cells (3 h) was observed by fluorescence microscopy (excitation/emission: 485/530 nm). Upper panel: light microscopy. Bottom panel: fluorescence microscopy. Control: *C. parapsilosis* cells treated with 0.15 M NaCl containing 5% DMSO. Green fluorescence indicates ROS overproduction caused by Nicotianin-I. **(b)** DCFH-DA fluorescence quantification of yeast cells treated with Nicotianin-I (IC_{50} and $2xIC_{50}$)

(excitation/emission: 485/530 nm). The results are presented as mean ± standard deviation. Distinct letters denote significantly different groups ($p < 0.05$) relative to the control (0.15 M NaCl containing 5% DMSO).

Candida cells treated with Nicotianin-I exhibited green fluorescence (Figure 3a), indicating ROS accumulation. Similarly, a quantitative evaluation correlated greater oxidative stress with higher concentrations of Nicotianin-I (Figure 3b). Incrementations on fluorescence intensity accounted for Nicotianin-I at its IC_{50} and two-fold IC_{50}: 108%/303% for *C. albicans*, 220%/280% for *C. krusei*, 88%/281% for *C. parapsilosis*, and 81/238% for *C. tropicalis*. ROS overproduction may cause damage on multiple cellular components and lead to cell death[30]. In this case, the antifungal capacity of Nicotianin-I might act by membrane damaging and oxidative stress. These mechanisms of action have been related to other AMPs[33].

5.2.3 Structural characterization

The Circular Dichroism (CD) spectra of Nicotianin-I demonstrates variability across different pH values (Figure 4a and Table 1). At pH 5.0 and 7.0, the CD spectra of Nicotianin-I exhibits two typical characteristics of a polyproline II (PPII) helix: a pronounced negative band in the range of 190-200 nm and a weaker positive band at 220-230 nm[34]. The structure is identified as a left-handed helix, exhibiting a dihedral angle usual of β-strands, and possesses an overall shape like a triangular prism[35]. PPII was identified in the collagen triple helix. However, other circular dichroism studies indicate that PPII may be present in various folded proteins[35]. Nicotianin I notably lacks proline residues, but other studies indicate that proline residues are not strictly necessary for the formation of PPII, thereby establishing PPII as a distinct category within secondary structures[36].

A significant spectral shift was observed between pH 7.0 and pH 9.0, indicating a pH-sensitive conformational transition. This behavior can be explained by the ionization of side chains with pKa values in this range. The three cysteine residues in the peptide may experience deprotonation of their thiol groups (pKa 8.2), which could affect local conformation or facilitate disulfide bond formation under oxidizing conditions. Consequently, it remains uncertain whether one or all three cysteines are deprotonated as a result of the pH increase. The elucidation of Nicotianin-I's structure through NMR measurements would address this issue. Furthermore, other ionizable residues, including histidine (pKa 6.0), lysine (pKa 10.5), and

arginine (pKa 12.5), may contribute to these structural changes by modifying electrostatic interactions, solvation, and backbone flexibility. The pH-induced shifts demonstrate the structural plasticity of Nicotianin-I and its ability to adopt different conformations in response to changes in the microenvironment.

The thermal stability of Nicotianin-I and its renaturing capacity were evaluated and compared to the CD spectra of the control (peptide at pH 7.0 at 25 ºC). Heating to 95 °C resulted in changes to the CD spectrum, characterized by a significant reduction in ellipticity, suggesting a conformational transition. After cooling to 25 ºC, the CD spectrum was restored, demonstrating the reversibility of the conformational change (Figure 4b). A temperature ramp experiment was conducted to investigate this behavior in detail, revealing a gradual transition and a melting temperature (Tm) of approximately 48.8 °C (data not shown). The findings indicate a reversible structural adjustment rather than classical denaturation, likely attributed to moderate conformational flexibility, a characteristic also noted in other peptides[37]. The reversibility observed enhances the conformational resilience of Nicotianin-I and may indicate a beneficial characteristic for its antimicrobial activity.

When subjected to a membrane-mimetic environment, simulated by TFE, the Nicotianin-I spectrum established a typical α-helicoidal "W" pattern (Figure 4c), with minimum values around 209 and 221 nm[38]. Structural analysis revealed an increased percentage of α-helix content (29.4%) relative to aqueous solutions (Table 1). This change can be due to TFE's capacity to diminish peptide–water hydrogen bonding and enhance intramolecular hydrogen bonds, thus stabilizing helicoidal structures[39]. Nonetheless, TFE-induced helicity may not accurately represent the peptide's natural structure under physiological environments. Instead, it emphasizes the peptide's structural capacity to assume an α-helical conformation in hydrophobic or membrane-mimicking conditions. Comparable behavior has been seen in many AMPs[39,40], which assume helical conformations following membrane insertion, consequently augmenting their membrane-permeabilizing ability[39]. Conversely, exposure to SDS, an environment mimicking negatively charged micelles, did not yield a notable increase in α-helical content (Figure 4c). Therefore, the spectral profile in SDS indicates that Nicotianin-I may retain a certain level of organized extended structure similar to polyproline type II helix.

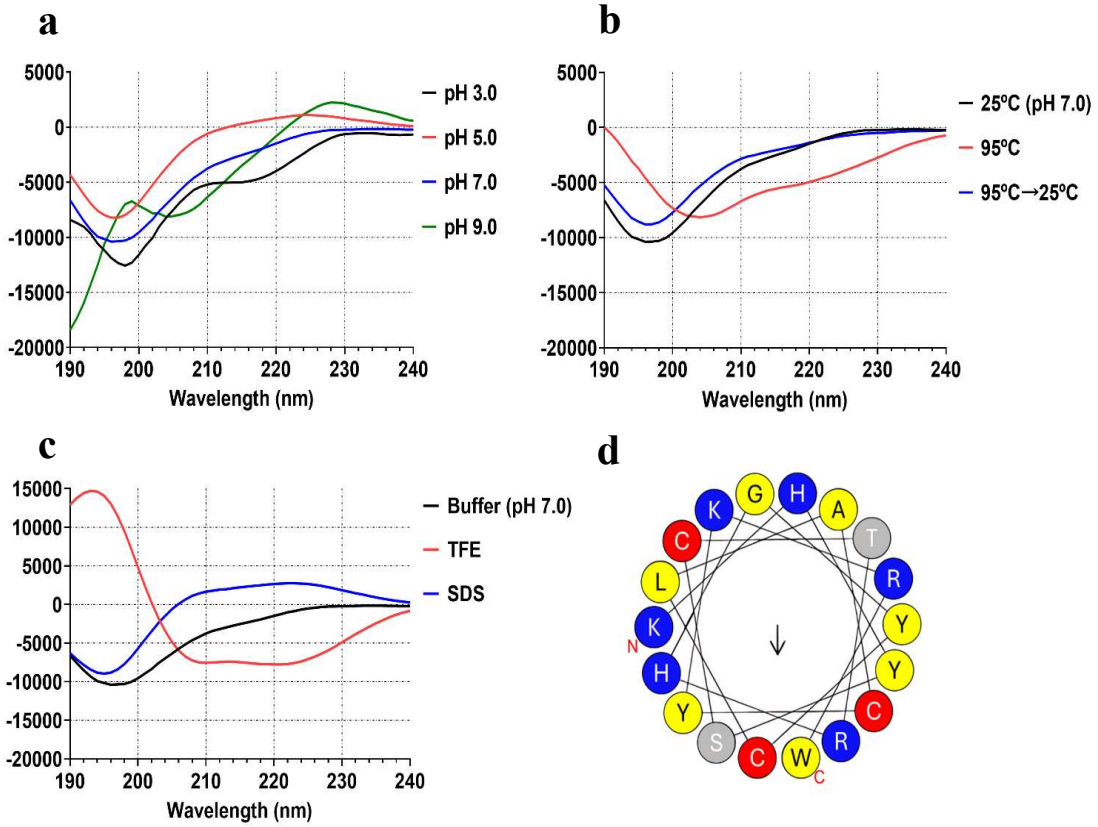

Fig. 4. Secondary structure of Nicotianin-I measured by circular dichroism (CD) under different conditions. **(a)** CD spectra of Nicotianin-I at pH 3.0, 5.0, 7.0, and 9.0. **(b)** CD spectra of Nicotianin-I at 25 ºC, after heating up to 95ºC, and after heating up to 95ºC and cooling to 25ºC. **(c)** CD spectra of Nicotianin-I in the presence of 20 µM SDS or 50% TFE. All analyses used the peptide at 50 µM. **(d)** Helical wheel projection was created using HeliQuest program (https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py). Black arrow indicates the direction and intensity of the hydrophobic moment. Amino acid residues are depicted as follows: hydrophobic (yellow), positively charged (blue), uncharged polar (gray), and cysteine (red).

