CARACTERIZAÇÃO BIOQUÍMICA E ATIVIDADES BIOLÓGICAS DE PEPTÍDEOS ANTIMICROBIANOS DE FOLHAS DE *Capsicum annuum* cv. Carioquinha

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JULHO DE 2023

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FICHA CATALOGRÁFICA
UENF - Bibliotecas
Elaborada com os dados fornecidos pela autora.

C521 Cherene, Milena Bellei.

Caracterização bioquímica e atividades biológicas de peptídeos antimicrobianos de folhas de *Capsicum annuum* cv. Carioquinha. / Milena Bellei Cherene. - Campos dos Goytacazes, RJ,2023.

120 f. : il.

Inclui bibliografia.

Tese (Doutorado em Biotecnologia Vegetal) - Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Biociências e Biotecnologia, 2023.

Orientadora: Valdirene Moreira Gomes.

1. Peptideos antimicrobianos. 2. *Capsicum annuum*. 3. bioprospecção. I. UniversidadeEstadual do Norte Fluminense Darcy Ribeiro. II. Título. CDD - 660.6

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APROVADA em 31 de julho de 2023.

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(Orientadora)

A todos os meus antepassados pela coragem e determinação para que eu pudesse existir, dedico. Que o melhor de cada um de vocês possa viver em mim.

AGRADECIMENTOS

Ao Criador, por ter feito tantas coisas maravilhosas para serem descobertas e estudadas. Que minha alma, meu coração e minha boca possam sempre bendizer o Teu nome.

A minha família, pelo apoio incondicional e por compartilharem comigo tanto os momentos de conquistas como os mais difíceis.

A minha querida orientadora Val, por me nortear nessa jornada, me incentivar, discutir ideias e apontar horizontes. Pés no chão, olhar no horizonte e umbigo na bancada!

À equipe do LFBM: professores, alunos e técnicos. Aos professores Gabriel Taveira e André Carvalho pelas valiosas contribuições e ensinamentos.

Às equipes do LQFPP e LSA, em especial aos professores Thiago, Elenir e Olney e aos alunos Fabrício, Sarah e Nina, pela maravilhosa parceria que tanto ampliaram os resultados da minha pesquisa.

Ao professor Miguel Castanho e toda equipe sua equipe, por todo carinho e atenção com que me acolheram e orientaram no estágio de doutoramento em Lisboa. Um agradecimento mais que especial a Marco e Vera por todos os ensinamentos e contribuições.

À UENF e ao PGBV o meu muito obrigada. Tenho muito orgulho em dizer que a UENF é parte da minha história há 30 anos.

Às agências de fomento CNPq, CAPES, FAPERJ e UENF.

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RESUMO

CHERENE, Milena Bellei, D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro, julho de 2023. **Caracterização bioquímica e atividades biológicas de peptídeos antimicrobianos de folhas de *Capsicum annuum* cv. Carioquinha.** Orientadora: Valdirene Moreira Gomes.

Os peptídeos antimicrobianos de plantas são uma ferramenta promissora para o desenvolvimento de novos fármacos, terapias e métodos de controle de pragas e patógenos devido a sua toxicidade seletiva, seu amplo espectro de atividades e características que dificultam o desenvolvimento de resistência microbiana. A cultivar Carioquinha de *Capsicum annuum* foi desenvolvida pelo Programa de Melhoramento Genético da UENF com o intuito de ter maior resistência a fitopatógenos. Vários peptídeos antimicrobianos obtidos de plantas do gênero *Capsicum* já foram caracterizados e suas atividades biológicas elucidadas. Neste sentido, esta tese apresenta um capítulo dedicado a descrever a purificação e caracterização bioquímica de três destes peptídeos obtidos de folhas de *C. annuum* cv. Carioquinha, bem como suas atividades contra leveduras do gênero *Candida*, seus possíveis mecanismos de ação e toxicidade para eritrócitos. Os peptídeos são um inibidor de protease, uma defensina-símile e, um peptídeo transportador de lipídeos do tipo 2 (LTP2), denominados *Ca*CPin-II, *Ca*CDef-like e *Ca*CLTP2, respectivamente. O segundo capítulo descreve a obtenção de uma fração enriquecida de *Ca*CPin-II e a atividade desta fração e do extrato de folhas de *C. annuum* sobre o desenvolvimento de larvas do inseto praga *Callosobruchus maculatus*. O terceiro e último capítulo avalia a toxicidade dos três peptídeos para o possível desenvolvimento de novos fármacos e terapias, utilizando testes *in vitro* com células de mamíferos e testes *in vivo* com o modelo invertebrado *Galleria mellonella*. Brevemente, os resultados indicam que os peptídeos causam diminuição da viabilidade celular e alterações morfológicas e nas membranas de leveduras do gênero *Candida*. O extrato de folhas e *Ca*CPin-II possuem capacidade de inibir importantes enzimas envolvidas no metabolismo de fungos e insetos, e interferem no desenvolvimento de *C. maculatus*. Os peptídeos, exceto *Ca*CPin-II, não são citotóxicos e não causam morte em *G. mellonella*. Conclui-se que *Ca*CPin-II, *Ca*CDef-like e *Ca*CLTP2 possuem potencial para o desenvolvimento de novos produtos biotecnológicos.

Palavras chave: Peptideos antimicrobianos; *Capsicum annuum*; bioprospecção.

ABSTRACT

CHERENE, Milena Bellei, D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro, July, 2023. **Biochemical characterization and biological activities of antimicrobial peptides from leaves of *Capsicum annuum* cv. Carioquinha.** Advisor: Valdirene Moreira Gomes.

Plant antimicrobial peptides are a promising tool for the development of new drugs, therapies and methods to control pests and pathogens due to their selective toxicity, their broad spectrum of activities and characteristics that hinder the development of microbial resistance. *Capsicum annuum* cv. Carioquinha was developed by the Programa de Melhoramento Genético from UENF in order to have greater resistance to phytopathogens. Several antimicrobial peptides obtained from *Capsicum* genus plants have already been characterized and their biological activities elucidated. In this sense, this thesis presents a chapter dedicated to describing the purification and biochemical characterization of three of these peptides obtained from leaves of *C. annuum* cv. Carioquinha, as well as its activities against yeasts of the genus *Candida*, its possible mechanisms of action and toxicity to erythrocytes. The peptides are a protease inhibitor, a defensin-like and a lipid transporter peptide type 2 (LTP2), called *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2, respectively. The second chapter describes the obtainment of an enriched fraction of *Ca*CPin-II and the activity of this fraction and of the *C. annuum* leaf extract on the development of larvae of the pest insect *Callosobruchus maculatus*. The third and final chapter evaluates the toxicity of the three peptides for the possible development of new drugs and therapies, using *in vitro* tests with mammalian cells and *in vivo* tests with the *Galleria mellonella* invertebrate model. Briefly, the results indicate that the peptides cause decreased cell viability and morphological and membrane changes in yeasts of the genus *Candida*. The leaf extract and *Ca*CPin-II were able to inhibit important enzymes involved in the metabolism of fungi and insects, and interfere with the development of *C. maculatus*. The peptides, except *Ca*CPin-II, are not cytotoxic and do not kill *G. mellonella*. We concluded that *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 have potential for the development of new biotechnological products.

Keywords: Antimicrobial peptides; *Capsicum annuum*; bioprospecting.

1 - INTRODUÇÃO GERAL

1.1 - Introdução

A utilização de elementos da natureza e seus compostos bioativos para o combate doenças e pragas e para defensa contra predadores é tão antiga quanto a própria humanidade, e as plantas constituem uma fonte abundante de tais compostos. Atualmente a biodiversidade vegetal conta tanto com espécies e variedades de ocorrência natural, como também com novas cultivares criadas ao longo dos anos pelo melhoramento genético. O Brasil é o país com a maior biodiversidade do mundo, e uma das plantas nativas muito utilizada pelos povos originários com fins medicinais e ritualísticos são as pimentas do gênero *Capsicum* (INSTITUTO SOCIOAMBIENTAL, 2016; MARINHO, 2014). Este gênero é originário das Américas e conta com 38 espécies catalogadas (CARRIZO GARCÍA *et al.*, 2016). Após a expansão marítima e colonização das Américas estas pimentas se espalharam pelo mundo e hoje possuem importância não só cultural como econômica (SILVAR; ROCHA; BARATA, 2022; TRIPODI *et al.*, 2021). A pesquisa com pimentas do gênero *Capsicum* iniciou-se na UENF em 1998, quando criou-se um banco de germoplasma a partir da coleta de frutos em mercados e feiras do estado do Rio de Janeiro, e hoje conta com vários acessos bem caracterizados e também com algumas cultivares desenvolvidas e protegidas. A cultivar Carioquinha é uma das linhagens de *C. annuum* desenvolvida pelo Programa de Melhoramento Genético de *Capsicum* da UENF (Figura 1).

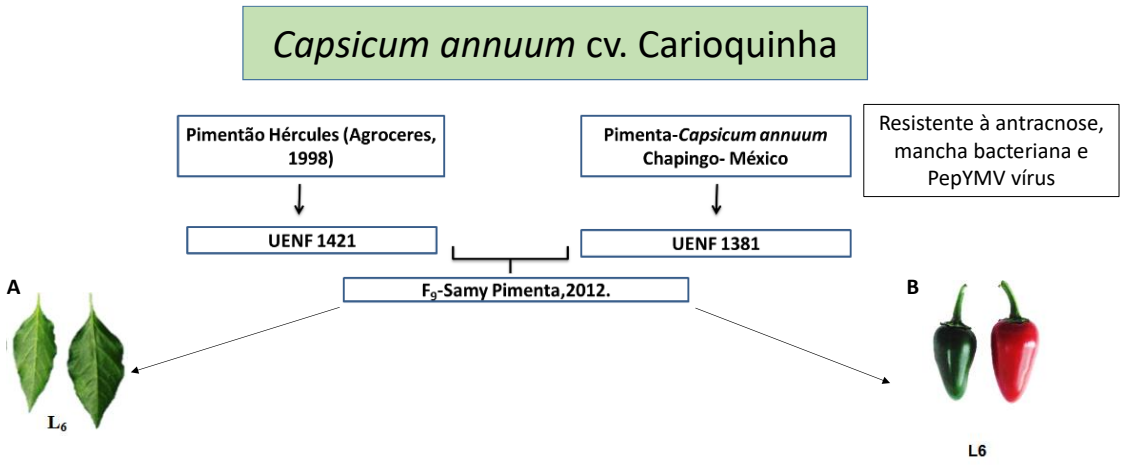

Figura 1. Genealogia do Programa de Melhoramento de *Capsicum* da UENF para obtenção de linhagens recombinantes resistentes à mancha bacteriana. Folhas sem sintomas de mancha bacteriana (**A**); forma e coloração de frutos (**B**) de *C. annuum* var. *annuum* cv. Carioquinha. Campos dos Goytacazes, UENF, 2015. (Adaptado de Pimenta, S., 2015).

Esta cultivar é proveniente do cruzamento entre os acessos UENF 1421 (Pimentão Hércules – Agroceres, 1998) e UENF 1381 (Pimenta Chapingo – México), e é resistente à mancha bacteriana causada por *Xanthomonas euvesicatoria*, um fitopatógeno bastante comum que afeta pimentas (BENTO *et al.*, 2017; PIMENTA *et al.*, 2016).

Vários compostos bioativos encontrados em pimentas do gênero *Capsicum* já foram isolados e caracterizados, dentre eles os peptídeos antimicrobianos, do inglês *Antimicrobial Peptides* (AMPs) (AFROZ *et al.*, 2020; OLIVEIRA *et al.*, 2022). Os AMPs são componentes da defesa inata de todos organismos vivos, sendo expressos de forma constitutiva, quando faz parte do programa de desenvolvimento normal do organismo, ou induzidos, quando se trata da defesa do organismo a algum fator de estresse. Essa moléculas são codificadas por genes, ou seja, são sintetizados nos ribossomos nas conformações linear ou cíclica, e são compostos de L-aminoácidos em sua cadeia polipeptídica (CARVALHO; GOMES, 2012). Essa característica difere os AMPs de outros peptídeos com atividade antimicrobiana, como gramicidinas, bacitracinas e polimixinas, que são sintetizados dentro das células por grandes complexos enzimáticos e possuem em sua cadeia polipeptídica aminoácidos não protéicos com diversos tipos de modificações (MARAHIEL; ESSEN, 2009; SIEBER; MARAHIEL, 2005). AMPs apresentam variedade quanto à sequência, estrutura e conformação, geralmente possuindo 12 a 100 resíduos de aminoácidos. São ricos em resíduos de cisteína, o que favorece na formação de pontes dissulfeto que promove a estabilidade, resistência a degradações decorrentes a temperatura, alteração de pH e ações proteolíticas (MOOKHERJEE *et al.*, 2020). O mecanismo de ação dos AMPs está interligado a vários fatores, como sua sequência de aminoácidos, sendo suas características catiônicas e anfifílicas determinantes na especificidade antimicrobiana. Portanto, apresentam baixa ou nenhuma toxicidade para células de mamíferos. Estudos têm demonstrado que em geral os peptídeos interagem com a membrana celular, através de rupturas ou permeabilização, mas também reagem com os elementos intracelulares, efetuam a inibição da síntese de DNA/RNA e de proteínas, controlando ou interrompendo o crescimento de microrganismos (AHMED; HAMMAMI, 2019; CAMPOS *et al.*, 2018).

A Figura 2 ilustra os principais mecanismos de ação dos AMPs tomando uma célula bacteriana como modelo. Os modelos de interação com a membrana descrevem o comportamento dos AMPs catiônicos, que interagem com as moléculas eletricamente carregadas da superfície das células-alvo sem a necessidade de receptores específicos;

são eles: o modelo de aduela de barril, o poro toroidal, o modelo de carpete e o modelo agregado (WEI; ZHANG, 2022).

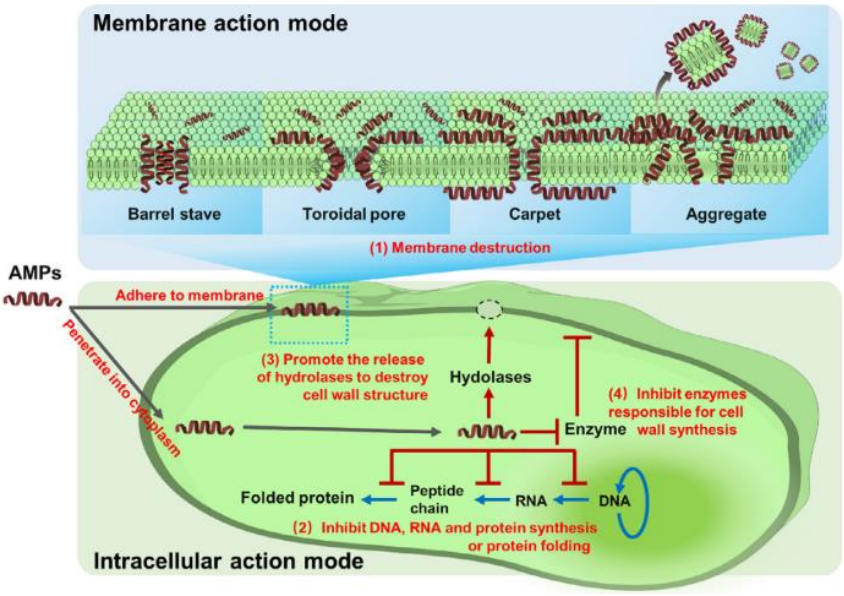

Figura 2. Mecanismos de ação de AMPs antibacterianos. Os mecanismos incluem o modo de ação nas membranas e o modo de ação intracelular. No modo de ação na membrana, os AMPs geralmente iniciam sua ação aderindo-se à superfície da membrana celular via interações eletrostáticas e hidrofóbicas, seguida da formação de poros transmembrana, que aumenta a permeabilidade celular e geralmente leva à morte da célula. Para os AMPs catiônicos, quatro principais modelos estão estabelecidos para descrever a formação de poros na membrana (1): as aduelas de barril, o poro toroidal, o modelo carpete e o modelo de agregados. No modelo de ação intracelular, os AMPs alcançam o citoplasma e interfere em alvos intracelulares e no metabolismo, ou seja, inibem a síntese de DNA, RNA e proteínas (2), promovem a liberação de liases que destroem estruturas celulares (3) e inibindo a ação de enzimas responsáveis pela síntese da parede celular (4). (Adaptado de Wei e Zhang, 2022).

Após a interação com os lipídeos aniônicos das membranas, os AMPs em solução aquosa assuem uma conformação em α-hélice anfifílica que promove a interação com a membrana. AMPs ricos em estruturas em folhas-β geralmente não passam por estas mudanças conformacionais, pois as folhas-β são estabilizadas por pontes dissulfeto (LEE; N. HALL; AGUILAR, 2015). O modelo de aduela em barril descreve a interação de AMPs paralelamente ao plano da membrana, que depois movem-se para um plano perpendicular à membrana e formam um poro de interior hidrofílico (KUMAR *et al.*, 2018; LEE; N. HALL; AGUILAR, 2015). No modelo de poro toroidal, os AMPs inserem-se verticalmente na membrana e interagem com os lipídeos para formar um poro com formato de anel. No modelo de carpete os AMPs não formam poros e não interagem com componentes hidrofóbicos da membrana, mas recobrem a superfície externa da

membrana formando clusters que lembram um carpete, o que culmina no colapso da membrana formado várias estruturas micelares. No modelo de agregados, os AMPs se ligam à superfície celular e quando atingem uma determinada concentração reposicionam-se e formam canais de vazamento de íons e água. Este modelo também explica a transferência de AMPs catiônicos para o citoplasma para atura em alvos intracelulares. Alguns AMPs podem assim alcançar o interior da célula sem causar ruptura direta da membrana, e levar a morte celular por inibir a síntese de DNA, RNA e proteínas, promovendo a liberação de enzimas líticas ou inibindo enzimas envolvidas no metabolismo (WEI; ZHANG, 2022).

1.2 - Peptídeos antimicrobianos de plantas

Existem várias classes estruturais de AMPs envolvidas na defesa das plantas. O primeiro AMP de planta foi uma tionina isolada do trigo e desde então, centenas de outros AMPs foram isolados de outras espécies vegetais. AMPs de plantas podem ser classificados de acordo com suas estruturas típicas ricas em cisteína, representados principalmente pelos grupos dos ciclotídeos, defensinas, tioninas, proteínas transportadora de lipídios (LTPs), snakinas, heveínas-símile e knottinas-símile (OJEDA; CARDOSO; FRANCO, 2019; ZASLOFF, 2019). Há também outras classes de AMPs, com estruturas atípicas contendo poucos ou nenhum resíduo de cisteína, como por exemplo, o caso das hairpinias e de peptídeos ricos em glicina (DE SOUZA CÂNDIDO *et al.*, 2014; LI *et al.*, 2021). Devido à natureza dos AMPs isolados de folhas de *C. annuum* neste trabalho, vamos abordar alguns aspectos bioquímicos e bioatividades do grupo das defensinas, das LTPs e dos inibidores de protease tipo Pin-II.

As defensinas de plantas são peptídeos básicos, contendo de 45 a 54 resíduos de aminoácidos, com 4 pontes dissulfeto e massa molecular de pelo menos 5 kDa (VAN LOON; REP; PIETERSE, 2006). Desde o isolamento da primeira defensina de planta do endosperma de trigo em 1990, várias outras já foram descritas em vários tecidos de dicotiledôneas e monocotiledôneas (PARISI *et al.*, 2019). O peptídeo maduro consiste em 5 segmentos de loops não conservados, ligando α-hélices e folhas-β e gerando estruturas mais complexas, e a assinatura molecular das defensinas é a presença do γ-core motif (GXCX3-9C). Diferenças nas sequências dos loops conferem diferentes funções às defensinas (SHER KHAN *et al.*, 2019; VAN DER WEERDEN; ANDERSON, 2013). As

características anfifílicas das defensinas permitem que elas interajam com lipídios de membrana específicos, porém, algumas defensinas requerem internalização nas células para exercerem suas funções. Estes peptídeos alcançam o citoplasma de diferente formas, incluindo endocitose dependente de energia, sistema de transporte de poliamina e transporte passivo (LUO *et al.,* 2021). Já foram descritas diversas atividades biológicas relacionadas às defensinas, como atividade antibacteriana, antifúngica, inseticida, e antitumoral e inibidora de proteases e α-amilases (CAMPOS *et al.*, 2018; KOVALEVA *et al.,* 2020) Defensinas são candidatos atrativos para o desenvolvimento de novas terapias antimicrobianas e antitumorais devido a sua alta seletividade e baixa indução de mecanismos de resistência de células tumorais e agentes infecciosos (HEIN *et al.*, 2022).

As LTPs são uma classe de peptídeos catiônicos, estruturalmente compactos, abundantes e solúveis (SALMINEN; BLOMQVIST; EDQVIST, 2016), isolados pela primeira vez de tubérculos de batata. É uma classe de AMP presente somente em vegetais, no entanto, não está presente na maioria dos vegetais inferiores. A função primárias das LTPs é a transferência de lipídeos (FINKINA *et al.*, 2016). Devido a sua baixa especificidade para substratos lipídicos, as LTPs de plantas são também chamadas de proteínas transportados de lipídios não-específica (nsLTP). Sua estrutura possui 4 pontes dissulfeto bem conservadas, 4 a 5 α-hélices dobradas para formar uma estrutura ajustada e resistente à desnaturação. Um motif de 8 cisteínas (C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C) está presente em todas as LTPs. Baseado no seu padrão de cisteínas e na sequência de aminoácidos, as LTPs são subdivididas em classes, sendo as LTPs tipo 1 e LTPs tipo 2 as mais estudadas. Os peptídeos do grupo das LTP1 possuem massa molecular de cerca de 10kDa, enquanto os que pertencem ao grupo das LTP2 possuem massa de cerca de 7 kDa (EDQVIST *et al.,* 2018). Cavidades hidrofóbicas são formadas para facilitar a ligação e o transporte de lipídios (BERECZ *et al.*, 2010). A cavidade hidrofóbica é extremamente flexível, podendo acomodar vários tipos de lipídeos, e possuem a forma de um túnel nas LTP1s e a forma cônica nas LTP2s (MELNIKOVA *et al.*, 2023). Foi proposto que as LTPs podem interagir com os lipídeos de membrana, translocando-os para o meio extracelular, levando à permeabilização de membranas e apoptose (FINKINA *et al.,* 2016). LTPs possuem uma variedade de atividades biológicas, tendo tanto um papel fisiológico importante na defesa de plantas, mas também possuem aplicações terapêuticas devido a suas atividades antitumoral e antimicrobiana, com baixa toxicidade para células de mamíferos (FINKINA *et al.,* 2016; REGENTE *et al.*, 2005). Estes

peptídeos possuem ainda aplicações biotecnológicas, no desenvolvimento de sistemas de drug-delivering, na indústria de alimentos, no desenvolvimento de cultivares resistentes a estresses bióticos e abióticos e no diagnóstico e tratamento de doenças alérgicas (MELNIKOVA *et al.*, 2023).

A família dos inibidores de protease (PIs, do inglês *Proteinase-Inhibitors*) possuem uma subclasse de inibidores de serino-proteases, muito comuns em plantas da família Solanaceae. Estes inibidores podem ser do tipo I, com 8kDa (Pin I – potato type I inhibitor) ou do tipo II, com 5 kDa (Pin II – potato type II inhibitor) (CHRISTELLER; LAING, 2005; DUNSE *et al.*, 2010; PAYNE *et al.*, 2020). Um inibidor do tipo Pin-II possui múltiplos domínios inibitórios de repetição (IRDs) unidos por 5 a 6 regiões ligantes (linkers), que são liberados após processamento proteolítico do Pin-II parental (Figura 3A). No gênero *Capsicum* a quantidade de IRDs é 5, mas em outros gêneros pode variar de 2 a 5. Cada IRD possui de 50 a 55 resíduos de aminoácidos e é estabilizada por pontes dissulfeto e uma região denominada RCL (do inglês reactive center loop), que é o sítio de interação primária com a serino protease alvo. A região ligante divide o IRD em cadeia pesada e cadeia leve, estando o RCL localizado nesta última cadeia. Os IRDs são classificados de acordo com sua região ligante em tipo 1, cuja sequência é DPNRP; tipo 2, cuja sequência é EEKKN; e tipo 3, que não tem região ligante (Figura 3B). Estas regiões específicas (linker, IRD e RCL) variam em número e sequência de aminoácidos entre os diferentes inibidores tipo Pin-II (YADAV; SAIKHEDKAR; GIRI, 2021).

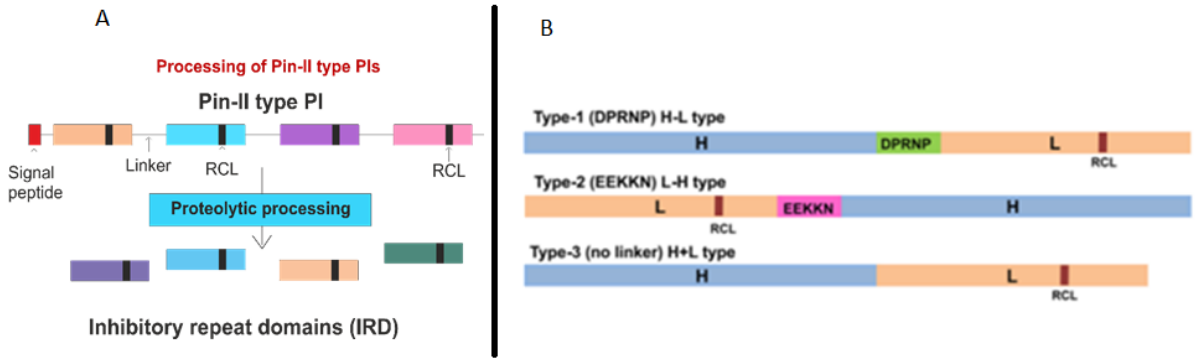

Figura 3. Estrutura dos inibidores de protease tipo Pin-II e seus domínios inibitórios de repetição (IRDs). (A) Modelo esquemático mostrando o processamento dos inibidores de protease tipo Pin-II em IRDs e (B) estrutura e classificação dos tipos de IRDs baseada na sequência das regiões ligantes: Cadeia pesada (H), Cadeia leve (L) e as diferentes regiões ligantes estão codificadas por cores (Adaptado de Yadav, Saikhedkar e Giri, 2021).

As proteases são enzimas que regulam a homeostase proteica, estando envolvidas na regulação de vários processos celulares, tais como expressão gênica, diferenciação, defesa imunológica, migração e morte celular, portanto inibidores de proteases possuem potencial para o desenvolvimento de fármacos e outros insumos. Inibidores tipo Pin-II são uma estratégia promissora como arcabouço para a engenharia de proteínas. Estes peptídeos podem ser usados como estratégia para o controle de insetos praga, como *Helicoverpa armigera* (DUNSE *et al.*, 2010) e como agentes antimicrobianos e antifúngicos (BÁRTOVÁ; BÁRTA; JAROŠOVÁ, 2019). Potamina-I é um inibidor tipo Pin-II que inibe quimiotripsina, tripsina e papaína, e possui atividades antimicrobianas, incluindo *Candida albicans* e *Rhizoctonia solani* (KIM *et al.*, 2005).

1.3 - Os AMPs como moléculas promissoras no combate a microrganismos resistentes

A descoberta da penicilina por Alexander Fleming e a criação de pesticidas para aumentar a produtividade agrícola nas lavouras trouxe mais qualidade de vida para a humanidade. Após muitos anos, o amplo uso de tais produtos gerou a seleção de microrganismos resistentes, fazendo com que muitos antimicrobianos não sejam mais efetivos (WANG, 2015). Com isso, a ciência hoje trava uma batalha contra o aparecimento de cepas de microrganismos com múltipla resistência aos antimicrobianos tradicionais. Segundo dados do CDC as infecções causadas por microrganismos resistentes matam anualmente 700 mil pessoas no mundo. Estima-se que até 2050 o número de mortes por microrganismos resistentes chegue a 10 milhões ao ano e que o custo destas infecções cheguem a 1 trilhão de dólares (NGUYEN-VIET *et al.*, 2017; O´NEILL, 2016). O CDC em 2019 criou uma lista para enfrentamento de patógenos resistentes, criando uma lista classificando patógenos resistentes de acordo com o nível de ameaça que eles representam para a saúde pública. O grupo “ameaças urgentes” refere-se aos patógenos cuja ameaça requerem ações urgentes e agressivas, que inclui a *Candida auris* e algumas bactérias resistentes aos antibióticos. O grupo “ameaças sérias” refere-se a patógenos cuja ameaça à saúde pública requer ações rápidas e duradouras, que e classificou como sérias as infecções por espécies do gênero *Candida* resistentes à fármacos, e algmas bactérias como MRSA (*Staphylococcus aureus* resistente à meticilina) e o bacilo da tuberculose resistente. O grupo “ameaças relativas” refere-se aos patógenos que requerem monitoramento cuidadoso e ações preventivas (CDC, 2019).

Os fungos patogênicos, apesar de muitas vezes ignorados, representam uma ameaça à saúde pública, à segurança alimentar e à biodiversidade (FISHER *et al.*, 2012). Segundo dados da GAFFI - Global Action Fund for Fungal Infection - mais de 300 milhões de pessoas sofrem de doenças graves causadas por fungos, e a mortalidade anual chega a 1.6 milhões. Dentro desta perspectiva, em 2022 a Organização Mundial de Saúde (WHO) criou uma lista de patógenos fúngicos prioritários para nortear as pesquisas e ações de saúde pública, uma vez que a população susceptível a tais infecções, como por exemplo pessoas imunucomprometidas e com comorbidades, vem aumentando nos últimos anos. Esta lista possui dezenove espécies que representam grave ameaça à saúde pública, divididas em três categoria: ameaça crítica, alta e média. As leveduras do gênero *Candida* destacam-se por terem representantes nas três categorias: *C. auris* e *C. albicans* (crítica); *C. parapsilosis* e *C.tropicalis* (alta); e *C. krusei* (média) (WHO, 2022). As espécies de *Candida sp* causam desde infecções cutaneomucosas até infecções sistêmicas. Um em cada quatro pacientes internados com candidemia vão a óbito. Cerca de 7% das candidemias são resistentes aos antimicrobianos disponíveis. A principal espécie causadora de candidemia é a *C. albicans*, porém com baixos níveos de resistência. No entanto, outras espécies não-*albicans*, incluindo *C. glabrata* e *C. parapsilosis*, são frequentemente resistentes e mais letais. Além disso, espécies altamente resistentes como *C. auris* têm causado muitos problemas (CDC, 2013, 2019).