Various antimicrobial peptides possess amphipathic α-helical structures, distinguished by different hydrophobic and hydrophilic regions, which are crucial for their activity[41]. To define these areas, the helical wheel projection has been used to show where the hydrophobic and hydrophilic residues are along the α-helix[42]. The helical wheel projection shows that Nicotianin-I's hydrophobic and hydrophilic residues are spread out along the α-helix (Figure 4d). It lacks a clearly defined hydrophobic face. This elucidates its low hydrophobic moment (0.215 µH) and supports a possible polyproline II helical structure, as the helical wheel projection suggests the absence of an amphipathic driver for an α-helix conformation. This

result is important as it will facilitate the development of novel analogs with better antimicrobial effectiveness. This could be done by adding positively charged amino acids, preferably to the hydrophilic side of the peptide, or by replacing polar amino acids on the hydrophobic side of the helix with hydrophobic ones. This method was utilized to synthesize five new peptides derived from the antimicrobial peptide Temporin F[43]. The peptides demonstrated improved activity against both Gram-negative and Gram-positive bacteria. Moreover, they demonstrated enhanced resistance to enzymatic degradation[43]. Similarly, to improve the antimicrobial activity of Uperin 3.6, three analogs were created by substituting less hydrophobic amino acids with cationic lysine residues. The net positive charge of peptides was augmented, maintaining their amphipathic structures, which led to a decrease in MIC from 64–128 mg/L to 16–64 mg/L. Peptides exhibited minimal hemolytic action and considerable toxicity towards two normal human epithelial cell lines[44].

Table 1. Nicotianin-I secondary structure distribution at different pHs, temperatures, and in the presence of SDS or TFE.

Solution	α-helix	β-structures	Coil
Sodium phosphate buffer pH 3.0	-1.0%	26.0%	75.2%
Sodium acetate buffer pH 5.0	-2.6%	30.5%	72.1%
Sodium phosphate buffer pH 7.0	0.3%	30.5%	69.2%
Tris – HCl buffer pH 9.0	3.3%	31.4%	65.3%
25ºC	0.3%	30.5%	69.2%
90ºC	8.9%	36.2%	54.9%
90ºC → 25ºC	0.6%	36.7%	62.6%
10 μM SDS	1.7%	35.6%	62.7%
50% TFE	29.4%	42.3%	28.3%

5.2.4 Cytotoxic activity

5.2.4.1 In vitro assays

Erythrocytes and mammal cell lineages are commonly used to evaluate indicatives of cytotoxicity by a possible new pharmacological candidate[45]. All the cytotoxicity assays of Nicotianin-I were conducted and compared to Pep1, which presented no anticandidal activity (Figure 1). The hemolytic assays using human erythrocytes showed that Pep1 and Nicotianin-I, both at 200 µM, presented a maximum percentual of hemolysis of 11.7% and 28.1% (Figure 5a), respectively. At concentrations between the calculated IC_{50} for the tested yeast, the hemolytic activity of Nicotianin-I was about 6.3%. These results, pair or surpass others regarding antimicrobial peptide cytotoxicity. For example, Rana frog's derived antimicrobial peptides have exhibited hemolysis values ranging from 20 to 100% at 100 µM[46].

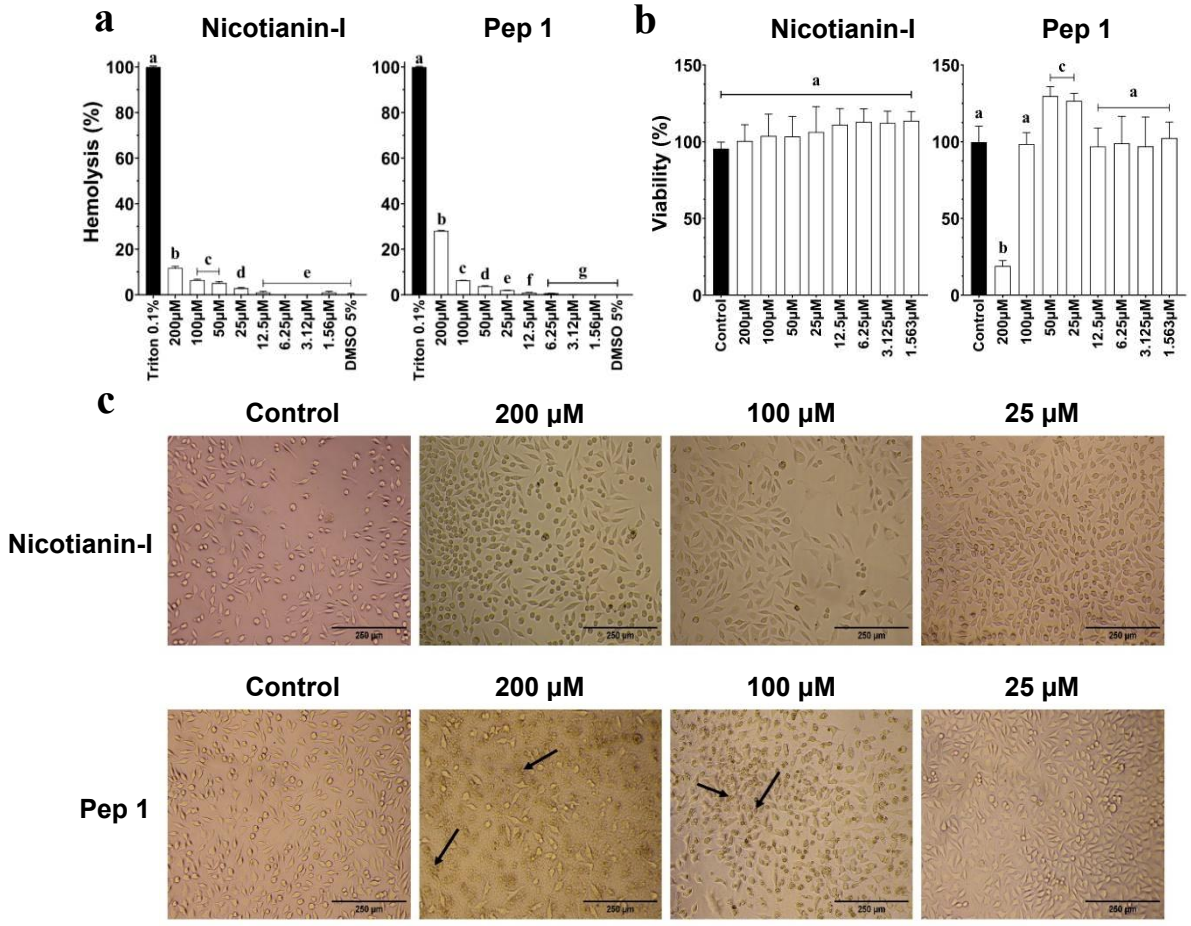

Fig. 5. Citotoxicity of Nicotianin-I against human erythrocytes and murine fibroblast cells. **(a)** Hemolysis percentage of Nicotianin-I and Pep1 regarding positive control (0.1% Triton-X-

100). **(b)** Murine fibroblast cell viability (MTT) results. **(c)** L929 cells treated with different concentrations of Nicotianin-I after 24 h of incubation. Images were obtained by a phase contrast optic microscope (200x magnification). Arrows points to annormal cells and cell debris. Control: cells treated with 0.15 M NaCl containing 5% of DMSO. The results are presented as mean ± standard deviation. Distinct letters denote significantly different groups ($p < 0.05$) relative to the control (0.15 M NaCl containing 5% DMSO).

The antiproliferative assay using L929 cells revealed that peptide Pep1 was capable of reduce the cellular viability up to 80.95% at 200 µM (Figure 5b). The cell damage was visually observed by the increased turbidity of media and presence of cell debris by phase contrast microscopy (Figure 5c). On the other hand, Nicotianin-I showed no cytotoxicity to fibroblasts at 200 µM (Figure 5b-c). Other studies have shown disparities between antiproliferative tests using fibroblast and hemolytic assays, as shown by THL-2-1, a de novo designed peptide[47].

5.2.4.2 In vivo assays

This is, to our knowledge, the third study assessing the toxicity of antifungal peptides in zebrafish. The first evaluated parameter was the impact of Nicotianin-I on the survival rate of zebrafish embryos. The survival rate following exposure to Nicotianin-I for 96 h was 90%, at 50 µg/mL (21 µM), comparable to the negative control rate of 95%. At the maximum dose evaluated (100 µg/mL or 42 µM), zebrafish embryos exhibited a survival rate of 40%. The only lethal effect detected was embryo coagulation. Embryo coagulation rates of up to 25% may be regarded as typical, reflecting the spontaneous mortality of zebrafish embryos occurring 24 h post-fertilization[48,49]. The LC_{50} for Nicotianin-I was determined to be 131 µg/mL or 54 µM. Substances exhibiting embryotoxicity at concentrations beyond 100 µg/mL in zebrafish are usually considered as having minimal toxicity, since testing higher concentrations may surpass the osmotic threshold[50].

In instance, three effective Mastoparan analog peptides targeting *C. albicans* demonstrated LC_{50} values in zebrafish embryos between 9.6 and 15.6 µg/mL[51]. Likewise, PepM3, an IsCT peptide analogue obtained from the venom of *Opisthacanthus madagascariensis* exhibiting anticandidal properties, revealed an LC_{50} of 125 µg/mL in zebrafish embryos, which the authors consider as indicative of minimal toxicity[52]. Nicotianin-I

exhibits low toxicity in this model, as its LC_{50} (131 μg/mL) is equivalent to or exceeds that of other antifungal peptides.

The LC_{50} of Nicotianin-I is clearly greater than that of commercial antifungal drugs. Amphotericin B (AmB) and nystatin (Nys) exhibited LC_{50} values of 1.3 μg/mL and 6.2 μg/mL, respectively, in zebrafish embryos at 120 hours post-fertilization[53]. However, the MICs for Nys (1 μg/mL) and AmB (0.25 μg/mL) against *C. albicans* and *C. parapsilosis* are significantly lower than those of Nicotianin-I, which are 292 μg/mL and 342 μg/mL, respectively. The therapeutic index (the ratio of toxicity to efficacy) of Nicotianin-I is 0.45 for *C. albicans* and 0.38 for *C. parapsilosis*, whereas for AmB and Nys, the indices are 5.2 and 6.2, respectively.