Estima-se que quase 4 milhões de pessoas no Brasil tenham infecções fúngicas a cada ano. Destas, 2,8 milhões são infecções causadas por *Candida sp* e 1 milhão por *Aspergillus sp*. Segundo estudo do Conselho Federal de Medicina, cerca de 14,5% das infecções em pacientes internados em UTIs são causadas por fungos, e este percentual tende a aumentar (CFM, 2015). No Brasil, segundo dados da ANVISA de 2018, as infecções por *Candida spp* são a quarta maior causa de internações em UTI por Infecção Primária de Corrente Sanguínea (IPCS) associada a uso de Cateter Venoso Central (CVC), estando o *Staphylococcus* coagulase negativa em primeiro lugar (ANVISA, 2018). Os principais antifúngicos atualmente usados na terapêutica médica de humanos e animais possuem muitos efeitos tóxicos colaterais e não podem ser usados a longo prazo, principalmente em infecções sistêmicas. Os principais antifúngicos disponíveis no mercado e aprovados pelas agências reguladoras pertencem três classes principais: triazóis, polieneos (representados pela anfotericina B) e equinocandinas. Muitas espécies do gênero *Candida* adquiriram resistência a estes antifúngicos usados como primeira e

segunda opção terapêutica, como o fluconazol e capsofungina (BEN-AMI; KONTOYIANNIS, 2021; TAVEIRA *et al.*, 2022). O uso indiscriminado de azóis como fármacos e pesticidas é um fator que leva ao aparecimento de resistência em fungos, incluindo espécies do gênero *Candida* (BASTOS *et al.*, 2019; BRILHANTE *et al.*, 2019).

As propriedade catiônicas e anfifílicas da maioria dos AMPs os torna seletivos para as superfícies eletricamente carregadas de microrganismos e células tumorais. Além da ação direta nas membranas das células-alvo, os AMPs podem atuar na modulação da imunidade do hospedeiro ajudando na resposta imune (Figura 4), o que torna estas moléculas uma alternativa promissora para o desenvolvimento de novas terapias. Os AMPs induzem a resposta imune do hospedeiro para combater parasitas e células tumorais modulando tanto a resposta humoral como a resposta celular. As defensinas são o grupo de AMPs com papel de destaque na imunorregulação (WEI; ZHANG, 2022).

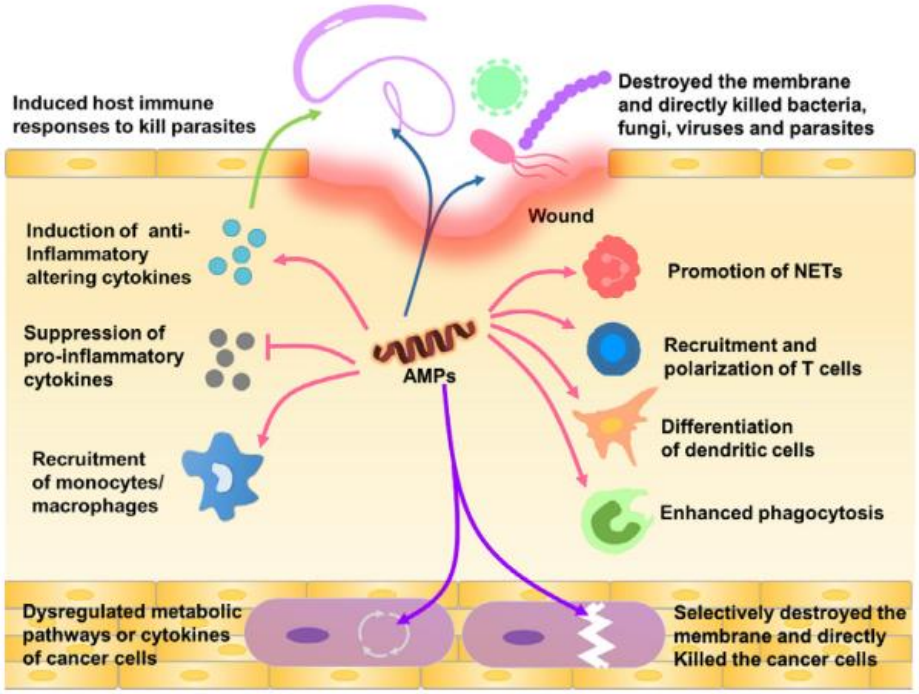

Figura 4. AMPs na modualção da resposta imune do hospedeiro. A bioatividade dos AMPs inclui regulação imunológica e ações de amplo espectro ou inespecíficas contra bactérias, vírus, parasitas ou células cancerígenas (Wei e Zhang, 2022).

Embora os AMPs sejam uma alternativa interessante para o desenvolvimento de fármacos, poucas pesquisas com este objetivo avançam para a fase pré-clínica de testes com animais. A instabilidade física e química dos peptídeos, a meia-vida curta, a degradação proteolítica e as características farmacocinéticas e farmacodinâmicas dos

AMPs são os principais obstáculos para sua aplicação clínica. Porém, uma solução é o encapsulamento destas moléculas para aumentar sua estabilidade em sistemas vivos (CARDOSO *et al.*, 2020; CHEN; LU, 2020). Mais de 3000 AMPs já foram identificados, mas poucos avançam para a fase de testes clínicos. Alguns poucos peptídeos antimicrobianos de síntese enzimática, como as gramicidinas e polimixinas, possuem aprovação pela agência reguladora U.S. Food and Drug Administration (FDA), e a maioria deles é utilizada para uso tópico em infecções bacterianas e fúngicas da pele (GRECO *et al.*, 2020; KOO; SEO, 2019). A toxicidade para células de mamíferos é o principal gargalo para a aprovação de peptídeos bioativos (GUPTA *et al.*, 2015). Encontrar o equilíbrio entre a bioatividade do AMP e a segurança para o paciente é o principal desafio para o desenvolvimento de fármacos. No entanto, AMPs de origem natural podem servir de arcabouço para o desenvolvimento de fármacos com toxicidade reduzida, mantendo ou aumentando suas bioatividades. Para isso, peptídeos sintéticos podem ser desenhados para ter maior toxicidade seletiva, através do ajuste de sua composição de aminoácidos e estrutura da cadeia peptídica, ou AMPs podem ser conjugados com outras moléculas, tais como biopolímeros, nanopartículas magnéticas, lipossomas e outros compostos para a criação de sistemas de drug-delivery (WEI; ZHANG, 2022).

1.4 - Os AMPs e outros inibidores de proteaese como alternativa no controle de insetos pragas

Além da problemática causada pelo uso irracional de antimicrobianos como fármacos, o uso de antimicrobianos e pesticidas em toda cadeia produtiva de alimentos é necessário, pois torna possível aumentar a produtividade agropecuária para satisfazer a demanda criada pelo crescimento populacional (SAATH; FACHINELLO, 2018). Tais insumos deixam resíduos nos alimentos, na água, no ambiente, que também acabam por contribuir para o aparecimento de microrganismos multirresistentes (BASTOS *et al.*, 2019; BRILHANTE *et al.*, 2019; O´NEILL, 2016). A pesquisa biotecnológica nos dias atuais deve ser pensada dentro do conceito de Saúde Única pois a saúde humana é interdependente de todas as outras espécies e do meio ambiente (MACKENZIE; JEGGO, 2019) exigindo a criação de novas substâncias de interesse biotecnológico para uso na agropecuária que proporcionem o desenvolvimento sustentável e impactos positivos na economia mundial e do Brasil.

O Brasil é o segundo país em exportação de alimentos segundo dados da FAO de 2020. O agronegócio é responsável por grande parte da economia do país, representando 21% do PIB e por metade das exportações no ano de 2019 segundo dados publicado no G1 em março deste ano (TOOGE, RIKARDY, 2020). Para exportar alimentos, os mesmos devem obedecer a uma série de normas sanitárias internacionais, que estabelecem a qualidade microbiológica, a presença de pesticidas, micotoxinas e outros parâmetros (SCUSSEL; BEBER; DE SOUZA KOERICH, 2016). Por isso, é importante investir em tecnologias que aumentem a produtividade mas mantenha o padrão que é exigido pelos países compradores. Ainda segundo a FAO, o Brasil é o quarto maior produtor de alimentos, mas também é um dos que mais desperdiça. Uma parte do desperdício ocorre pós-colheita, onde produtos agrícolas podem ser deteriorados por microrganismos e insetos. Fungos e oomicetos destroem um terço das colheitas mundiais anualmente, o que daria para alimentar 600 milhões de pessoas (FISHER *et al.*, 2012). No Brasil, as perdas anuais causadas por insetos em grãos armazenados chegam a 10% (LORINI, 2015). É preciso buscar substâncias que aumentem a durabilidade dos alimentos, garantindo a segurança alimentar do consumidor do campo até a mesa.

Os grãos são uma importante fonte nutricional devido ao fato de armazenarem nutrientes (POUTANEN *et al.*, 2021). Dentre os grãos usados na alimentação, as leguminosas representam uma importante fonte de proteínas em locais onde a proteína animal não é acessível para toda a população (FAO, 2016). O feijão de corda (*Vigna unguiculata*) é uma fonte de proteínas relevante para países em desenvolvimento (ABEBE; ALEMAYEHU, 2022) e o *Callosobruchus maculatus* (Coleoptera: Chrysomelidae: Bruchinae) é a principal peste que afeta *V. unguiculata* no período pós-colheita (CARDOSO *et al.*, 2017), podendo afetar também outras espécies do gênero *Vigna* e outras espécies de leguminosas, como soja (*Glicine max*) e ervilhas (*Lens culinaris*) (TUDA *et al.*, 2014). A Figura 5 mostra a morfologia de um macho e uma fêmea de *C. maculatus*. A infestação pelo inseto é difícil de controlar devido à grande quantidade de ovos colocados pelas fêmeas e ao seu curto ciclo de vida (BECK; BLUMER, 2011). O inseto bruquídeo *C. maculatus* foi o modelo de estudo deste trabalho para avaliar a atividade inseticida de extrato de folhas de *C. annuum* e correlacioná-la com um AMP inibidor de protease presente neste extrato.

Figura 5. Vista dorsal de um macho e uma fêmea de *Callosobruchus maculatus*. Fotos do macho e da fêmea estão na mesma escala. Os quadrados medem 1mm. (Adapatado de Beck e Blumer, 2011).

Muitos compostos de origem vegetal com atividade inseticida e repelente já foram descritos na literatura. Dentre eles destacam-se os metabólitos secundários, como piretrinas, rotenonas, azadiractina e óleos essenciais, e os compostos de natureza protéica, como AMPs do grupo dos ciclotídeos, defensinas e inibidores de protease, além de compostos com maior massa molecular, como vicilinas e lectinas (VELASQUES *et al.*, 2017). Dentre os AMPs, os ciclotídeos merecem destaque devido ao fato de já haver no mercado um inseticida denominado "Sero X" desenvolvido na Austrália e formulado à partir de extrato de *Clitoria ternatea* contendo estes peptídeos, que é usado como biopesticida no combate da larva do lepidóptero *Helicoverpa armigera*, a principal praga de lavouras de algodão (GROVER *et al.*, 2021). Esta família compreende peptídeos com 28 - 37 resíduos de aminoácidos ligados em uma estrutura cíclica cabeça-cauda, contendo três ligações dissulfeto que conferem um arranjo estrutural característico denominado de nó de cistina cíclico (Cyclic Cystine Knot – CCK) (CRAIK *et al.*, 2012). Os mecanismos de ação dos compostos protéicos no intestino médio dos insetos estão representados na Figura 6.

As defensinas possuem atividade inibidora de amilases e reduzem a biodisponibilidade de açúcares para o desenvolvimento do inseto, assim como os inibidores de protease reduzem a biodisponibilidade de aminoácidos provenientes das proteínas da dieta. Ciclotídeos, vicilinas, lectinas e subunidades da albumina atuam diretamente na membrana peritrófica do intestino médio do inseto, interferindo na absorção de nutrientes (VELASQUES *et al.*, 2017). Também já foi descrita uma lectina isolada de peçonha de aranha que atravessa a parede do intestino médio do inseto chegando a hemolinfa e exercendo sua atividade inseticida (FITCHES *et al.*, 2004).

Devido a um dos peptídeos isolados neste trabalho ser um inibidor de protease tipo Pin-II, vamos abordar com mais detalhes a atividade inseticida dos inibidores de protease.

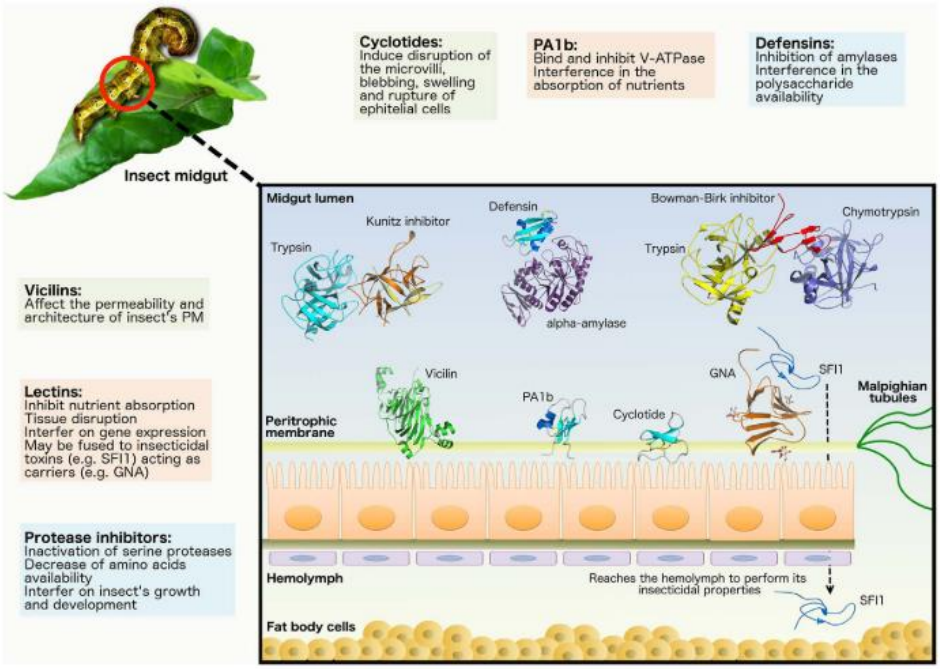

Figura 6. Mecanismos de ação de moléculas inseticidas de natureza proteica. Os mecanismos de ação de compostos proteicos no intestino médio de insetos principalmente como antinutrientes, interferindo na absorção de nutrientes. Sua natureza constitutiva os torna uma classe interessante de compostos de defesa a serem expressos por engenharia genética (Adaptado de Velasques et al., 2017).

Os inibidores de protease (PIs) são um grupo promissor para a criação de novas cultivares através da expressão heteróloga, e vários estudos com plantas transgênicas já foram feitos para aumentar a resistência de plantações aos insetos e fitopatógenos resistentes aos pesticidas disponíveis no mercado (VELASQUES *et al.*, 2017). Estes inibidores estão presentes em todos os tubérculos e sementes de leguminosas, mas também são expressos em vários outros órgãos de plantas de diferentes grupos taxonômicos (MOSOLOV; VALUEVA, 2005). PIs possuem papel importante na defesa de plantas contra insetos herbívoros, pois inibem suas enzimas do trato digestório reduzindo a biodisponibilidade de aminoácidos para seu crescimento e desenvolvimento. A massa molecular dos PIs varia de 3 a 25 kDa e seu mecanismo de ação envolve a formação de um complexo estável com a protease alvo bloqueando seu sítio ativo. Antigamente eram classificados de acordo com o tipo de protease que inibiam (serino, cisteíno, metaloproteases e aspartato proteases), no entanto, alguns PIs podem inibir mais

de uma classe de protease (HABIB; FAZILI, 2007; RAWLINGS; TOLLE; BARRETT, 2004). Atualmente a classificação proposta por Rawlings et al. 2004, agrupa os PIs em pelo mesmo 16 famílias baseado na sua similaridade de sequência, e disponíveis em um banco de dados de PIs de plantas (LEO *et al.*, 2002; VELASQUES *et al.*, 2017). Os inibidores de proteases tipo Pin-II são encontrados principalmente em plantas da família Solanaceae, tendo vários deles isolados de plantas do gênero *Capsicum*. Estes peptídeos possuem atividade inseticida em larvas de *Helicoverpa armigera* (MISHRA *et al.*, 2010, 2012).

1.5 - Plantas do gênero *Capsicum* como fonte para obtenção de AMPs

O gênero *Capsicum* pertence à família Solanaceae. Esse gênero se destaca pela sua importância comercial e econômica, sendo amplamente utilizado no setor alimentício, ornamental e medicinal. Engloba as plantas popularmente conhecidas como pimentas e pimentões (MOSCONE *et al.*, 2007). A pimenta é descrita como um alimento funcional devido as suas propriedades biológicas que favorecem a prevenção e até mesmo o tratamento de doenças como diabetes, câncer, problemas cardiovasculares, sendo também utilizada como matéria-prima na formulação de remédios caseiros que aliviam dores musculares, reumatismo, desordens gastrointestinais e na prevenção de arteriosclerose (ALVAREZ-PARRILLA *et al.*, 2011).

No Brasil as pimentas são cultivadas principalmente nos estados de Minas Gerais, Bahia e Goiás. O gênero *Capsicum* origina-se de zonas tropicais e úmidas da América Central e do Sul. Das espécies do *Capsicum*, três são muito propagadas e tem frutos com muita pungência, o *C. annuum*, *C. frutescens* e o *C. chinense*. A pungência do fruto *Capsicum* é devida a um grupo de compostos chamados capsaicinóides que estão presentes em diferentes quantidades nas variedades de pimentas (AL OTHMAN *et al.*, 2011).

Nos últimos 20 anos um grande número de AMPs de plantas foram isolados e caracterizados (LI *et al.*, 2021; SANTOS-SILVA *et al.*, 2020), incluindo aqueles isolados de plantas do gênero *Capsicum* (AFROZ *et al.*, 2020; OLIVEIRA *et al.*, 2022). Já foram isolados e caracterizados vários AMPs de *Capsicum* sp, como: defensinas (ANAYA-LÓPEZ *et al.*, 2006; GEBARA *et al.*, 2020; MARACAHIPES *et al.*, 2019); proteínas transportadoras de lipídeos (LTPs) (DIZ *et al.*, 2006); inibidores de proteases (DA SILVA

PEREIRA *et al.*, 2018; DIZ *et al.*, 2006; RIBEIRO *et al.*, 2007); AMPs tionina-símile (TAVEIRA *et al.*, 2014); γ-tionina (DÍAZ-MURILLO *et al.*, 2016); e heveína-símile (GAMES *et al.*, 2016). Os mecanismos de ação e atividades biológicas destes peptídeos de *Capsicum* têm sido elucidados apresentando efeito similar a apoptose em fungos (RIBEIRO *et al.*, 2007) com indução de espécies reativas de oxigênio e nitrogênio, permeabilização de membranas de microrganismos (SILVA *et al.*, 2017; TAVEIRA *et al.*, 2014, 2018). Além disso, AMPs de *Capsicum* sp têm sido expressos em sistemas heterólogos para obtenção de plantas com maior resistência a pragas e fitopatógenos (MOGUEL-SALAZAR *et al.*, 2011).

As defensinas isoladas e caracterizadas de plantas do gênero *Capsicum* geralmente estão envolvidas na defesa contra fitopatógenos, principalmente fungos (ANAYA-LÓPEZ *et al.*, 2006). Defensinas de *Capsicum* sp. possuem atividade contra fungos filamentosos, como *Fusarium oxysporum*, *Botrytis cinerea* (MEYER *et al.*, 1996) e *Colletotrichum gloeosporioides* (MARACAHIPES *et al.*, 2019), mas também já foi descrita atividade contra leveduras do gênero *Candida* e contra *Mycobacterium tuberculosis* (AGUIEIRAS *et al.*, 2021; GEBARA *et al.*, 2020). Atividades antifúngicas também já foram descritas para LTPs isoladas de *Capsicum* sp., como por exemplo, contra as leveduras *C. albicans*, *Saccharomyces cerevisiae* e *S. pombe* (DIZ *et al.*, 2006), e fungos filamentosos *C. lindemunthuianum* e *F. oxysporum* (CRUZ *et al.*, 2010). Até o momento não foram descritas atividades antimicrobianas de inibidores de protease tipo Pin-II isolados de plantas do gênero *Capsicum*, sendo descritas somente atividades inseticidas para esta classe de AMP.

A Figura 7 resume os diferentes peptídeos antimicrobianos e os genes dos peptídeos antimicrobianos já identificados em plantas do gênero *Capsicum* até o ano de 2022 (OLIVEIRA *et al.*, 2022).

Gene name	Peptide name	Number of amino acid residues	Molecular mass	Family	Plant organ	Species	Antimicrobial activity in vitro	Reference
PepThi	-	-	-	Thionin-like	Fruits, Leaves, Stems, and Roots	*C. annuum*	not determined	Oh et al. (1999)
CATHION1	-	-	-	Thionin	Leave	*C. annuum*	not determined	Lee et al. (2000a)
-	*Ca*Thi	-	~6 kDa	Thionin	Fruit	*C. annuum*	*F. solani* *C. parapsilosis* *C. pelliculosa* *C. buinensis* *C. mogii* *S. cerevisiae* *C. albicans* *C. tropicalis* *E. coli* *P. aeruginosa*	Taveira et al. (2014; 2016; 2017)
J1	J1	48	~5 kDa	Defensin	Fruit	*C. annuum*	*F. oxysporum* *B. cinerea*	Meyer et al. (1996)
j1-1 and j1-2	-	-	-	Defensin	-	*C. annuum*	not determined	Meyer et al. (1996); Houlné et al. (1998); Oh et al. (1999)
CADEF1	-	-	-	Defensin	Stem, Root, and Fruit of unripe peppers	*C. annuum*	not determined	Do et al. (2004)
CDef1	CDef1	47	5,2 kDa	Defensin	Fruit	*C. annuum*	not determined	Maarof et al. (2011)
-	IIFF7*Ca*	-	~5 kDa	Defensin	Fruit	*C. annuum*	*C. gloeosporioides*	Maraca-hipes et al. (2019a)
-	Cadef1	~40	5 kDa	Defensin	Fruit	*C. annuum*	*C. gloeosporioides*	Maraca-hipes et al. (2019b)
CanThio1	CanThio1	64	7,22 kDa	Defensin	Flowers	*C. annuum*	not determined	Nikte et al. (2019)
CanThio2	CanThio2	72	8,36 kDa					
CanThio3	CanThio3	63	7,13 kDa					
CanThio4	CanThio4	81	9,32 kDa					
CanThio5	CanThio5	59	6,73 kDa					
CanThio6	CanThio6	59	6,70 kDa					
CanThio7	CanThio7	59	6,73 kDa					
CanThio8	CanThio8	59	6,81 kDa					
-	CaDeF2.1	40	5 kDa	Defensin	Fruit	*C. annuum*	*C. buinensis* *C. tropicalis* *C. parapsilosis* *M. tuberculosis*	Gebara et al. (2020)
-	CaDeF2.2	40	~6 kDa					
-	*Cc*Def3	-	~6,5 kDa	Defensin	Fruit	*C. chinense*	*C. albicans* *C. buinensis* *C. tropicalis*	Aguiciras et al. (2021)
CALTPI	-	-	-	LTP	Leaves, Stalks, Fruit	*C. annuum*	not determined	Jung et al. (2003)
CALTPII	-	-	-					
CALTPIII	-	-	-					
CcLTP	CcLTP-27	136	13.5 kDa	LTP	Fruit	*C. chinense*	not determined	Liu et al. (2006)
	CcLTP+27	145	14.3 kDa					
-	F1	-	~10 kDa	LTP	Seed	*C. annuum*	*C. albicans* *S. cerevisiae* *S. pombe*	Diz et al. (2006)
-	CM1	-	9 kDa	LTP	Seed	*C. annuum*	*F. oxysporum* *C. lindemunthianum* *S. cerevisiae* *P. membranifaciens* *C. tropicalis* *C. albicans*	Cruz et al. (2010)

Gene name	Peptide name	Number of amino acid residues	Molecular mass	Family	Plant organ	Species	Antimicrobial activity in vitro	Reference
-	*Ca*-LTP$_1$	-	9 kDa	LTP	Seed	*C. annuum*	*C. lindemunthianum* *C. tropicalis*	Diz et al. (2011)
CaMF2	CaMF2	94	9,65 kDa	LTP	Anther	*C. annuum*	not determined	Chen et al. (2011)
-	PSI-1.1	55	6,05 kDa	PI	Seed	*C. annuum*	not determined	Antcheva et al. (1996)
-	PLPIs	-	6 kDa	PI	Leaves	*C. annuum*	not determined	Moura and Ryan (2001)
-	PSI-1.2	52	-	PI	Seed	*C. annuum*	not determined	Antcheva et al. (2001)
- -	CapA1 CapA2	- -	~12 kDa ~12 k Da	PI	Leaves	*C. annuum*	not determined	Tamhane et al. (2005)
CanPI-7 *CanPI-15*	CanPI-7 CanPI-15	- -	25 kDa 6 kDa	PI	-	*C. annuum*	not determined	Mishra et al. (2013)
-	PEF2 -A	-	5 kDa	PI	Seed	*C. chinense*	*C. albicans* *P. membranifaciens* *S. cerevisiae* *C. tropicalis* *K. marxiannus*	Dias et al. (2013)
-	PEF2 -B	-	8,5 kDa	PI	Seed	*C. chinense*	not determined	
-	PEF3	-	5 kDa	PI	Seed	*C. chinense*	not determined	
-	HyPep	-	6 kDa	PI	Seed	*C. annuum* Hybrid (Ikedu X UENF 1381)	*S. cerevisiae* *C. albicans* *C. tropicalis* *K. marxiannus*	Bard et al. (2015)
-	CaTI	-	6 kDa	PI	Seed	*C. annuum*	*S. cerevisiae* *C. tropicalis* *C. albicans* *K. marxiannus* *C. gloeosporioides* *C. lindemuthianum* *F. oxysporum*	Ribeiro et al. (2012); Silva et al. (2017a, b)
-	H4	-	9 kDa	Vicilin-like	-	*C. baccatum*	*S. cerevisiae* *C. albicans* *C. tropicalis* *K. marxiannus*	Bard et al. (2014)
-	Ca-Alb	-	-	2 S albumin	Seed	*C. annuum*	*K. marxiannus* *C. tropicalis* *C. albicans* *S. cerevisiae*	Ribeiro et al. (2012)
-	HEV-CAN	40	4,258 kDa	Hevein-like	Leaves	*C. annuum*	*Clavibacter michiganensis ssp. michiganensis* *Ralstonia solanacearum*	Games et al. (2016)

Figura 7. Peptídeos antimicrobianos e genes de peptídeos antimicrobianos identificados no gênero *Capsicum* (Adaptado de Oliveira et al., 2022).

O conhecimento das propriedades dos AMPs facilita seu isolamento, classificação, além da criação de AMPs , que permite aos pesquisadores desenvolverem novos produtos com potencial agronômico e farmacológico, que causem menos impacto ao meio ambiente e à saúde dos usuários (CAMPOS *et al.*, 2018; PORTO *et al.*, 2018). Os AMPs constituem uma estratégia com muitas vantagens para o desenvolvimento de novos antimicrobianos: possuem um amplo espectro de atividade, baixa capacidade de induzir resistência, podem agir em sinergia com antimicrobianos tradicionais e podem modular a resposta imune e neutralizar endotoxinas (BOTO; DE LA LASTRA; GONZÁLEZ, 2018; MISHRA *et al.*, 2017). A pesquisa de novas alternativas ao combate de pragas e patógenos, como o uso de AMPs isolados de plantas, colabora em atender vários objetivos

dispostos na Agenda 2030 da ONU para o Desenvolvimento Sustentável (ONU, 2015), como por exemplo o objetivo 2 (Acabar com a fome, alcançar a segurança alimentar e melhoria da nutrição e promover a agricultura sustentável) e o objetivo 3 (Assegurar uma vida saudável e promover o bem-estar para todos, em todas as idades).

Conhecer e preservar a flora e os conhecimentos etnofarmacológicos de nosso país, como é o caso do uso das plantas do gênero *Capsicum*, é importante para a criação de novos produtos biotecnológicos e para a conservação da biodiversidade, ajudando a conter doenças infecciosas e pragas. Esta tese teve como objetivo isolar e caracterizar peptídeos antimicrobianos de folhas da cultivar Carioquinha de *C. annuum* var. *annuum* e avaliar suas atividades antifúngicas e inseticidas e sua toxicidade *in silico*, *in vitro* e *in vivo* para animais. A tese está organizada em três capítulos referentes a artigos científicos produzidos durante o doutorado. Cada capítulo possui uma introdução, resultados e discussão, além de descrever os métodos utilizados. No final da tese são apresentadas as principais conclusões do trabalho.

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2 - OBJETIVOS

Objetivo Geral

Isolar e caracterizar peptídeos antimicrobianos de folhas da cultivar Carioquinha de *C. annuum* var. *annuum* e avaliar suas atividades antifúngicas e inseticidas e sua toxicidade *in silico*, *in vitro* e *in vivo* para animais.

Objetivos Específicos

Purificar e caracterizar bioquimicamente peptídeos antimicrobianos de folhas de *C. annuum*;

Avaliar o efeito antifúngico dos peptídeos caracterizados e os possíveis mecanismos de ação destes em leveduras do gênero *Candida*;

Obter a fração contendo inibidor de protease no extrato de folhas de *C. annuum*;

Comparar o efeito da fração contendo o inibidor de protease com o do extrato total de folhas de *C. annuum* no desenvolvimento do inseto *C. maculatus*;

Avaliar a citotoxicidade *in vitro* dos peptídeos contra células de mamíferos e seus possíveis mecanismo de ação nestas células;

Avaliar a toxicidade *in vivo* dos peptídeos usando larvas de *Galleria mellonella* como modelo de estudo.