Concerning the non-lethal effects, hatching delay (hd) and granules around the embryo (gae) were noted in the presence of Nicotianin-I (Fig. 6j-l), but exclusively at the highest dose examined (100 μg/mL). Thus, the lowest observed adverse effect level (LOAEL), defined as the minimal concentration or quantity of a drug identified by experimentation or observation to induce an undesirable impact, was 100 μg/mL (Table 2). Notwithstanding the occurrence of these effects, they were transient and hence did not influence embryo development further. On the other hand, Nys and AmB induced inner organ toxicity and various developmental abnormalities in a dose-dependent manner, leading to lethal outcomes. Zebrafish embryos exhibited cardiac abnormalities, including pericardial edema and reduced heartbeat, following exposure to 3 μg/mL of Nys (3 × MIC) and 0.8 μg/mL of AmB (3.2 × MIC). Furthermore, the administration of both drugs at sub-therapeutic doses (<1 × MIC) resulted in a reduction of yolk absorption, suggesting a potential hepatotoxic effect[53]. While both polyenes exhibit significantly higher therapeutic indexes compared to Nicotianin-I, it is important to note that cytotoxicity remains a common and undesirable side effect of antifungal agents. In addition, zebrafish embryos exhibit greater sensitivity to toxicants compared to traditional mammalian models, potentially resulting in an overestimation of toxicity levels. In future studies, we will evaluate the toxicity of Nicotianin-I in rodents to develop a comprehensive safety profile, facilitating a more precise calculation of its therapeutic index.

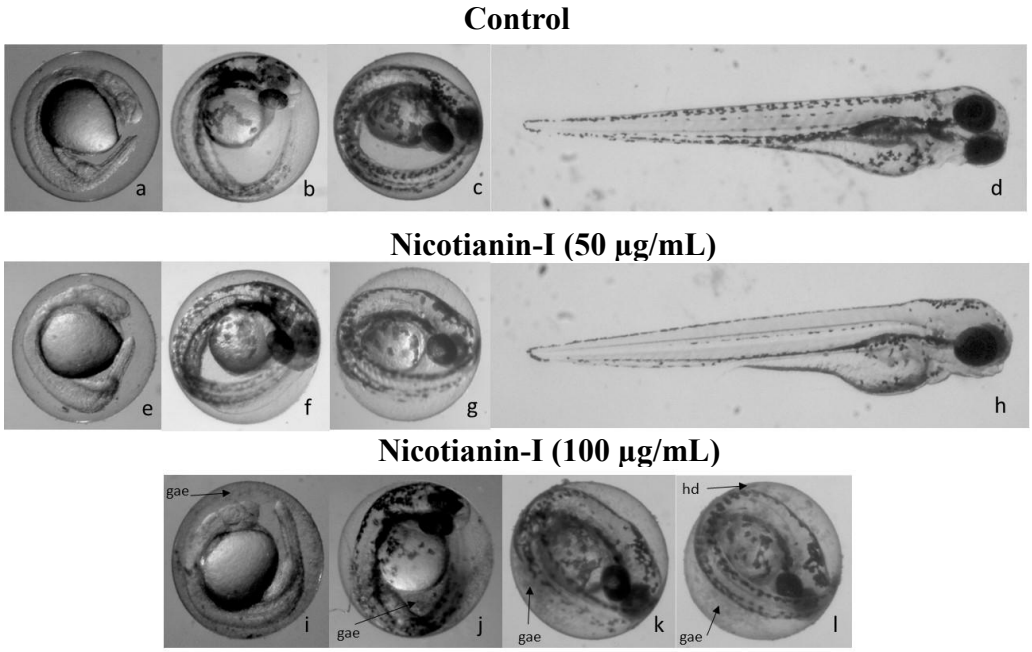

Fig. 6. Effect of Nicotianin-I on zebrafish embryos and larvae. **(a)**, **(b)**, **(c)** and **(d)** Zebrafish embryos and larva with normal development after 24, 48, 72, and 96 hpf exposed only to E3 medium (Control), respectively. **(e)**, **(f)**, **(g)** and **(h)** Zebrafish embryos and larva with 24, 48, 72, and 96 hpf exposed to 50 µg/mL of Nicotianin-I, respectively. **(i)**, **(j)** and **(k)** Zebrafish embryos with 24, 48, and 72 hpf, respectively, exposed to 100 µg/mL of Nicotianin-I showing granules around the embryos (gae). **(l)** Zebrafish embryo with 96 h exposed to 100 µg/mL of Nicotianin-I, showing granules around the embryos (gae) and hatching delay (hd).

Table 3. Toxicological effects of Nicotianin-I on zebrafish embryos.

Embryo toxicological effects	Nicotianin-I
Eye malformation	n.e.*
Otolith malformation	n.e.
Mouth malformation	n.e.
Spine malformation	n.e.
Body pigmentation	n.e.
Hatching delay	**100 mg/L**
Yolk sac edema	n.e.
Pericardial edema	n.e
Blood clotting	n.e.
Undersize	n.e.
Granules around the embryo	**100 mg/L**
Mortality (LC_{50})	131.85 mg/L

Bold values are LOAL (Lowest-observed-adverse-effect level)

* n.e.: no effect or less than 20% of embryos affected.

5.3 Conclusions

Our findings were able to assert the antifungal efficacy of the ornamental tobacco's AMPs, with a notable distinction for Nicotianin-I, which exhibited antifungal potential against all Candida strains. Nicotianin-I promoted membrane permeabilization, possibly creating pores or inducing cell lysis, resulting in the leakage of cell contents. Additionally, it prompted the overproduction of ROS, indicating its diverse mechanisms of action. In terms of cytotoxicity to non-target cells, it demonstrated a significantly low toxic profile to human erythrocytes, murine fibroblasts, and zebrafish embryos. Nicotianin-I exhibits several attributes that warrant additional exploration regarding its potential as a therapeutic candidate. This AMP may also offer a promising amino acid framework for further bioinformatic enhancement, optimizing its advantages and mitigating its deficiencies.

5.4 Material and Methods

5.4.1 Nectar peptides

Nearly 800 peptides were found in the floral nectar of ornamental tobacco, a cross of *Nicotiana sanderae* and *N. langsdorffii*. Comprehensive bioinformatics and *in vitro* analyses identified six peptides with antimicrobial activity against phytopathogenic fungi and bacteria[11]. Here, we evaluated the same six peptides against different Candida species of clinical interest. All peptides were synthesized by Synpeptide Co. Ltd. (China) with purity above 95%, using solid-phase peptide chemistry. The purity of the peptides was determined by reversed-phase high-pressure liquid chromatography (RP-HPLC) and mass spectrometry. The peptides were Pep1 (VCPCACCSTPRRV), Pep2 (EYCSAKSAKPGVHCRSCALVNMYK), Pep3 (LHSGEGSTCYFFKTNPCCCGTWNCT), Pep4 (WKCGSTPAPRKYCTCVAKMW), Pep5 (HRFSYMCFVAQVLNKDYCSCCKF), and Pep6 (KHYSCTRHGYC LACYKRWF).

5.4.2 Anticandidal activity

The anticandidal activity was performed against following species: *Candida albicans* (ATCC 10231), *C. krusei* (ATCC 22019), *C. parapsilosis* (ATCC 6258), and *C. tropicalis* (INCQS 40042). The cell concentration was standardized by OD_{600nm} to achieve a

concentration of 1.5 x 10^3 CFU/mL. The assays were performed using 96-well microplates, mixing the cell suspensions (100 µL) with 100 µL of each peptide serially diluted (200 to 1.56 µM, final concentrations) in 0.15 M NaCl containing 5% DMSO (Dimethyl sulfoxide), which was used as control. The plates were incubated for 24 h at 37 ºC under agitation (150 rpm) and the reading were done at 600 nm using a Microtiter Plate Reader[54]. The results are shown as percentage of growth, considering OD_{600nm} of the control as 100%.

Cell viability assays also were performed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent[13]. For that, cell suspensions (25 µL) were incubated with 25 µL of the most promising peptide (200 – 6.25 µM). After 24 h at 37 °C, 50 µL of 5 mg/mL MTT was transferred to each well and kept in dark for 3 h. Afterward, 100 µL of 100% DMSO was transferred into each well, homogenized, and the OD_{540nm} was measured using a Microtiter Plate Reader.

5.4.3 Membrane damage evaluation

The fluorophore Propidium Iodide (PI) was used to determine damage in the fungal cell membrane. First, Nicotianin-I was incubated with cell suspensions at 37 ºC for 24 h, as described before. After, the samples were centrifuged at 5,000 x g at 4 ºC for 5 min and the pellets (cells) resuspended with 50 µL of 0.15 M NaCl containing 10 µL of 0.1 mg/mL PI. After 30 min at 25 °C in the dark, the samples were again centrifugated and then the pellets rewashed and rinsed in 50 µL of 0.15 M NaCl. The fluorescence was observed using fluorescence microscope (Olympus System BX 41, Tokyo, Japan - excitation wavelength of 488 nm and an emission wavelength of 525 nm)[55].