3 - CAPÍTULO 1

Structural and biochemical characterization of three antimicrobial peptides from *Capsicum annuum* L. var. *annuum* leaves for anti-*Candida* use

Chapter 1: Manuscript published

Structural and biochemical characterization of three antimicrobial peptides from *Capsicum annuum* L. var. *annuum* leaves for anti-*Candida* use

Milena Bellei Cherene[1], Gabriel Bonan Taveira[1], Fabricio Almeida-Silva[2], Marciele Souza da Silva[1], Marco Calvinho Cavaco[3], André Teixeira da Silva-Ferreira[4], Jonas Enrique Aguilar Perales[4], André de Oliveira Carvalho[1], Thiago Motta Venâncio[2], Olney Vieira da Motta[5], Rosana Rodrigues[6], Miguel Augusto Rico Botas Castanho[3], Valdirene Moreira Gomes[1*].

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Type of article: Research article

Situation: Published

Journal: Probiotics and Antimicrobial Proteins

Impact factor: 5.265

Resumo

O surgimento de microrganismos resistentes reduziu a eficácia dos antimicrobianos atualmente disponíveis, necessitando o desenvolvimento de novas estratégias. Peptídeos antimicrobianos de plantas, do inglês *Antimicrobial Peptides* (AMPs), são candidatos promissores para o desenvolvimento de novos medicamentos. Neste estudo, objetivamos isolar, caracterizar e avaliar as atividades antimicrobianas de AMPs isolados de *Capsicum annuum*. O potencial antifúngico foi testado contra espécies de *Candida*. Três AMPs de folhas de *C. annuum* foram isolados e caracterizados: um inibidor de protease, uma proteína defensina-símile e uma proteína transportadora de lipídeos, respectivamente denominados *Ca*CPin-II, *Ca*CDef-like e *Ca*CLTP2. Todos os três peptídeos tinham massa molecular entre 3,5 e 6,5 kDa e causaram alterações morfológicas e fisiológicas em quatro diferentes espécies do gênero *Candida*, como formação de pseudohifas, aumento de volume e aglutinação celular, inibição do crescimento, redução da viabilidade celular, estresse oxidativo, permeabilização de membrana, e ativação da metacaspase. Com exceção da *Ca*CPin-II, os peptídeos apresentaram baixa ou nenhuma atividade hemolítica nas concentrações utilizadas nos ensaios de levedura. *Ca*CPin-II inibiu a atividade da α-amilase. Juntos, esses resultados sugerem que esses peptídeos têm potencial como agentes antimicrobianos contra espécies do gênero *Candida* e podem servir como arcabouços para o desenvolvimento de peptídeos sintéticos para esse fim.

Palavras chave: antifúngicos; peptídeos bioativos; gênero *Candida*; *Capsicum annuum*

Highlights

. antimicrobial resistance has increased the fungal infections incidence

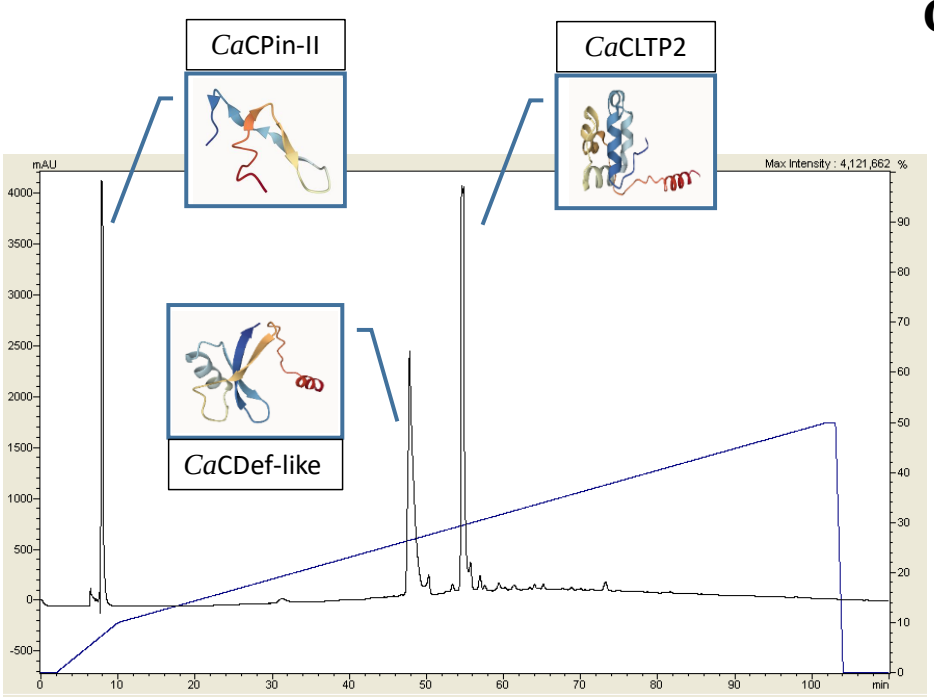
. antimicrobial peptides (AMPs) can be used for the development of new drugs

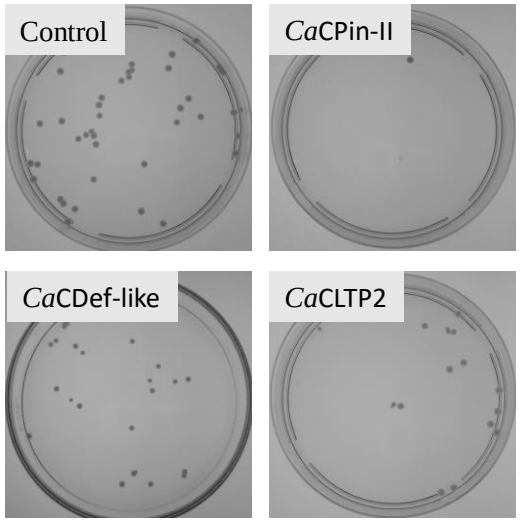
. AMPs were isolated from *Capsicum annum* extract with a single step methodology

. *C. annuum* AMPs with antifungal potential were isolated and characterized

Graphical Abstract

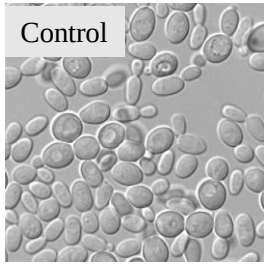
(Resumo gráfico)



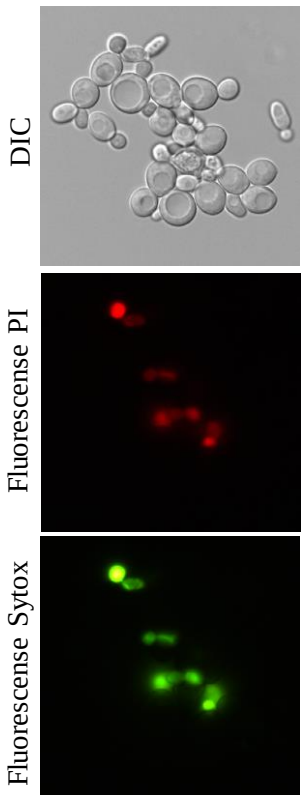


Reduce yeast viability

Induce morphophysiological changes



Treated yeasts



Probiotics and Antimicrobial Proteins
https://doi.org/10.1007/s12602-023-10112-3

RESEARCH

Structural and Biochemical Characterization of Three Antimicrobial Peptides from *Capsicum annuum* L. var. *annuum* Leaves for Anti-*Candida* Use

Milena Bellei Cherene[1] · Gabriel Bonan Taveira[1] · Fabricio Almeida-Silva[2] · Marciele Souza da Silva[1] · Marco Calvinho Cavaco[3] · André Teixeira da Silva-Ferreira[4] · Jonas Enrique Aguilar Perales[4] · André de Oliveira Carvalho[1] · Thiago Motta Venâncio[2] · Olney Vieira da Motta[5] · Rosana Rodrigues[6] · Miguel Augusto Rico Botas Castanho[3] · Valdirene Moreira Gomes[1]

Accepted: 14 June 2023

Abstract
The emergence of resistant microorganisms has reduced the effectiveness of currently available antimicrobials, necessitating the development of new strategies. Plant antimicrobial peptides (AMPs) are promising candidates for novel drug development. In this study, we aimed to isolate, characterize, and evaluate the antimicrobial activities of AMPs isolated from *Capsicum annuum*. The antifungal potential was tested against *Candida* species. Three AMPs from *C. annuum* leaves were isolated and characterized: a protease inhibitor, a defensin-like protein, and a lipid transporter protein, respectively named *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2. All three peptides had a molecular mass between 3.5 and 6.5 kDa and caused morphological and physiological changes in four different species of the genus *Candida*, such as pseudohyphae formation, cell swelling and agglutination, growth inhibition, reduced cell viability, oxidative stress, membrane permeabilization, and metacaspase activation. Except for *Ca*CPin-II, the peptides showed low or no hemolytic activity at the concentrations used in the yeast assays. *Ca*CPin-II inhibited α-amylase activity. Together, these results suggest that these peptides have the potential as antimicrobial agents against species of the genus *Candida* and can serve as scaffolds for the development of synthetic peptides for this purpose.

Keywords Antifungal · Bioactive peptides · *Candida* genus · *Capsicum annuum*

Introduction

Antimicrobial peptides (AMPs) play a role in the defense and can be expressed constitutively as part of the organism's normal developmental program or induced upon exposure to a stress factor. AMPs contain 12–100 amino acid residues and vary in their conformation and sequence [1, 2]. Many structural classes of AMPs are involved in plant defense, mainly represented by groups of defensins, thionins, lipid transport proteins (LTPs), and circular

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Published online: 26 June 2023

peptides (i.e., cyclotides) [3, 4]. Structural diversity is the most striking feature of plant AMPs; however, they share some common characteristics such as a relatively low molecular weight (less than 10 kDa) and a variable number of cysteine residues that form disulfide bonds and contribute to the stabilization of the tertiary structure. Different structural classes of AMPs have compact structures and are resistant to chemical and proteolytic degradation. They also have amphipathic properties, with both a positively charged hydrophilic region capable of interacting with anionic residues and a hydrophobic region that interacts with lipids [5, 6]. In general, AMPs have broad inhibitory activity against the growth and development of other organisms such as filamentous fungi, yeasts, bacteria, viruses, protozoa, and insects [1]. AMPs can disrupt and permeabilize membranes by interacting with cell membrane components and/or intracellular targets, inhibiting DNA/RNA and protein synthesis, and controlling or interrupting the growth of microorganisms [7, 8].

The indiscriminate use of antimicrobials has resulted in the emergence of microorganisms that are resistant to conventional drugs. Although often overlooked, pathogenic fungi pose a threat to public health, biodiversity, and food safety [9]. More than 300 million people suffer from serious fungal diseases with an estimated annual mortality of 1.6 million [10]. Almost 4 million people in Brazil are expected to have fungal infections each year. Of this total, 2.8 million are infections caused by *Candida* sp. and 1 million by *Aspergillus* sp., which mainly affect immunocompromised individuals [11]. *Candida* sp. can cause mucosal, cutaneous, and systemic infections, and resistant *Candida* infections are classified as serious by the Center for Disease Control and Prevention (CDC) [12]. Approximately 7% of candidemia cases are resistant to available antimicrobials, and the lethality in hospitalized patients is estimated at 25%. *Candida albicans* is the most prevalent species in candidemia, although it has low resistance levels. Conversely, other species, such as *Candida glabrata* and *Candida parapsilosis* are often resistant and lethal [12, 13].

Because AMPs have diverse mechanisms of action, broad-spectrum activity, and biochemical characteristics that hinder the evolution of resistance, the development of new drugs derived from them is a promising strategy [14, 15]. Additionally, AMPs have the advantage of low or no toxicity to animal cells [16, 17]. In recent years, the genus *Capsicum* has been regarded as an important source of molecules that stand out as possible candidates to overcome microbial resistance to conventional drugs [18]. Several *Capsicum* AMPs have been isolated and characterized, including defensins [19–21], lipid transport proteins (LTPs) [22], proteinase inhibitors [22–24], thionin-like peptides [25], ɣ-thionins [26], and hevein-like peptides [27]. The mechanisms of action of the AMPs isolated from *Capsicum* have not been completely elucidated; however, they have been shown to trigger events that activate programmed cell death [28], including morphological changes, increased membrane permeabilization, production of reactive oxygen species (ROS), and dissipation of mitochondrial potential [24, 25, 29]. In previous work, our research group purified and characterized several AMPs isolated from *C. annuum*. Here, we isolated, characterized, and evaluated the antifungal activity and mechanisms of action of three AMPs isolated from *C. annuum* leaves.

Materials and Methods

Biological Material

Seeds of *Capsicum annuum* L. var. *annuum* (cv. Cario-oquinha) were provided by the Laboratório de Melhoramento Genético Vegetal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil. Seeding was carried out in polyestirene foam of 72 cells with commercial substrate and kept in a growth chamber with a controlled temperature of 28 °C and a photoperiod of 12 h. When the seedlings exceeded 10 cm in height, they were transplanted into 5 L pots and transferred to the greenhouse. At 60 days, the leaves were cut close to the petiole and used for peptide extraction.

The yeasts *Candida tropicalis* (CE017), *Candida albicans* (CE022), and *Candida parapsilosis* (CE002) were provided from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The yeast *Candida buinensis* (3982) was provided from Micoteca URM from Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil. *Candida* yeasts were kept on Sabouraud 2% glucose agar (Merck) at the Laboratório de Fisiologia e Bioquímica de Microrganismos, from Centro de Biociência e Biotecnologia, UENF, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Extraction, Purification, and Biochemical Characterization of Peptides

C. annuum leaves (10 g) were macerated and extracted with 30 mL of 60% methanol (v/v) at room temperature over a period of 24 h. The extract was filtered to remove debris and partitioned with dichloromethane (1:1, v/v) at room temperature for a period of 24 h and the aqueous phase were separated and concentrated by lyophilization.

The leaves extract (0.1 g) was dissolved in 10 mL of 20% acetonitrile, filtered with 0.22 µm pore size Millex® syringe

filter and injected onto a high-performance liquid chromatography (HPLC) VP-ODS (250×4.6 mm i.d.) C_{18} reverse-phase column (Shim-pack, Shimadzu) with a C_8 guard column (*Pelliguard*, Sigma). The column was equilibrated with 10 mL of solution A (0.1% trifluoroacetic acid (TFA)), and eluted with 100% solution A for the first 2 min followed by a gradient of solution B (90% acetonitrile containing 0.1% TFA). The concentration of solution B was maintained at 0% for 2 min; increased to 10% from 2 to 10 min; to 50% from 10 to 102 min and maintained at 50% for 1 min. Then, the concentration of solution B was reduced to 0% and was kept at 0% until the end of the run at 110 min. The chromatography was developed at a flow rate of 0.5 mL min^{-1} at a temperature of 37 °C. The presence of proteins was determined by online absorbance measurement at 220 and 280 nm.

Proteins quantifications were done by the bicinchoninic acid (BCA) method described by Smith et al. [30], with modifications, using ovalbumin as the protein standard. SDS-tricine-gel electrophoresis was performed according to the method described by Schagger and von Jagow [31].

Amino acid sequence analysis was done using time of flight mass spectrometry (MALDI-TOF) on peptides isolated from HPLC fractions P_0, P_1, and P_2. The fractions were subjected to SDS-tricine-gel electrophoresis and the single band from each fraction was excised from the gel, digested with trypsin, and subjected to a mass spectrometry evaluation. Tryptic peptide fragments were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The instrument used was Orbitrap Q Exactive Plus (Thermo Scientific). Sequence similarity searches were performed using BLASTp and the nonredundant protein database (nr) [32] for P_1 and P_2, while P_0 was analyzed using the PINIR (Pin-II type PIs Information Resources) database [33]. Selected sequences were aligned with ClustalW [34].

Circular dichroism (CD) spectra of the three peptides were acquired in a J-815 CD spectropolarimeter (Jasco, Japan) at 25 °C in the 190–260 nm wavelength range, with a bandwidth of 0.50 nm and a scan speed of 50 nm/min, using a 0.1 cm quartz cell. Further, 200–400 µg mL^{-1} peptide solutions were prepared in ultra-pure water. The final spectra for each peptide were the average of ten consecutive scans per sample after subtraction of buffer baselines. Results were expressed as mean residue ellipticity ($[\theta]_{MRW}$) (deg×cm^2×$dmol^{-1}$), as follows:

$$\mathrm{MRW} = \frac{\theta_{oBS} \times \mathrm{MRW}}{10 \times d \times c} \quad (1)$$

where Θ_{obs} is the observed ellipticity in degrees, MRW is the mean residue weight, d is the cell path length, and c is the peptide concentration.

The peptide fragments were searched against the nonredundant protein sequence database using BLASTp [32], revealing XP_016579689.1 and KAF3615994.1 as best hits (defensin and non-specific LTP 2, respectively) and the sequence P56615 (UniProt ID) with 100% similarity with *C. annuum* protease inhibitor. These sequences were searched against the AlphaFold DB [35] using the EBI Protein Similarity Search tool (https://www.ebi.ac.uk/Tools/sss/fasta/). The AlphaFold model of the best orthologs (Q9FFP8 for defensin and A0A0R0FHG0 for nsLTP2) and P56615 (for protease inhibitor) were used as templates for homology modeling with Phyre2 [36] using one-to-one threading mode. Modeling of the 35 amino acid residue fragment RLCTNCCAGRKGCNYYSADGTFICEGESDPNNPKA found in the mass spectrometry analysis of peptide P_0 (*Ca*CPin-II) was done with Phyre2.

In Silico Prediction of Cell-Penetrating Potential

For increased confidence, we used three independent machine learning-based approaches that use random forest classifiers to predict the cell-penetrating potential of peptides: SkipCPP-Pred [37], MLCPP [38], and CellPPD-Mod [39]. Results obtained from SkipCPP-Pred analyses have confidence scores ranging between 0 and 1 (1 is the maximum confidence). MLCPP probability scores is the probability of permeabilizing membranes, which ranges from 0 to 1 (1 is the greatest ability to permeabilize). Additionally, we performed a BLAST search for similar cell-penetrating peptides in the CPPsite 2.0 database [40] using P56615, XP_016579689.1 and KAF3615994.1 as queries. Predictions of cell-penetrating potential of the defensin were performed under three scenarios: mature peptide sequence, γ-core, and γ-core plus two upstream and downstream residues, based on antimicrobial activity evidence from a previous work [41].

Effect of Peptides on Yeast Growth

An inoculum from each *Candida* stock was transferred to Petri dishes containing Sabouraud agar and incubated at 30 °C for 24 h. After this period, the yeast cells were transferred to a liquid culture medium Sabouraud dextrose broth (10 mL). Yeast cells were count in a Neubauer chamber (Optik Labor) for further calculation of appropriate dilutions. A yeast growth inhibition quantitative assay was performed following the method described by Broekaert et al. [42], with some modifications. Yeast cells (1×10^4 cells mL^{-1}) were treated with peptides at increasing concentrations from 1.56 to 200 µg mL^{-1} diluted in 100 µL of Sabouraud dextrose broth. The assay was performed on cell culture microplates (96 wells; Nunc) at 30 °C for 48 h. Absorbance measurement at 620 nm (EZ Read 400, Biochrom) were taken at zero hour and at every 8 h for the following 48 h. Yeast growth controls without addition of peptides were also determined. Assays were performed in triplicate.

Yeast Cell Viability Assay

To evaluate whether the inhibition of yeast growth was caused by the fungistatic or fungicidal effect of the peptides, the control cells (without peptide treatment) and treated cells (with 200 μg mL^{-1} peptide treatment for 24 h) were washed and diluted 1000-fold. An aliquot of yeast cells were quantified in a Neubauer chamber and an aliquot with 10^2 cells mL^{-1} was spread with a Drigalski spatula on the surface of a Petri dish containing Sabouraud agar and grown at 30 °C for 48 h. After this period, the colony-forming units were quantified and Petri dishes were photographed. The assays were performed in triplicate, and the results are presented assuming that the control represents 100% cell viability.

Cell Death Monitoring Assays and Plasma Membrane Permeabilization

Cell death monitoring was done by propidium iodide (PI) staining and the plasma membrane permeabilization of cells was measured by Sytox green uptake, according to the methodology described by Deere et al. [43] and by Thevissen et al. [44], respectively. Yeasts cells were incubated with peptides (200 μg mL^{-1}) for 24 h. After this time, a 100 μL aliquot of each yeast cell suspension was incubated with 0.5 μg mL^{-1} of PI and 0.2 μM of Sytox green in 1.5 mL microcentrifuge tubes for 10 min at 25 °C. Cells were analyzed by DIC optical microscope (Axiovison 4, Zeiss) equipped with a fluorescent filter set for detection of the PI (excitation wavelength, 561 nm, emission wavelength 630 nm) and for detection of fluorescein (excitation wavelength, 450–490 nm, emission wavelength 500 nm). Negative controls (untreated yeast cells) were also done to determine the baseline level of membrane permeability and cell death. Cell counts were performed using the ImageJ software tool [45]. The results represent triplicate experiments.

Increase of Intracellular ROS Detection

To determine whether the mechanism of action of peptides involves cell oxidative stress, the fluorescent probe 2,7-dichlorofluorescein diacetate (H_2DCFDA) was used to measure intracellular reactive oxygen species (ROS) according to the protocol described by Mello et al. [46]. Yeast cells were incubated with peptides (200 μg mL^{-1}) for 24 h and after this time, 100 μL of yeast cell suspension was incubated with 50 μM of H_2DCFDA in microcentrifuge tubes of 1.5 mL for 30 min at 25 °C. Yeast cells were visualized by DIC optical microscope (Axiovison 4, Zeiss) equipped with a fluorescent filter set for detection of the fluorescein (excitation wavelength 450–490 nm, emission wavelength 500 nm). Cell counts were performed using the ImageJ software tool [45]. The experiments were performed in triplicate.

Metacaspase Activity Detection

Metacaspase activity detection was performed using the CaspACE FITC-VAD-FMK marker (Promega) as described by the manufacturer. Yeast cells were incubated with peptides (200 μg mL^{-1}) for 24 h and after this incubation, an aliquot of 100 μL of yeast cell suspension was incubated for 20 min at 30 °C with 0.5 μL of probe solution (supplied by the kit) containing 50 μM of the FITC-VAD-FMK marker. Cells were analyzed by DIC using an optical microscope equipped with a fluorescence filter to detect fluorescein (excitation wavelength 450–490 nm, emission wavelength 500 nm). Cell counts were performed using the ImageJ software tool [45]. The experiments were performed in triplicate.

Enzymes Inhibition Assays

The enzymatic activity assay for *Tenebrio molitor* α-amylase and human salivary α-amylase (EC 3.2.1.1) was performed as previously described by Bernfeld [47]. Activity assay for porcine pancreatic α-amylase was done with the same method, but using PBS buffer. Peptides at increasing concentrations were incubated with 10 U of α-amylase, and each enzyme mixture was equilibrated in a water bath for 15 min at 37 °C. After that, 25 μL of 1% starch solution (Sigma-Aldrich Co.) were added, and the samples were incubated in a water bath at 37 °C for 30 min. The samples were removed from the water bath and mixed with 400 μL of 3.5 dinitrosalicylic acid (DNS). Each sample was boiled for 5 min and, after cooling, mixed with 400 μL of water. Substrate hydrolysis was determined by absorbance reading at 540 nm (Chameleon V, Hidex). EDTA (5 mM) was used as a negative control. All inhibition assays were performed in triplicate and the results were shown in activity relative to the positive control (100% activity).

Trypsin and chymotrypsin inhibitory activities were quantified by measuring the hydrolytic activity of porcine trypsin and bovine chymotrypsin toward the substrates BapNa (*N*-benzoyl-Dl-arginyl-*p*-nitroanilide) and BatNa (*N*-benzoyl-L-tirosil-*p*-nitroanilide) respectively. Inhibitory activity was determined by incubating peptides at increasing concentrations from 25 to 300 μg mL^{-1} with enzymes and their respective substrates in 50 mM Tris–HCl buffer (pH 8.0) at 37 °C in a final volume of 200 μL. A control sample without peptides was included in each assay. The reaction was stopped with 100 μL of 30% (v/v) acetic acid. Substrate hydrolysis was quantified by measuring the absorbance of *p*-nitroaniline at 405 nm [48]. All inhibition assays were

performed in triplicate and the results were shown in activity relative to the positive control (100% activity).

Papain inhibitory activity was performed according to Michaud et al. [49] with modifications, using azocasein as substrate. Azocasein 1% was prepared as solution in citrate–phosphate buffer (100 mM sodium citrate pH 5.6; 100 mM sodium phosphate, 0.1% triton X-100, and 1.5 mM DTT). *Ca*CPin-II at increasing concentrations from 30 to 240 μg mL^{-1} were incubated with papain (Fluka) 10 μg mL^{-1} and azocasein solution to a final volume of 120 μL. Samples were incubated at 37 °C for 1 h. The reaction was stopped with 300 μL of 10% trichloroacetic acid (TCA). Samples were centrifuged at 2000 × *g* for 5 min. To the supernatant were added 300 μL of NaOH 1 M. The absorbances were measured at 440 nm. All inhibition assays were performed in triplicate and the results were shown in activity relative to the positive control (100% activity).

Hemolytic Activity

Peptides hemolytic activity was measured using defibrinated sheep red blood cells (sRBC) according to the methodology described by Oren and Shai [50] with modifications. Fresh defibrinated sRBC with EDTA were washed with saline solution (0.15 M NaCl) by centrifugation for 10 min at 2400 × *g* and resuspended in saline solution (final erythrocyte concentration, 1% v/v). The peptides dilutions were prepared in microtubes containing saline solution. Then, peptides at concentrations of 400, 200, and 100 μg mL^{-1} (50 μL) were incubated with sRBC suspension (50 μL) at 37 °C for 1 h. After this incubation time, the samples were centrifuged at 2400 × *g* for 10 min, and the supernatant was transferred to a well in a 96-well microplate. Free hemoglobin content was measured by absorbance at 405 nm. Positive hemolysis control (C^+) was performed using a solution containing 1% Triton X-100 and a negative hemolysis control (C^-) was performed using erythrocytes in saline. Hemolytic activity percentual was calculated assuming that the positive control represents 100% hemolysis according to the formula: % of hemolytic activity = $100 \times (\text{peptide}_{ABS405} - C^-_{ABS405})/(C^+_{ABS405} - C^-_{ABS405})$. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of peptide (μg mL^{-1}) that caused 50% hemolysis and was estimated by nonlinear regression of a log (inhibitor) vs. normalized response equation to the experimental data. The results shown are average values obtained in three experiments, performed in triplicate. Dose–response curves were constructed.

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism software (version 8.0 for Windows) and one-way analysis of variance (ANOVA); $p < 0.05$ was considered statistically significant. The half maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression of a log (inhibitor) vs. normalized response equation to the experimental data.

Results

Purification and Characterization of Peptides from *C. annuum* Leaves

Extracts from *C. annuum* leaves were separated into three fractions using reversed-phase chromatography: peak 0 (P_0), peak 1 (P_1), and peak 2 (P_2) (Fig. 1). On gel electrophoresis, the three fractions presented a single band each with a molecular mass between 3.5 and 6.5 kDa (Fig. 1 insert).

The 35 amino acid residues of the band obtained from fraction P_0 (Fig. 2A), termed *Ca*CPin-II (RLCTNCCAGRKGCNYYSADGTFICEGESDPNNPKA), were compared with a specific Pin-II type protease inhibitor database and revealed 100% similarity with the primary structure of some inhibitory repeated domains (IRDs), such as IRD 100, IRD 118, IRD 84, and IRD 85, all of them from *C. annuum*. The 29 amino acid residues of the band obtained from fraction P_1 (Fig. 2B), termed *Ca*CDef-like (VPTTPFLCTNDPQCK—-VNYEDGHCFDILSK), were compared with the protein database and revealed 85, 85, 85, and 82% similarity with the primary structure of plant AMPs stress-induced peptide from *C. annuum* (ID: AHI85724.1), flower specific defensin-like peptide from *C. annuum* (ID: XP_016579689.1), defensin-like hypothetic protein from *C. annuum* (ID: KAF3614338.1), and defensin-like hypothetic protein from *C. chinense* (ID: PHT96586.1), respectively. The sequence of 33 amino acid residues of the band from fraction P_2 (Fig. 2C) were named *Ca*CLTP2 (GQQSCLCGYM—KQYVNSPNARKVVGQCGVSVPNC), and revealed 87, 82, 82, and 55% similarity with the primary structure of plant AMPs belonging to the non-specific lipid transfer proteins type 2 class (nsLTP2) peptides from *C. annuum* (ID: KAF3615994.1), *C. annuum* (ID: KAF3615995.1), *C. baccatum* (ID: PHT56658.1), and *C. chinense* (ID: PHU06864.1), respectively. Homology modeling of *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 protein tertiary structures is shown in Fig. 3A–C. Homology modeling of *Ca*CPin-II structure showed that it was composed only of a β-sheet with four antiparallel strands. *Ca*CDef-like protein had a compact globular structure consisting of an α-helix and a β-sheet composed of three antiparallel strands, in a βαββ configuration. The spatial structure of *Ca*CLTP2 included three α-helices and a region containing single helical coils. The first and second helices were arranged parallel to each other, and the third helix was formed at an angle of 90° with respect to the second helix. Red regions represent signal peptides. Circular