Further analyses were performed to determine the possible pore formation in the fungal cell membrane. For that, the same assays above were performed adding Fluorescein Isothiocyanate (FITC) linked to 4 kDa dextran polymer instead of PI. The reading was done with the aid of a fluorescence microplate reader (Synergy MX, Biotek, Winoosky, VT, USA) emission/excitation wavelengths of 490/520nm as described before[55]. The pore formation also was evaluated quantifying the extracellular protein content of the fungal cells treated with Nicotianin-I. The cell-free-supernatant (CFS) was collected after centrifugation (5,000 x g at 4 ºC for 5 min) and the soluble proteins were determined using Bradford method[56], for both the treated and non-treated cells[57].

5.4.4 Ultrastructural analysis by scanning electron microscopy (SEM)

The yeasts were incubated with Nicotianin-I as described before. After, the cells were washed three times with 0.15 M potassium phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde. Finally, the material was dehydrated with ethanol serial concentrations (10-100%) before adding hexamethyldisilazane (HMDS) solutions (1:1 ratio and pure) and then transferred to sterilized circular coverslips (~13 mm)[57]. The cells were coated on gold (QT150 ES, Quorum) and the captures were done (WD = 10 µm / mag = 10.000x/20.000x) using a Scanning Electron Microscope (Quanta 450-FEG, FEI).

5.4.5 Reactive Oxygen Species (ROS) overproduction

To measure the ROS accumulation inside the cells treated with Nicotianin-I, 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was used as fluorescence probe. The peptide was incubated with cell suspensions (1.5 x 10^6) at 37 ºC for 3 h, using IC_{50} or 2-fold IC_{50}. After, the samples were centrifuged at 5,000 x g at 4 ºC for 5 min and the pellets resuspended with 50 µL of 0.15 M NaCl containing 9 µL of 0.1 mg/mL DCFH-DA. After 20 min at 25 °C in the dark, the samples were again centrifugated and then the pellets rewashed and rinsed in 50 µL of 0.15 M NaCl. The fluorescence was evaluated qualitatively by fluorescence microscope (Olympus System BX 41, Tokyo, Japan - excitation wavelength of 485 nm and an emission wavelength of 530 nm) and quantitatively using a fluorescence microplate reader (Synergy MX, Biotek, Winoosky, VT, USA), under the same excitation and emission wavelengths[30].

5.4.6 Structural characterization

Circular Dichroism CD spectra of Nicotianin-I (50 µM) was achieved using a Jasco-815 spectropolarimeter (Jasco Inc., Tokyo, Japan) in a quartz cuvette (0.1 cm), ranging of 190-240 nm at 25 ºC with 4 replicate scans per analysis. The structural stability was investigated under different pH values (3.0, 5.0, 7.0, and 9.0). The effect of temperature was evaluated by applying a temperature ramp (25 – 95°C), wherein the temperature was incrementally raised by 3°C per minute, with ellipticity monitored at a wavelength of 210 nm. Upon reaching 95°C, the temperature was reduced back to 25°C, and a new scan was performed to assess the potential renaturation of peptide. Additionally, structural stability also was assessed in the presence of 20

μM Sodium Dodecyl Sulphate (SDS) and 50% Trifluoroethanol (TFE)[58]. Helical wheel projection was created using HeliQuest program (https://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py)[42].

5.4.7 In vitro toxicity assays

5.4.7.1 Hemolysis assay

To assess a possible indicative of cytotoxicity, 150 μL of Nicotianin-I, serially diluted with 0.15 M NaCl containing 5% DMSO, was incubated at 37 ºC for 1 h with the same volume of 1% human erythrocyte (Type O), which was obtained from Hematology and Hemotherapy Center of Ceará, Brazil. Samples were centrifuged for 5 min at 2,500 x g and the supernatant transferred to 96-well microplates. Released hemoglobin content was measured at OD_{414nm}. 0.1% Triton-X-100 and 0.15 M NaCl containing 5% DMSO were used as the positive and negative controls, respectively[59].

5.4.7.2 Cytotoxicity assay

L929 (murine fibroblast) cell lineage, kindly conceded by Biological Physics Laboratory (Physics Department, Federal University of Ceará), was cultured on Dulbecco's modified Eagle's media (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 10,000 U/mL penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA), at 37 ºC and 5% CO_2 atmosphere saturation[60].

Cell viability of peptide-treated fibroblast cells was tested by MTT method. Shortly, L929 cells were trypsinized (Trypsin-EDTA solution) and seeded into 96-well plates with DMEM media (100μL) at 1 x 10^5 cells/mL for 48 h, followed by the addition of same volume of serially diluted Nicotianin-I (200 - 1.56 μM) and negative control (0.15 M NaCl). The images were captured with the aid of an optic microscope coupled with phase contrast (AE200, Motic, Barcelona, CT, ES) under 200x magnification. Finally, 10 μL of 5 mg/mL MTT was added in each well and incubated at 37 ºC for 3 h. Formazan was solubilized with DMSO (150 μL) and the OD_{570nm} was measured in a microplate reader[61].

5.4.8 In vivo toxicity assays

5.4.8.1 Zebrafish embryos

Zebrafish embryos (AB wild-type strain) with approximately one-hour post-fertilization (hpf) were provided by the Production Unit for Alternative Model Organisms (UniPOM), Federal University of Paraiba (UFPB), João Pessoa, Brazil. A day before the experiment, zebrafish adults (male to female ratio of 2:1) were transferred to a 7-L spawning tank with a bottom mesh and a quick-opening valve for embryo collection. Embryos were collected on the day of the experiment and cultured in adapted embryonic medium E3 (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, and 0.33 mM MgSO4) containing 0.005% methylene blue. Only spawning with a fertilization rate ≥ 90% were used. Viable embryos (normal cleavage pattern and without morphological changes) were selected using an inverted light microscope (Televal 31, Zeiss, Germany), at 50x magnification. The experiments were approved by the Ethics Committee on the Use of Animals at UFPB, with authorization documented by protocol No. 4460140920.

5.4.8.2 Acute toxicity test

The Fish Embryo Acute Toxicity (FET test) test was conducted according to OECD's guideline number 236[50] adapted for 96-well plate, as described by Muniz et al.[62]. Zebrafish embryos with up to 3 hpf were exposed to five concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) of Nicotianin-I, being the last concentration adopted as a limit concentration. Twenty wells were filled with 0.3 mL of E3 medium and 1 embryo. In the same conditions, E3 medium and 0.15 M NaCl containing 5% DMSO were used as controls.

Lethal and non-lethal effects were observed for 96 h. Embryos showing lethality endpoints (embryo coagulation, lack of somite formation, non-detachment of the tail bud, and lack of heartbeat) were considered dead. This number was used to determine the survival rate % [number of alive organisms/total number of organisms x 100] per tested concentration. The exposures were under static conditions (without renovation of the exposure solution). Observations were performed using a stereomicroscope at 50x magnification. After 96 h, surviving larvae were euthanized with eugenol and properly discarded.

5.4.9 Statistical analysis

All experiments were conducted three times, with each biological replicate executed in triplicate. All results were presented as the mean and corresponding standard deviation (SD). The ANOVA test was employed for multiple mean comparisons. Dunnet's test was conducted to compare the means individually. All statistical analyses used a $p < 0.05$. Graphpad Prism 8.0 was used for statistical analyses and graphical production.

DECLARATION OF COMPETING INTEREST

All experiments were conducted three times, with each biological replicate executed in triplicate. All results were presented as the mean and corresponding standard deviation (SD). The ANOVA test was employed for multiple mean comparisons. Dunnet's test was conducted to compare the means individually. All statistical analyses used a $p < 0.05$. Graphpad Prism 8.0 was used for statistical analyses and graphical production.

CONTRIBUTIONS

J.M.M. Neto, T.K.B. Aguiar, M.F. Oliveira, Q.L.S.G. Chaves, D.R.A.L. Mourão, V.O. Silva, M.T.V. Nascimento, R.F.Carneiro, D.F Farias, B.F. Sousa, J.S. Sousa: Methodology and validation; C.D.T. Freitas: Conceptualization, project administration, funding acquisition. J.M.M. Neto, D. Farias, R.F. Carneiro, C.D.T Freitas: Data curation, formal analysis, writing - original draft, writing - review & editing.

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6. CAPÍTULO III - ARTIGO CIENTÍFICO II

Artigo Científico sob estruturação para submissão: Qualis A / IF >3.0

Elucidating Nicotianin-I cell-penetrating properties and synergistic effects against an azole-resistant *Candida tropicalis* strain

ABSTRACT

Several non-*albicans* yeasts, as *Candida tropicalis*, are capable of causing persistent invasive infections, which hamper medical treatment and increase mutant selection window, leading to greater mortality rates and resistance-acquisition. Combined therapy is a common and effective strategy used in such cases, yet multidrug-resistance (MDR) hinder the establishment of these tactics. Therefore, the investigation of the synergistic properties of novel antifungals in association with market-stablished drugs is essential to fight MDR. The current research focused in investigating the membrane-targeting properties and synergistic effects of Nicotianin-I in association with three antifungal drugs: Amphotericin B (AMB), Caspofungin (CAS), and Fluconazole (FCZ) in an azole-resistant strain of *C. tropicalis*. A Minimum Inhibitory Concentration (MIC) value of 25 μM for Nicotianin-I was obtained using standardized CLSI protocols, a 4-fold reduction when compared to our previous study. The investigation of peptide's membrane interaction revealed that Nicotianin-I was able to strongly neutralize the negative membrane charge at growing concentrations, proving that Nicotianin-I's action is intimately connected with its membrane-association properties, being capable to penetrate the cytosol during its action. It was also observed that Nicotianin-I presented no synergism with AMB (FICI = 1.5), but it was capable to synergize with CAS (FICI = 0.1915) and FCZ (0.1406), causing the strain to reacquire the drug-sensibility in the last case, even at low concentrations. Thus, the current study demonstrates the potential of this nectar peptide as a candidate in the development of new combined-therapy and its penetrating properties also hints its potential as a cytosol-delivering molecule for further studies.