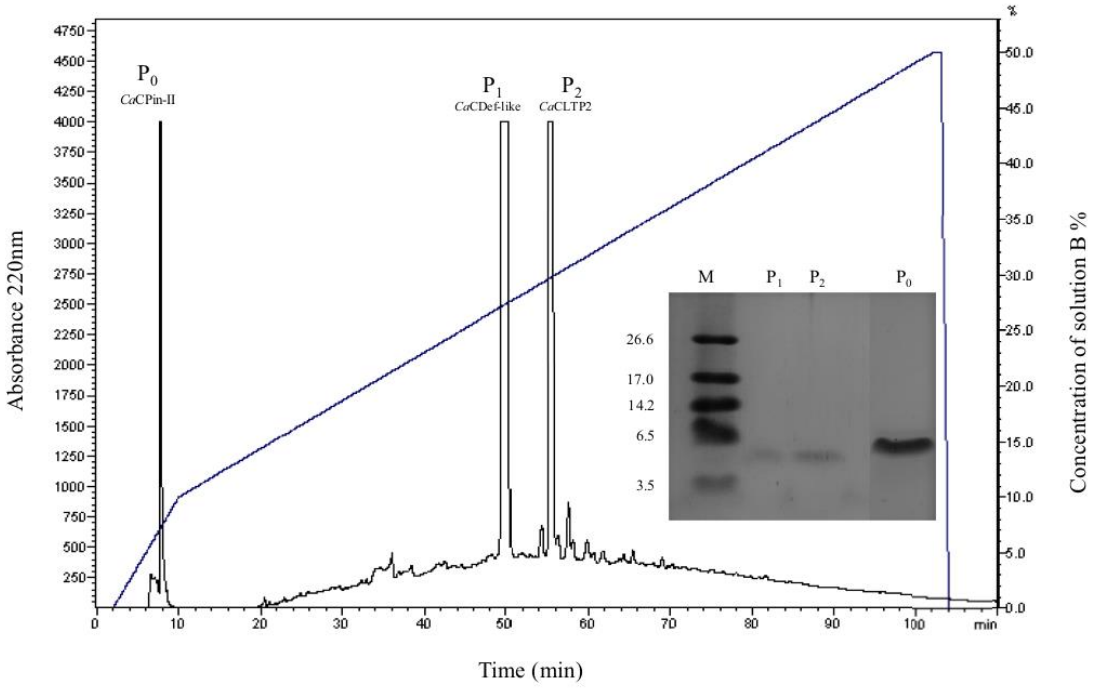


Fig. 1 Fractionation of *C. annuun* extract. Chromatogram of the extract of *C. annuum* leaves in reversed-phase C_{18} column. The column was equilibrated and eluted with 0.1% trifluoroacetic acid (TFA) (solution A) and eluted using a gradient of 90% acetonitrile in 0.1% (solution B). The flow used was 0.5 mL min^{-1}. (Insert) Fractionation in a SDS-tricine-gel of peptides enriched fractions obtained by the fractionation of the extract of *C. annuum* leaves (cv. Carioquinha) by reversed-phase C_{18} column in HPLC. **P_0** Peak 0 (*Ca*CPin-II). **P_1** Peak 1 (*Ca*CDef-like). **P_2** Peak 2 (*Ca*CLTP2). **M** Molecular mass (kDa) markers: 26.6, 17.0, 14.2, 6.5 and 3.2 kDa

dichroism spectroscopy of the *Ca*CDef-like and *Ca*CLTP2 peptides (Fig. 3E) revealed a random coil structure compatible with the structure predicted by homology modeling (Fig. 3B, C). However, circular dichroism spectroscopy of the *Ca*CPin-II peptide revealed a secondary structure pattern different from that of the β-sheet-rich structure predicted by homology modeling (Fig. 3A). Therefore, we performed structural modeling of one of the 35 amino acid residue fragments identified in *Ca*CPin-II (Fig. 3D). This fragment was modeled with 99.9% confidence using the single highest-scoring template. In the structure predicted for this fragment, a disordered conformation predominated, also containing a β-sheet and an α-helix, which is consistent with the circular dichroism results.

Antifungal Activity of the Peptides Against *Candida* Species

To analyze the effect of peptides on the growth of yeasts *C. albicans*, *C. buinensis*, *C. tropicalis*, and *C. parapsilosis*, we used *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 at increasing concentrations from 1.56 to 200 μg mL^{-1} (Fig. 4). *Ca*CPin-II inhibited *C. albicans* growth at all concentrations, reaching 39% inhibition at the concentration of 200 μg mL^{-1}. However, no significant difference was observed between 25 and 200 μg mL^{-1} (i.e., inhibition was not dose-dependent). *C. buinensis* growth showed 37 and 70% inhibition at concentrations of 100 and 200 μg mL^{-1}, respectively, and the IC_{50} value for *C. buinensis* was 54.2 μg mL^{-1}. *Ca*CPin-II had no significant effect on the growth of *C. tropicalis* or *C. parapsilosis* at any of the concentrations tested. *Ca*CDef-like peptide also inhibited the growth of *C. albicans* at all tested concentrations, with no dose-dependent effect, reaching 44% inhibition at 50 μg mL^{-1}, although there was no significant difference between 200 and 3.12 μg mL^{-1}. The growth of *C. buinensis* and *C. tropicalis* was not significantly affected by *Ca*CDef-like at the concentrations tested, while that of *C. parapsilosis* was inhibited by only 10% at 50 μg mL^{-1}. *Ca*CLTP2 peptide also inhibited the growth of *C. albicans* at all tested concentrations with no dose-dependent effect, reaching 45% inhibition at 200 μg mL^{-1}, and there was

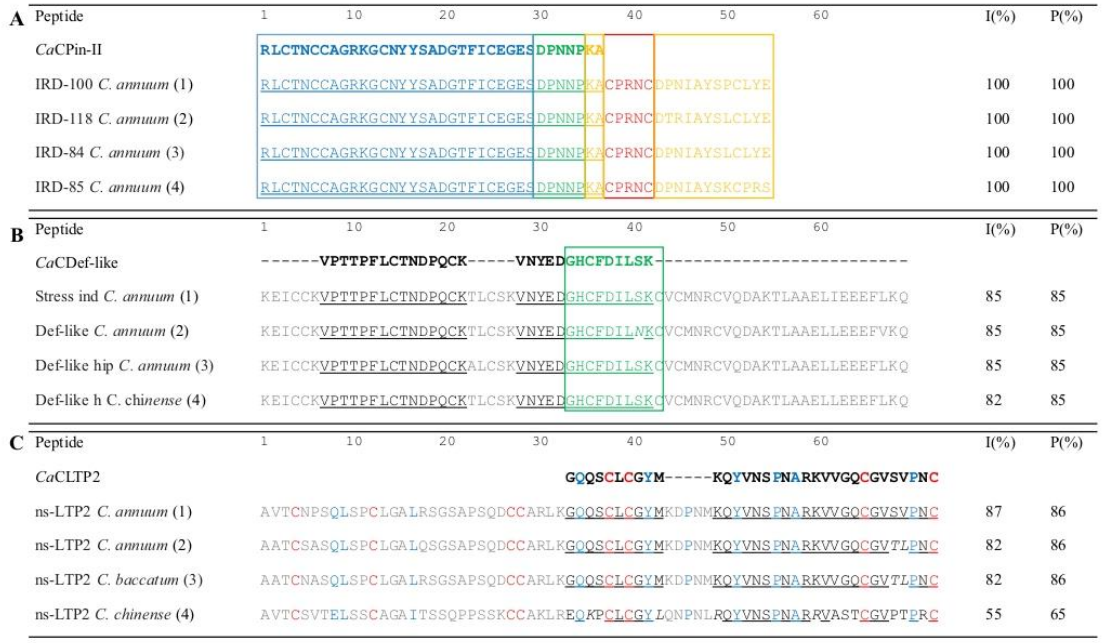


Fig. 2 **A** Alignment of the 35 amino acid residues obtained from the peptide of the P_0 fraction with the following peptide sequences similar to the inhibitory repeated domains (IRDs) from Pin-II type protease inhibitor. Heavy chain amino acids residues are color-coded in blue, linker region (DPNNP) is color-coded in green, light chain amino acids residues are color-coded in yellow with the reactive center loop (RCL) highlighted in red. **B** Alignment of the 29 amino acid residues obtained from the peptide of the P_1 fraction with the following peptide sequences similar to the defensins-like. The green highlighted region represents the γ-core motif (GXCX3-9C). *C. annuum* stress induced protein (1) (ID: AHI85724.1). *C. annuum* flower specific defensin-like, predicted protein (2) (ID: XP_016579689.1). *C. annuum* defensin-like, hypothetic protein FXO38_35740 (3) (ID: KAF3614338.1). *C. chinense* defensin-like, hypothetic protein BC332_34488 (4) (ID: PHT96586.1). **C** Alignment of the 33 amino acid residues obtained from the peptide of the P_2 fraction with the following peptide sequences similar to the ns-LTP2. The cysteines highlighted in red represent the 8-cysteine motif found in LTP2. Residues highlighted in blue are those highly conserved in LTP2. *C. annuum* ns-LTP2 (1) (ID: KAF3615994.1). *C. annuum* ns-LTP2 (2) (ID: KAF3615995.1). *C. baccatum* ns-LTP2 (3) (ID: PHT56658.1). *C. chinense* ns-LTP2 (4) (ID: PHU06864.1). I% indicates the percentage of identical residues and amino acids highlighted in underline (including Cys residues). P% indicates the percentage of positive amino acid residues (with the same physicochemical characteristics) highlighted by italicized. I% and P% were made based on the amino acids of the tryptic fragments obtained in mass spectrometry. Spaces (-) have been introduced for better alignment. The numbers above the sequence indicate the size of the peptides in amino acid residues. The sequences shown were obtained from Blast-p (b/c) and from Pin-II type PIs Information Resources (PINIR) (a), and aligned using Clustal Omega

no significant difference in inhibition between 200 and 3.12 μg mL^{-1}. *Ca*CLTP2 at the concentrations tested did not significantly affect *C. tropicalis* and *C. parapsilosis* growth and inhibited only 18% of the *C. buinensis* growth.

The viability assay (Fig. 5) demonstrated that the species most susceptible to *Ca*CPin-II were *C. albicans* and *C. buinensis*, with viability losses of 38.3 and 96.6%, respectively. *C. albicans*, *C. buinensi*, and *C. parapsilosis* were susceptible to *Ca*CDef-like, with approximately 37% viability loss, and the species most susceptible to *Ca*CLTP2 were *C. albicans* and *C. buinensis*, with viability losses of 49.6 and 66.2%, respectively. These data suggest that the inhibitory effect of the peptides is fungistatic but *Ca*CPin-II (200 μg mL^{-1}) is lethal to 96.6% of *C. buinensis* yeasts.

Toxic Effects of Peptides Against Yeasts

The antifungal effects of the peptides were characterized with respect to their mechanism of action. For this purpose, fluorescent probes were used (Table 1). Because the effects of the peptides on the different *Candida* species were similar, microscopic photos of *C. buinensis* are presented in Fig. 6, and those of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* are presented in Supplementary Information.

C. albicans, *C. buinensis*, *C. tropicalis*, and *C. parapsilosis* were used to test membrane permeabilization using a SYTOX Green probe after 24 h in the presence of the peptides (200 μg mL^{-1}). *Ca*CPin-II and *Ca*CDef-like proteins induced membrane permeabilization. *Ca*CLTP2 was not significantly

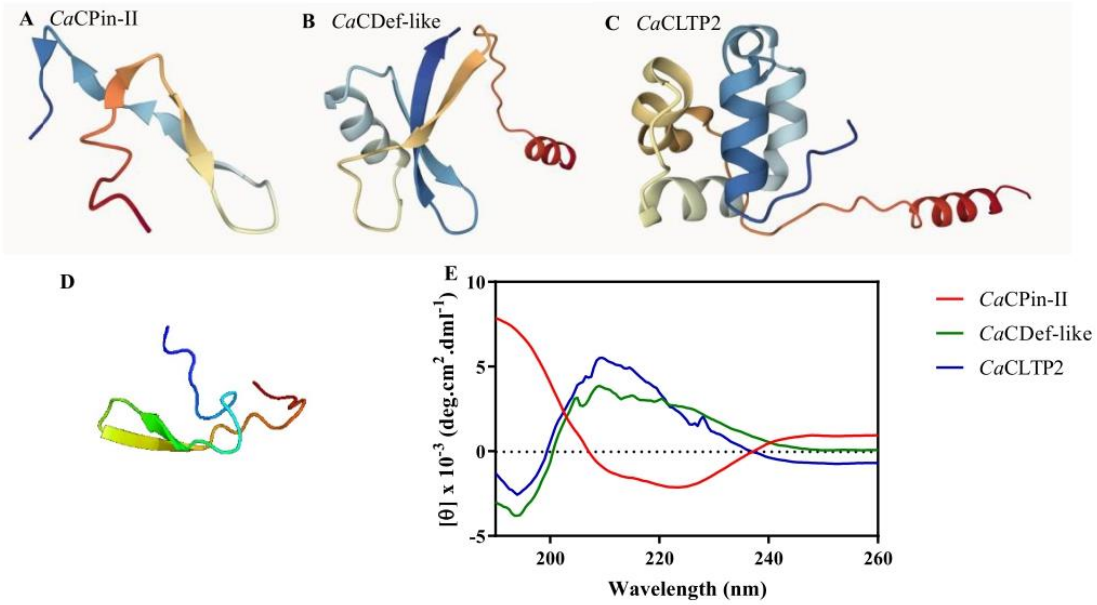


Fig. 3 Tertiary and secondary structures of peptides. Homology modeling of protein tertiary structures of *Ca*CPin-II (**A**), *Ca*CDef-like (**B**), and *Ca*CLTP2 (**C**) from *C. annuum* leaves. These sequences were searched against the AlphaFold DB (Jumper et al. 35) using the EBI Protein Similarity Search tool (https://www.ebi.ac.uk/Tools/sss/fasta/). The AlphaFold model of sequence P56615 (Pin-II) and of the best orthologs (Q9FFP8 for defensin and A0A0R0FHG0 for nsLTP2) were used as templates for homology modeling with Phyre2 (Kelley et al. 36) using one-to-one threading mode. **D** Modeling of one of the 35 amino acid residue fragments of *Ca*CPin-II. **E** Secondary structure of *Ca*CPin-II (red line), *Ca*CDef-like (green line), and *Ca*CLTP2 (blue line). Circular dichroism (CD) spectra of 200–400 μg mL^{-1} peptide solutions prepared in ultra-pure water

effective in permeabilizing the membranes of the tested yeasts. This assay showed that *Ca*CPin-II and *Ca*CDef-like peptides could compromise the structure of the plasma membrane, as shown in Fig. 6A for *C. buinensis*, possibly causing the membrane to be permeable enough for the probe to enter the cells; however, this effect was not observed in *C. albicans* and *C. parapsilosis* cells (Supplementary Information).

We also observed using DIC microscopy that in the presence of peptides, yeast growth displayed morphological changes that were not observed in the controls, such as cell agglomeration, increased cell volume, vacuole formation, difficulty in bud liberation, and the emergence of pseudohyphae.

The effect of peptides on the induction of ROS production in different species of *Candida* resulted in labeling with the probes of *C. buinensis* grown in the presence of *Ca*CPin-II (10.6% of cells) (Fig. 6B) and *C. tropicalis* cells grown in the presence of *Ca*CPin-II and *Ca*CDef-like (30.0 and 44.6% of cells respectively) (Table 1). Positive labeling was also observed for *C. parapsilosis*, but it was less intense. These results indicated increased ROS production in these yeasts. The same effect was not observed in *C. albicans* treated with the same peptides. *Ca*CLTP2 induced less intense ROS production in *C. parapsilosis* and *C. albicans* than the other tested peptides.

The rate of yeast cell death was determined using a propidium iodide assay with 200 μg mL^{-1} peptides. The cells labeled with the SYTOX probe was the same labeled with propidium iodide, as shown for *C. buinensis* in Fig. 6. *Ca*CPin-II caused 20 and 25% cell death in *C. buinensis* and *C. tropicalis* respectively, whereas *Ca*CDef-like and *Ca*CLTP2 only caused 20% cell death in *C. tropicalis* (Table 1). To determine if apoptosis-like programmed cell death (PCD) was the cause of cell death, we performed metacaspase activity detection (Fig. 6C). *Ca*CPin-II induced metacaspase activity in approximately 9 and 16% of *C. buinensis* and *C. tropicalis* cells, respectively, while *Ca*CDef-like and *Ca*CLTP2 respectively induced metacaspase activity in 19 and 12% of *C. tropicalis* cells (Table 1). Collectively, these results suggest that apoptosis-like PCD might be involved in cell death in these cases.