Keywords: Combined Antimicrobial Therapy; Cell-penetrating peptide; Membrane-binding peptide; Synergism.

6.1 Introduction

Candidemia cases continue to be main concerns from a clinical point of view, as they present high mortality rates associated with the emergence of resistant isolates cases [1]. *Candida tropicalis*, a pathogenic yeast recently highlighted by the World Health Organization (WHO) as a high priority pathogen [2] reaches mortality rates of up to 60% in invasive candidiasis, far surpassing fungi of the same genus [1,3].

C. tropicalis is commonly associated with mortality rates due to the presence multiple virulence factors associated with its infection, such as virulence-associated enzymes (*e.g.* hemolysin and proteases), high capacity of biofilm formation [4,5] and enhanced adhesion capacity due to pseudohyphae formation [6]. Such characteristics lead to longer infections, impairing proper treatments and allowing resistance acquisition [1,7]. As a result, some reports have shown an increased prevalence of resistant (mainly azole-resistance) and multidrug-resistant (MDR) *C. tropicalis* in medical centers, reaching up to 42% of fluconazole resistance in some cases [8–10].

Throughout the years reports suggest an increasing in *Candida* resistance associated with anticandidal front-line drugs (azoles and echinocandins) [11,12], resulting in the adoption of alternative approaches to better address infection management, as drug combination. Combined antimicrobial therapy entails greater efficacy, especially for MDR organisms, and a reduced selective pressure for resistant mutants, leading to lower resistance acquisition due to the complementarity of different acting mechanisms [13–15]. Although the most common clinical applications involve the combination of already market-established antifungal drugs [15,16], numerous studies in the literatures denotes the synergistic profile of commercial antifungals with various non-usual molecules, such as non-antibiotics [17,18], medicinal plant extracts [19,20], essential oils [21,22] and antimicrobial peptides [23,24].

Antimicrobial peptides (AMPs) inherently demonstrate their potential when it comes to infection control as they are biocompatible broad-spectrum antimicrobial compounds that multitarget different cell structures, hindering resistance development by microorganisms [25]. Despite this, several snags call into question in the widespread application of AMPs, such as high production costs and cytotoxicity [26], both complications being alleviated by using drug-combination approaches, which reduce the need for high peptide concentrations and, consequently, therapy cost and the probability of developing undesired side-effects associated with cytotoxicity [25].

Nicotianin-I, an antimicrobial peptide identified from *Nicotiana spp.* floral nectar has already demonstrated its antimicrobial effect against phytopathogenic fungi [27] and human pathogenic yeasts of the genus *Candida* (unpublished results). Nevertheless, their mechanisms of action and combinatory potential with commercial drugs were not fully elucidated. Therefore, the current study aims to comprehensively understand the physicochemical mechanisms associated with Nicotianin-I fungal membrane-permeabilization to evidence and explain possible synergistic effects with fluconazole (FCZ), amphotericin B (AMB), and caspofungin (CAS) against a fluconazole-resistant *C. tropicalis* strain.

6.2 Results and Discussion

6.2.1 C. tropicalis responses to Nicotianin-I and antifungals exposure

AMPs are small cationic molecules that require membrane binding to target fungi and bacteria, meaning that peptides should be available to exert their function. In other terms, peptide molecular interactions with macromolecules composing complex media [28] can partially impede the peptide full potential. Previous studies have shown discrepancies between antifungal susceptibility testing of *Candida* isolates when using complex media (Sabouraud Dextrose Broth - SDB) or defined media (RPMI 1640) [29]. This clear up our acquired data, which re-evaluated Nicotianin-I's inhibitory concentration for *C. tropicalis* with a four-fold reduction (25μM) when compared to our prior study (86.9μM), which used SDB in peptide testing [30]. Furthermore, as other studies using the same strain corroborate [31,32], *C. tropicalis* INCQS 40042 susceptibility testing (CLSI M27/M59/M60) confirmed strain susceptibility to AMB and CAS, but a strong resistance to FCZ, as detailed in Table 1.

MTT, a membrane permeable tetrazolium salt that is reduced through cellular metabolism to a quantifiable purple product (formazan) [33], was used to infer planktonic cells metabolic response to antifungal treatment. Nicotianin-I and AMB completely inhibited metabolism with a later metabolic reestablishment when sub-inhibitory concentrations were reached. CAS impeded more strongly the yeast metabolism in all concentrations, while FCZ only caused a significant metabolic reduction at the maximum tested concentration (Fig. 1).

Table 1. Susceptibility testing results of *C. tropicalis* for AMB, CAS, FCZ and Nicotianin-I (N-I). Inhibitory concentrations are presented in the first row. Interpretive categories, Epidemiological Cutoff Values and Breakpoints are presented as detailed in Clinical and Laboratory Standards Institutes (Documents: M27, M59 and M60).

	***C. tropicalis* INCQS 40042**				***C. parapsilosis* ATCC 22019**	
	N-I	**AMB**	**CAS**	**FCZ**	**AMB**	**FCZ**
MIC	25µM	0.125*	0.03*	128*	0.25*	1*
ICt	-	*S*	*S*	*R*	*S*	*S*
ECV	-	2*	-	1*	1*	2*
BP	-	-	0.25*	2*	-	2*

*Antifungal concentration in µg/mL
MIC = Minimum Inhibitory Concentrations; ICt = Interpretive Categories; ECV = Epidemiological Cutoff Values; BP = Breakpoints.
Interpretive Categories: Susceptible (S), Intermediate (I), Susceptible-dose dependent (SDD) or Resistant (R).

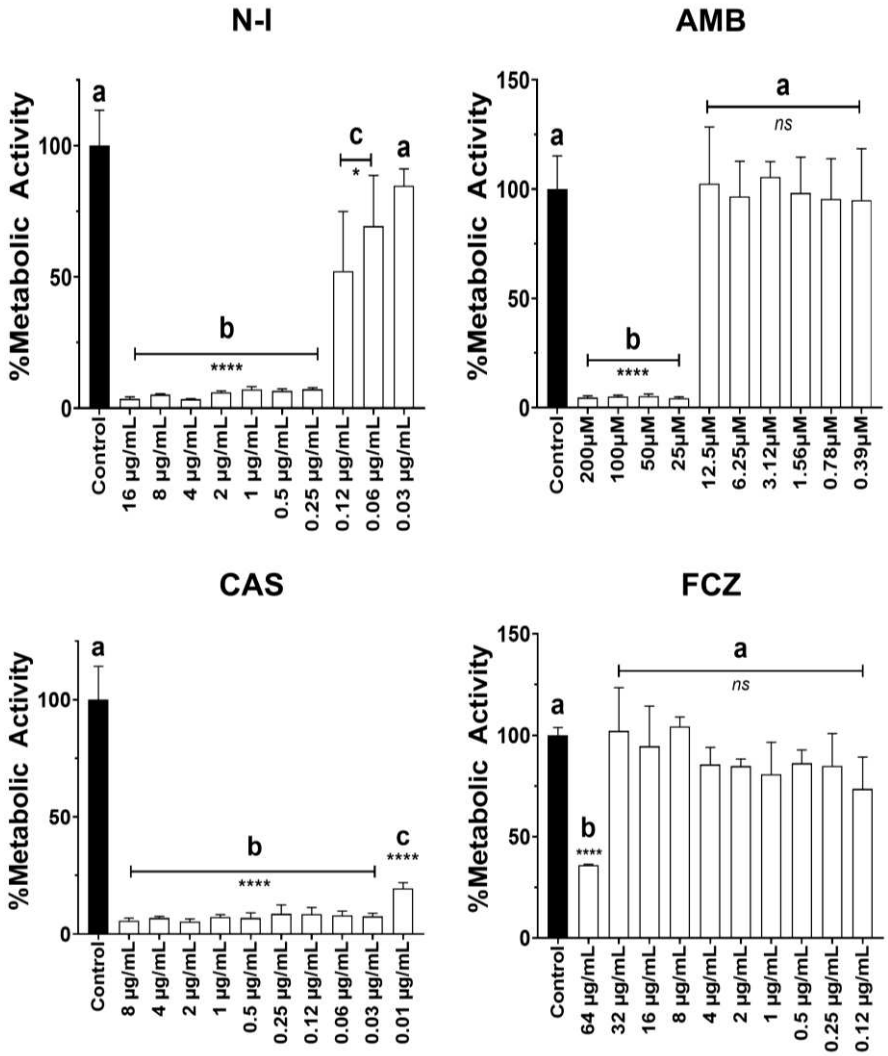

Figure 1. *C. tropicalis* metabolic activity assay results. Nicotianin-I (N-I), AMB, CAS and FCZ, at varying concentrations, metabolic inhibitory effects were evaluated. The results are

present as the mean of three triplicates ± standard deviation. Different letters denote significantly distinct groups point by One-way ANOVA ($p < 0.05$). RPMI was used as positive growth control.

The strain's metabolic withstanding to FCZ indicates an existing resistance-associated mechanism on the strain due to the overexpression of efflux pumps (*CDR1* or *CDR2*) or azole-targets (*ERG11*) all being proven azole-resistance mechanisms in *C. tropicalis* [34,35]. AMB and AMPs act through similar mechanisms, requiring a threshold concentration to cause microbial harm [36,37], partially explaining the rapid function-loss in both cases. Lastly, CAS strongly inhibits cell wall glucan synthesis, leading to severe osmotic damage to yeast [38]. *C. tropicalis* cell wall is mainly composed of glucans (62.9%) [39] and the susceptibility denotes the absence of strain's alternative resistance mechanisms.

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6.2.2 Nicotianin-I presents penetrating properties

Nicotianin-I antifungal acting mechanism is, at least partially, related to its ability to permeabilize membranes, leading to secondary damage through oxidative stress [30]. Zeta potential (ζ) measurements were conducted to assess the capacity of Nicotianin-I to bind into the microbial membrane, which would be translated into an increased superficial charge of yeasts in contact with the AMP. *Candida* blastospores exhibited a ζ of -31.5 ± 0.56 mV under control conditions. In response to increasing concentrations of Nicotianin-I, growing electrical yeast surface charges were obtained: -13.8 ± 1.52 mV (MIC) and 4.32 ± 0.39 mV (4-fold-MIC). Interestingly, under MIC concentrations, a mild downward tendency was observed after the first minutes of incubation (Fig. 2), implying that some peptide molecules are being detached from cell surface and, most likely, internalized into the cytosol.

Other studies also pointed to an increased zeta potential after peptide exposure [41,42], demonstrating that Nicotianin-I molecules are capable of neutralizing anionic charges of membrane acidic molecules, which further leads to a membrane collapse [43]. Surface zeta potential neutralization also points to a possible secondary effect of Nicotianin-I and other AMPs, since there is a direct correlation between adhesion and membrane electrical potential [44]. Thus, since adhesion is a key process in the infection establishment and persistance [45], Nicotianin-I exposure may reduce the development and permanence of invasion-related virulence traits associated with microbial adhesion.

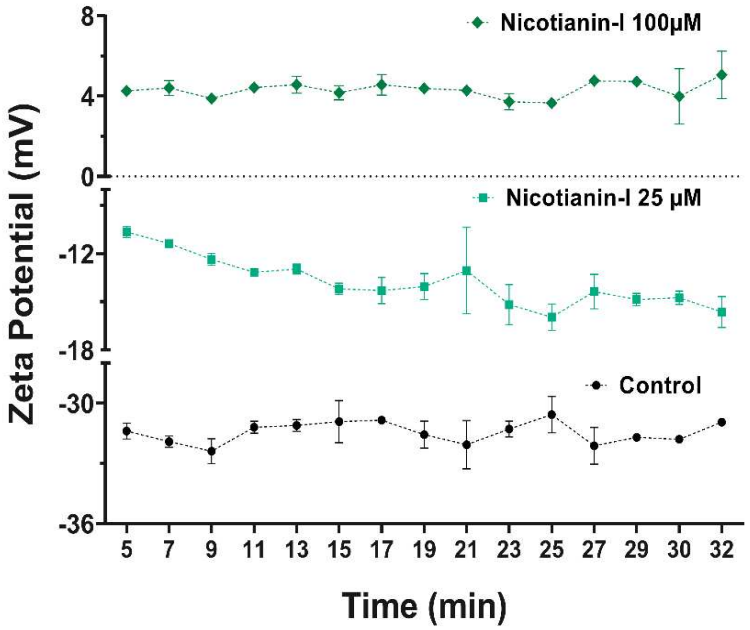

Fig 3. *C. tropicalis* cells surface ζ-potential evaluation. *C. tropicalis* cells (10^6 cells/mL) were tested at 25 µM (MIC) and 100 µM (4-fold-MIC) and 15 measurements were performed. Data are shown as mean ± σ of two independent replicates. Untreated (control) yeasts presented a mean ζ-potential of -31.5±0.56 mV.

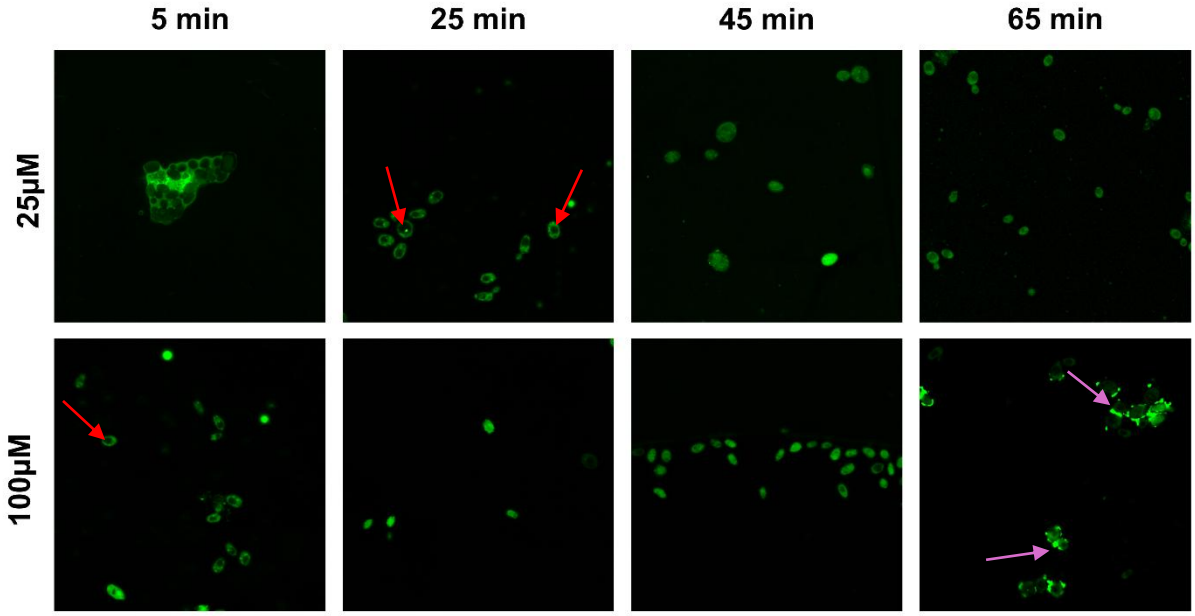

Fig. 4. Cell-penetrating assay. CLSM images of *C. tropicalis* cells exposed to different concentrations (MIC and 4-fold-MIC) of FITC labeled Nicotianin-I at different exposure times (0, 20, 40 and 60min). Red arrows point to nucleus opacity and purple arrows to extracellular artifacts.

Cell-penetrating properties of Nicotianin-I were verified via fluorescence labeling of peptides. Blastospores exposed to Nicotianin-I, at MIC (25μM) and 4-fold-MIC (100μM), were visualized through Confocal Laser Scanning Microscopy (CLSM) at different time intervals (Fig. 3). A progressive diffusion of Nicotianin-I into the cell was observed at 25 μM. Firstly, peptides bind to blastospores membrane (5 min) progressively invading cytosol (25 min) and conquering the yeast nucleus at last, since nucleic opacity is observed in some cells (red arrows) in the early incubation times. This partially explains the decline in zeta potential at the same concentration and confirms our hypothesis of Nicotianina-I's cell-penetrating properties. The incubation with Nicotianin-I (25μM) after 45 or 65 min does not presents significant changes in fluorescence and cell structure, pointing to a certain stability in the given incubation times.

Cytosolic presence of Nicotianin-I (100μM) was observed in all incubation times, with artifacts formation after 60 min incubation (purple arrows). These highly fluorescent artifacts are indicative of the bilayer collapse, forming extracellular membrane micelles associated with peptides. This is an indicative that Nicotianin-I acts through carpet-like mechanisms, also referred as detergent-like, characterized by peptidic accumulation on the outer microbial membrane, forming micelles at high concentrations [40]. In short, once peptide accumulation reaches a critical concentration, transitory curvatures are formed on the membrane which may form peptide-containing micelles [36]. The absence of significant changes in cell fluorescence under 100μM also corroborates our zeta potential results, since no major fluctuations were observed in this concentration regarding all incubation times. Other studies have also observed AMPs exhibiting cell-penetrating properties, for example P-113, a histatin-derived peptide, and CATH-2 were capable of penetrating *C. albicans* [46] and *Escherichia coli* cells [47], respectively, and were capable of present both mechanisms (membrane damaging and penetration), as they are closely linked processes [36].

6.2.3 Azoles and Echinochandin synergism with Nicotianin-I

Regardless of peptides' membrane-targeting mechanism, they are capable of exert synergistic properties with other antifungal classes. To investigate the synergistic potential of Nicotianin-I with AMB, CAS and FCZ, a checkerboard assay was performed (Table 2), denoting Nicotianin-I synergism with CAS and FCZ, with Fractional Inhibitory Concentration

Indexes (FICI) lower than 0.5, and an indifferent effect when combined with AMB (0.5 ≤ FICI ≤ 4.0).

As already mentioned, *C. tropicalis* azole-resistance is mainly associated with mutations in efflux pumps genes, reducing the cytosolic presence of drugs, and 14-α-lanosterol demethylase gene mutations, which reduce drug-protein affinity or lead to target overexpression [34,35]. On the other hand, Nicotianin-I's membrane permeabilization leads to a certain degree of bilayer function-loss [25,36], meaning that extracellular compounds may enter cells unrestrictedly. Thus, the synergism between these molecules (FICI = 0.1406) might be explained by the peptides promoting a greater cytosolic presence of drugs – via membrane permeabilization – while FCZ more readily inhibited ergosterol synthesis, implying a biophysical fragilization of membranes, since sterols attenuate membrane perturbation and are capable of impeding membrane binding of AMPs [48], consequently facilitating the Nicotianin-I binding properties.

Furthermore, CAS inhibits the FKS1 subunit of a transmembrane enzymatic complex (β-1,3-glucan synthase), through an intracellular non-competitive site, leading to cell wall destructuring, osmotic stress and, ultimately, cell death [49]. Echinocandin-peptide synergism (FICI = 0.1915) might also be explained by a two-way street between these molecules: firstly, CAS promotes yeast cell wall degradation of yeasts, which then reduces the physical barrier between the peptide and the membrane. This leads to a greater peptide association with the membrane and a consequent increased membrane fluidity, causing a greater exposure of FKS1 CAS-interaction sites, with a consequent enhancement in the antimicrobial potential of both molecules.

Table 2. Checkerboard assays synergism interpretations. Combinations MICs, FICs and FICIs are below detailed. AMB and CAS evaluated total growth-inhibition and FCZ a 50% inhibition. FICIs of drug combinations are interpreted as presenting SYN (Synergistic), IND (Indifferent) or ANT (Antagonistic) effects.

	***C. tropicalis* INCQS 40042**				
	MIC_{solo}	**MIC_{comb}**	**FIC**	**FICI**	**INTPN**
AMB	0.12 µg/mL	0.063µg/mL	.5	1.5	IND
N-I	25 µM	25µM	1		
CAS	0.03 µg/mL	0.004 µg/mL	.0625	.1915	SYN
N-I	25 µM	1.56 µM	.1295		
FCZ	128 µg/mL	2 µg/mL	.125	.1406	SYN
N-I	25 µM	3.12 µM	.0156		

MIC_{solo} = Minimum Solo Inhibitory Concentrations; MIC_{comb} = Minimum Combined Inhibitory Concentrations; FIC = Fractional Inhibitory Concentrations; FICI = Fractional Inhibitory Concentration Index; CSt = Combination Status.

Lastly, AMB's pore-forming mechanism is related to its capacity to hijack membrane ergosterol [37,50] and Nicotianin-I has an implied detergent-like mode of action. Since both molecules directly target membranes, additive (FICI = 0.5) properties of polyene-peptide were already expected, as one process does not intensify the other.

Due to the reciprocal interaction between peptides and azoles or echinocandins, multiple studies have also reported similar results with these drug classes: as AMP-17, a *Musca domestica* peptide, exhibited synergic effect with FCZ with a FICI of 0.28 in *C. albicans* biofilms [51]; and Cdc50-inspired AMPs, which exhibited synergistic profiles with multiple azoles in *Cryptococcus neoformans* [52]. Despite this, most FICI values were far higher than our findings, which revealed an 8-fold MIC reduction for CAS MIC, 64-fold for FCZ and 8- to 16-fold for Nicotianin-I. This underscores the potential of Nicotianin-I/FCZ and Nicotianin-I /CAS combinations for further studies. AMP-polyenes synergistic potential related in literature are mostly associated with multiple non-membrane targeting mechanisms, as P*v*D1-derived synthetic peptides, which also caused DNA damaging in *C. albicans* [53], with other studies also finding a non-synergistic profile between AMPs and AMB: as hLF(1-11) a human lactoferrin-derived peptide, which exhibited no AMB synergism against multiple *Malassezia spp.* strains [54].

Metabolic activity (MTT) of planktonic cells subjected to checkerboard assay revealed important variations in the combined profile of drug-peptide interactions (Fig. 5). Supra-inhibitory concentrations of AMB and CAS demonstrated a total inhibition of metabolic activity in yeasts with a partial/total metabolic reestablishment in sub-inhibitory concentrations. On the other hand, FCZ exhibited major metabolic inhibitions even at sub-inhibitory concentrations, counter to solo MTT results, which demonstrated no alteration up to 64 µg/mL (Figure 1), redoubling the disclosure of Nicotianin-I-FCZ synergism, encouraging further studies involving proteomic and gene expression investigation of *Candida* strains subjected to Nicotianina-I.

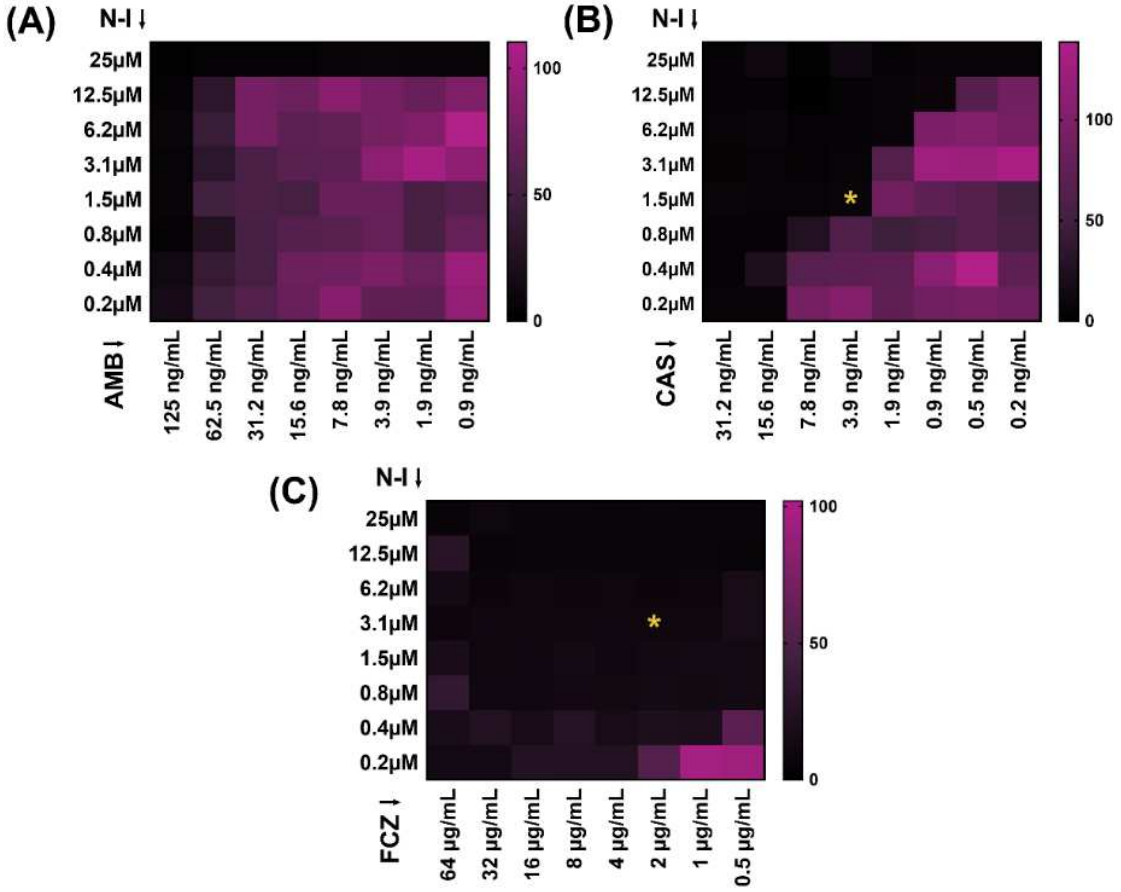

Figure 5. Checkerboard metabolic activity heatmaps. MTT assay evaluation are presented.by each drug combination: (A) AMB/N-I. (B) CAS/N-I and (C) FCZ/N-I. Results are expressed as the average percentual of metabolic activity of two technical replicates, related to growth-positive control (RPMI). Asterisks mark MIC_{comb} spots with lowest FICIs.

6.3 Conclusions

The current study was able to redefine inhibitory concentrations for Nicotianin-I, using standardized protocols, finding a much lower MIC value for the same molecule. The membrane-binding properties and membrane-disrupting mechanisms underlying Nicotianin-I's antifungal properties were also elucidated, concluding that this antimicrobial peptide derived from ornamental tobacco's nectar could damage *C. tropicalis* cells through membrane aggregation on the outer membrane, leading to bilayer micellization and, finally, cell death. During this process, the peptide permeated the phospholipidic bilayer, leading to self-internalization, confirming its duality as AMP and cell penetrating peptide (CPP). Lastly, the checkerboard assay revealed an additive profile of Nicotianin-I-AMB combination, a synergistic profile in the Nicotianin-I-CAS combination and a strong synergism caused by Nicotianin-I-FCZ combination, even in an azole-resistant strain. The current research demonstrates the potential of Nicotianin-I in the development of combined therapies, owing to its azole and echinocandin synergism, potentially mitigating the perpetuation of MDR infections. Even so, complementary studies involving proteomic and transcriptomic investigations of Nicotianin-I-treated yeasts are still required to understand more thoroughly the molecular mechanisms involved in the fungicidal process. In addition, its cell-penetrating properties can be explored for other antimicrobial approaches, as antisense PNA conjugation.

6.4 Methodology

6.4.1 Antimicrobial Peptide and Antifungal Substances

Nicotianin-I (KHYSCTRHGYCLACYKRWF) was obtained by Synpeptide Co. Ltd (China). The synthesis was carried out by solid-phase methods and the purification was done using reversed-phase High Performance Liquid Chromatography (RP-HPLC), with further ESI-MS analysis to grant purity above 95%. Peptide stock (1mM) solutions were prepared in sterile water and stored at -20 ºC. Stock solutions of Amphotericin-B (AMB, Sigma-Adlrich Co.) were prepared in DMSO, while fluconazole (FCZ, Pfizer™) and caspofungin (CAS, Merck™) were diluted in sterile water and stocked at -20 ºC.

6.4.2 Strains, Media and culture conditions

Candida tropicalis INCQS 40042 was obtained by the National Institute of Health Quality Control from the Oswaldo Cruz Foundation (Fiocruz). The strain was plated on Sabourad Dextrose Agar (SDA) and the cultures were adjusted by turbidity (McFarland scale 0.5) and further diluted in RPMI 1640 supplemented with L-Glutamine, absent of sodium bicarbonate (Gibco™) buffered with 0.165M MOPS (Sigma-Aldrich Co.) until pH 7.0. *Candida parapsilosis* ATCC 22019 was used as susceptibility testing control.

6.4.3 Determining Minimum Inhibitory Concentrations

Broth microdilution assays were performed according to Clinical and Laboratóry Standards Institute (CLSI, 2020). Antifungal and peptide final concentration in each test ranged as follows: N-I (200 - 0.4 µM), AMB (16 - 0.031 µg/mL), FCZ (64 – 0.125 µg/mL) and CAS (8 – 0.015 µg/mL). Microplates were incubated at 35 ºC for 24 h. MICs were determined as the lowest drug/peptide concentration in which no significative growth was observed.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assays were also performed, following an adapted protocol [55,56], using the preceding plates. Microplates were centrifuged for 5 min at 2.000 x *g*, 150µL of the supernatant was discarded and 50 µL of 0.1%$_{(w/v)}$ MTT in Phosphate Buffered Saline (PBS) 1%$_{(w/v)}$ added with 2 %$_{(w/v)}$ Dextrose. Plates were reincubated at 35 ºC for 3h and 100µL of DMSO was added. The plates were maintained at a 200rpm shaking for 30min before transferring 100µL of the solution to a new flat-bottomed 96-well plate, which was then read at OD_{570nm}.

6.4.4 Cell Penetrating Assay

6.4.4.1 Peptide FITC Labeling

FITC Isomer I (Sigma-Aldrich, Saint Louis, Missouri, EUA) labeling was performed according to manufacturer's manual. Briefly, a solution of Nicotianin-I (2mg/mL) prepared in 100mM Sodium Carbonate Buffer (pH 9.0) was mixed with 50µL of 1 mg/mL FITC solution in 5µL intervals and incubated overnight under agitation at 4ºC, in the absence of light. Then, 200µL of 312.5mM NH_4Cl was added to stop reaction, and the solution was reincubated

for further 30min before adding 250µL of 30% Glycerol. Liquid chromatography (ÄKTA Pure™, Cytiva) coupled with a Superdex® Peptide 10/300 GL was used to purify the marked peptide, which was lyophilized for further use.

6.4.4.2 Penetrating Assays by CLSM (Confocal Laser Scanning Microscopy)

Labeled Nicotianin-I cellular localization assay was adapted from a tumoral cell methodology [57]. FITC-(N-I) was resuspended in sterile water and evenly mixed with *C. tropicalis* cells (1.0 x 10^6) to achieve final concentrations of 25 and 100µM. After incubation, aliquots were taken at intervals (5, 25, 45 and 65min) and washed thrice in PBS 0.1M (5.000 x *g* at 4ºC for 5min). Aliquots were transferred to glass slides, sealed and subjected to Confocal Laser Scanning Microscopy analysis (CLSM) (LM710, Zeiss, Jena, Germany) under excitation/emission values of 495 and 525nm, respectively.

6.4.5 Zeta Potential Measurements

Zeta potential measurements were done by a Zetasizer Nano (Malvern, UK) using disposable cells (optical path = 10 mm). Briefly, 12 h cultures of *C. tropicalis* (1.0 x 10^6) were washed (5.000 x *g*, 5 min), resuspended in HEPES Buffer (200µM) and combined with N-I to achieve final concentrations of 25 and 100µM. Untreated cells were used as negative control. 15 measurements were performed at room temperature (25 ºC) at 120s intervals for 15 minutes after 5 min of equilibration. An applied current of 40mV was applied for analysis [41,42].

6.4.6 Checkerboard Assay

Nicotianin-I synergism with antifungals (AMB, FCZ and CAS) was evaluated by a checkerboard assay. A duplicate of the microdilution assay for each antifungal-peptide combination were done to assess the synergistic, non-synergistic or antagonistic effects. Combinations were performed starting at combined MIC concentrations. The combinations used were N-I+AMB, N-I+FCZ and N-I+CAS, at the following concentrations: N-I (25 µM - 0,195 µM), AMB (0,125 µg/mL - 0,001 µg/mL), FCZ (64 µg/mL - 0,5 µg/mL) and CAS (0,031 µg/mL - 0,24 ng/mL). Negative control used RPMI 1640 without antimicrobials. MIC_{syn} was considered the lowest concentrations in which the fungal growth wasn't visible. Fractional

Inhibitory Concentrations (FICs) were individually calculated for each combination and the fractional inhibitory concentration index (FICI) defined the synergistic (FICI ≤ 0.5), non-synergistic (0.5 ≤ FICI ≤ 4.0) or antagonistic (FICI > 4.0) effect of the peptide-drug combination [58]. MTT assays of each plate were also performed as described at Section 4.2.

6.4.7 Statistical Analysis

All experimental data were collected by two to three independent assays, each being executed in triplicate. All results were presented as the mean and corresponding standard deviation (SD). The ANOVA test was employed for multiple mean comparisons when homoscedasticity and gaussian distributions were confirmed. Dunnet's test was conducted to compare the means individually. All statistical analyses used a confidence interval of 95 % ($p < 0.05$). Graphpad Prism 8.0 was used for statistical analyses and graphical production.

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ANEXO A – ARTIGO CIENTÍFICO III

Artigo científico publicado na revista *Future Microbiology*

Qualis: A3

Fator de Impacto: 2,9

Review

Rise and fall of Caspofungin: the current status of Caspofungin as a treatment for *Cryptococcus neoformans* infection

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Antifungal infections are becoming a major concern to human health due to antimicrobial resistance. Echinocandins have been promising agents against resistant fungal infections, primarily caspofungin, which has a more effective mechanism of action than azoles and polyenes. However, fungi such as *Cryptococcus neoformans* appear to be inheritably resistant to these drugs, which is concerning due to the high clinical importance of *C. neoformans*. In this review, we review the history of *C. neoformans* and the treatments used to treat antifungals over the years, focusing on caspofungin, while highlighting the *C. neoformans* problem and possible explanations for its inherent resistance.

Plain language summary: Caspofungin is a drug used to treat several types of fungal infections. Resistance to caspofungin is a huge problem, especially in those that are immunocompromised. It is important to understand the history of caspofungin discovery, its clinical applications and its mechanism of action, as well as if a new drug target could be used overcome resistance. This review may perform guide new studies combining caspofungin with other drugs and indicate new potential targets for caspofungin.

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Keywords: *Cryptococcus neoformans* • echinocandins • glucan synthesis • resistance

ANEXO B – ARTIGO CIENTÍFICO IV

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RESEARCH ARTICLE

Purification, Characterization, and Antimicrobial Activity Against *Candida parapsilosis and Staphylococcus aureus* of a Highly Stable Type-1 Cystatin from *Terminalia catappa* L. Seeds

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Abstract: ***Introduction*:** Clinic infections caused by various microorganisms are a public health concern. The rise of new strains resistant to traditional antibiotics has exacerbated the problem. Thus, the search for new antimicrobial molecules remains highly relevant.

***Methods*:** The current study purified, characterized, and assessed the antimicrobial activity of a papain inhibitor from *Terminalia catappa* L. seeds.

***Results*:** The inhibitor was purified by heating the crude extract at 80°C for 30 min, followed by ion exchange chromatography on a DEAE cellulose column. The purification index was 9-fold, yielding 2.3%. SDS-PAGE and size exclusion chromatography revealed that the protease inhibitor (*Tc*PI) is a 15.9 kDa monomeric protein. The inhibition kinetics showed that *Tc*PI is a competitive inhibitor specific to papain (Ki = 1.02 x 10^{-4} M). *Tc*PI remained active even after heating at 100°C for 120 min and at pH conditions varying from 2.0 to 10.0. Even after 60 min, *Tc*PI was resistant to papain proteolysis. *Tc*PI exhibited antimicrobial activity against *Candida parapsilosis* and *Staphylococcus aureus*.

***Conclusion*:** Here, we show that *Tc*PI is a highly stable type-1 cystatin with the potential to combat infections caused by *C. parapsilosis* and *S. aureus*. Additional investigations into *Tc*PI's structural aspects and mechanism of action, as well as safety assessments, are essential prerequisites for its potential application as a novel therapeutic intervention.

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