Effects of Peptides on α-Amylase and Protease Activities

To elucidate the toxic effects of the peptides against yeasts, we investigated their capacity to inhibit hydrolases. *Ca*CPin-II inhibited *T. molitor* α-amylase (Fig. 7A), human salivary

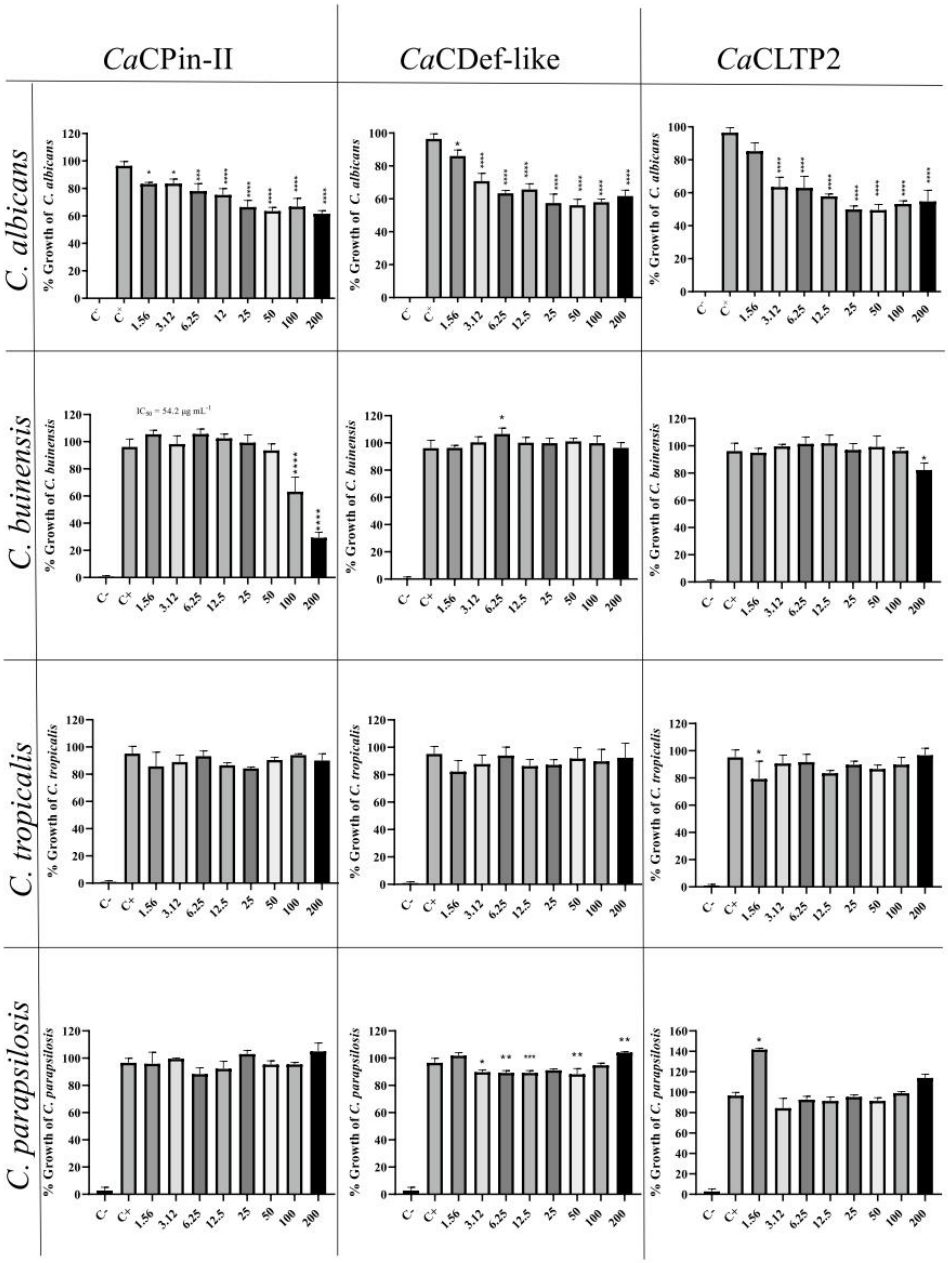
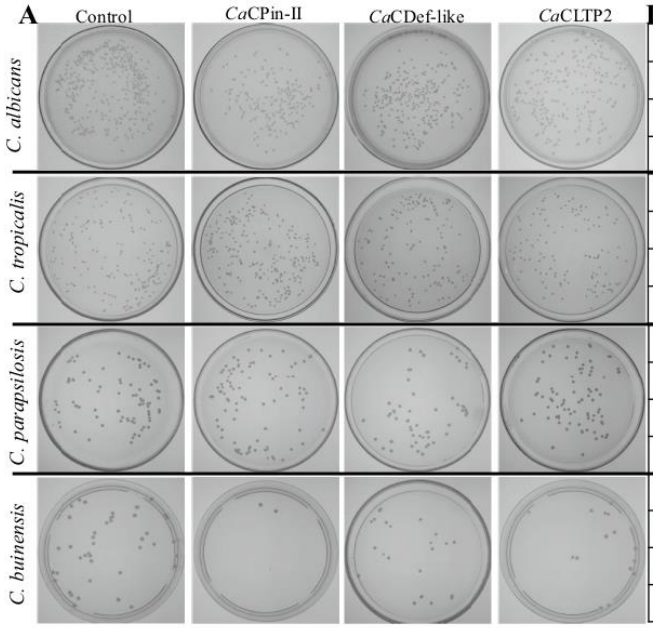


Fig. 4 Effect of peptides on yeast growth. Yeasts suspensions (1×10^4 cell mL^{-1}) of *Candida* sp. were treated or untreated with *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 from *C. annuum* leaves (200, 100, 50, 25, 12.5, 6.26, 3.12, or 1.56 µg mL^{-1}) for 24 h. Yeasts growth in the resulted cultures was quantified by optical density (620 nm). Data are presented as a percentage of yeasts growth of treated culture compared to the growth of corresponding untreated culture (100%). Negative control (C^-)- Sabouraud Broth; positive Control (C^+) untreated yeasts suspensions. The results presented are mean values obtained over three experiments, each done in triplicate. Dose response curves were constructed and $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared to positive control (0 µg mL^{-1}) determined by Tukey test



B

Peptide	CFU	Viability loss (%)
Control	364.5 ± 10.6	0
*Ca*CPin-II	229.5 ±23.3	38.3**
*Ca*CDef-like	237.0 ±24.0	36.3*
*Ca*CLTP2	187.5 ±19.1	49.6**
Control	157.7 ±15.4	0
*Ca*CPin-II	193.0 ±18.5	0
*Ca*CDef-like	140.3 ±16.4	11.0
*Ca*CLTP2	115.0 ±24.9	27.1
Control	88.0 ±3.5	0
*Ca*CPin-II	81.3 ±15.0	7.6
*Ca*CDef-like	55.7 ±7.0	36.7*
*Ca*CLTP2	72.7 ±19.7	17.42
Control	44.3 ±7.5	0
*Ca*CPin-II	1.5 ±0.7	96.6***
*Ca*CDef-like	28.0 ±6.6	36.8*
*Ca*CLTP2	15.0 ±3.0	66.2***

Fig. 5 Cell viability. **A** Photographs of the Petri dishes for analysis of yeasts cells viability after the treatment with *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 (200 µg mL^{-1}) for 24 h. **B** Table presenting the percentage of viability loss of yeast cells after the treatment with *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 (200 µg mL^{-1}) for 24 h. Colony-forming unit (CFU). The experiments were carried out in triplicate and $*p<0.05$; $**p<0.01$; $***p<0.001$ compared to control (untreated) determined by *t* test

α-amylase (Fig. 7B), and porcine pancreatic α-amylase (Fig. 7C), with an IC_{50} of 19.4 µg mL^{-1}, 4.9 µg mL^{-1}, and 1.8 µg mL^{-1}, respectively. *Ca*CDef-like and *Ca*CLTP2, at the same concentrations, were unable to inhibit α-amylases (data not shown). We also tested the ability of these peptides to inhibit protease activity. None of the peptides inhibited the serine proteases trypsin and chymotrypsin at concentrations up to 300 µg mL^{-1} (data not shown). Because *Ca*CPin-II shares identities with protease inhibitor peptides, we tested its ability to inhibit cysteine proteases. This peptide inhibited papain (Fig. 7D) with an IC_{50} of 84.36 µg mL^{-1}. These data suggest that the ability to inhibit α-amylase and cysteine proteases may be related to the toxicity of CaCPin-II against yeasts.

Hemolytic Activity

To evaluate the possibility that the inhibitory effects of the peptides against *Candida* species were associated with toxicity in mammalian cells, we monitored the hemolytic activity against defibrinated sheep red blood cells. *Ca*CPin-II showed hemolytic activity at a concentration of 200 µg mL^{-1}, causing 28% hemolysis ($p<0.0001$ compared to the positive control). The *Ca*CPin-II CC_{50} was calculated as 270 µg mL^{-1} (Fig. 8A). *Ca*CDef-like had no hemolytic effect at concentrations up to 400 µg mL^{-1} ($p<0.0001$ compared with the positive control) (Fig. 8B), and *Ca*CLTP2 showed a weak hemolytic effect at a concentration of 200 µg mL^{-1}, causing 1.7% hemolysis and presenting a $CC_{50}>400$ µg mL^{-1} ($p<0.0001$ compared with the positive control) (Fig. 8C).

Discussion

Over the past 20 years, many AMPs have been purified and characterized from numerous plants [51, 52], including hot pepper (*C. annuum*), a species with well-described medicinal importance [18]. Some peptides purified from hot peppers show antifungal activity, including a thionin-like peptide with activity against *C. albicans* and *C. tropicalis* [25], defensins with activity against *Candida* species [19], LTP-1 with activity against *C. tropicalis* [53], and a hevein-like peptide with activity against *Fusarium oxysporum* and *Colletotrichum gloesporioides* [54]. However, the mechanism of action of plant antimicrobial peptides against pathogens requires further investigation. We isolated and characterized three peptides from *C. annuum* leaves that caused a considerable increase in the incidence of *Candida* spp.

Table 1 Cell density and fluorescent cell percentage of *Candida* sp. treated with *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 peptides from *Capsicum annuum* leaves

Yeast	Treatment	Cell count in DIC[a]	Cell count in flourescence (PI)[a] and % of fluorescense cells[b]		Cell count in fluorescence (Sytox)[a] and % of fluorescence cells[b]		Cell count in DIC[a]	Cell count in fluorescence (ROS)[a] and % of fluorescence cells[b]		Cell count in DIC[a]	Cell count in fluorescence (caspase)[a] and % of fluorescence cells[b]	
		a	a	b	a	b	a	a	b	a	a	b
C. albicans	Control	426.0 ± 31.3	1.8 ± 1.5	0.4	1.5 ± 1.3	0.4	137.4 ± 26.8	16.6 ± 11.8	12.1	73.4 ± 15.0	0.0 ± 0.0	0.0
	*Ca*CPin-II	475.3 ± 51.4	0.7 ± 0.6	0.1	1.3 ± 1.5	0.3	92.7 ± 46.6	3.0 ± 2.1	3.2	169.8 ± 10.1	0.0 ± 0.0	0.0
	*Ca*CDef-like	397.6 ± 53.9	2.8 ± 1.9	0.7	3.2 ± 1.3	0.8	162.6 ± 17.0	5.0 ± 2.1	3.1	100.4 ± 11.4	0.0 ± 0.0	0.0
	*Ca*CLTP2	483.0 ± 46.7	6.3 ± 1.5	1.3	6.5 ± 3.0	1.3	131.4 ± 12.7	8.0 ± 1.9	6.1	82.8 ± 15.3	0.0 ± 0.0	0.0
C. buinensis	Control	643.0 ± 116.3	11.0 ± 7.1	1.7	11.8 ± 4.8	1.83	261.0 ± 27.8	5.6 ± 2.9	1.6	117.4 ± 37.6	0.0 ± 0.0	0.0
	*Ca*CPin-II	35.0 ± 12.7	6.8 ± 4.1	19.5	6.8 ± 4.6	19.5	83.0 ± 30.9	8.7 ± 4.8	10.5	61.2 ± 12.8	5.4 ± 1.1	8.8
	*Ca*CDef-like	265.6 ± 5.8	4.4 ± 2.5	1.7	6.0 ± 2.5	2.3	120.8 ± 8.3	2.5 ± 1.7	2.1	101.0 ± 5.0	0.6 ± 0.9	0.6
	*Ca*CLTP2	203.8 ± 32.5	0.6 ± 0.9	0.3	0.6 ± 0.9	0.3	155.8 ± 23.2	3.2 ± 1.6	2.1	103.4 ± 7.8	0.0 ± 0.0	0.0
C. tropicalis	Control	72.2 ± 8.5	5.4 ± 7.8	7.5	8.0 ± 3.1	11.1	113.8 ± 26.6	14.0 ± 8.12	12.3	434.0 ± 82.6	3.6 ± 1.7	0.8
	*Ca*CPin-II	83.0 ± 8.2	21.0 ± 2.5	25.3	15.4 ± 3.2	18.6	127.6 ± 13.5	38.2 ± 9.6	30.0	446.6 ± 105.9	70.6 ± 29.8	15.8
	*Ca*CDef-like	113.7 ± 24.7	22.6 ± 8.3	19.9	21.7 ± 8.2	19.1	98.6 ± 24.1	44.0 ± 21.1	44.6	345.6 ± 50.1	65.4 ± 25.9	18.9
	*Ca*CLTP2	69.4 ± 8.0	14.2 ± 4.4	20.5	12.4 ± 5.0	17.9	74.6 ± 8.0	5.6 ± 3.6	7.5	477.2 ± 86.6	59.4 ± 10.0	12.4
C. parapsilosis	Control	170.8 ± 44.1	17.4 ± 2.7	10.2	17.6 ± 5.9	10.3	156.0 ± 12.8	1.4 ± 1.9	0.9	152.6 ± 16.8	0.6 ± 0.9	0.4
	*Ca*CPin-II	87.7 ± 8.5	5.5 ± 2.8	6.3	11.3 ± 3.3	12.9	109.6 ± 8.5	4.6 ± 2.9	4.2	89.2 ± 19.9	0.4 ± 0.5	0.4
	*Ca*CDef-like	95.2 ± 15.2	9.6 ± 2.9	10.1	12.4 ± 3.4	13.0	190.3 ± 28.8	15.8 ± 5.9	8.3	311.4 ± 43.5	1.0 ± 1.4	0.3
	*Ca*CLTP2	92.6 ± 18.4	6.8 ± 2.4	7.3	11.2 ± 4.3	12.1	145.5 ± 15.2	5.3 ± 2.9	3.7	210.2 ± 30.9	1.4 ± 1.9	0.7

[a]Cells number determination in five random fields of the DIC and fluorescence views of the samples obtained from microscopy assay

[b]The total cell number in DIC of each yeast (in control and test) was assumed as 100%

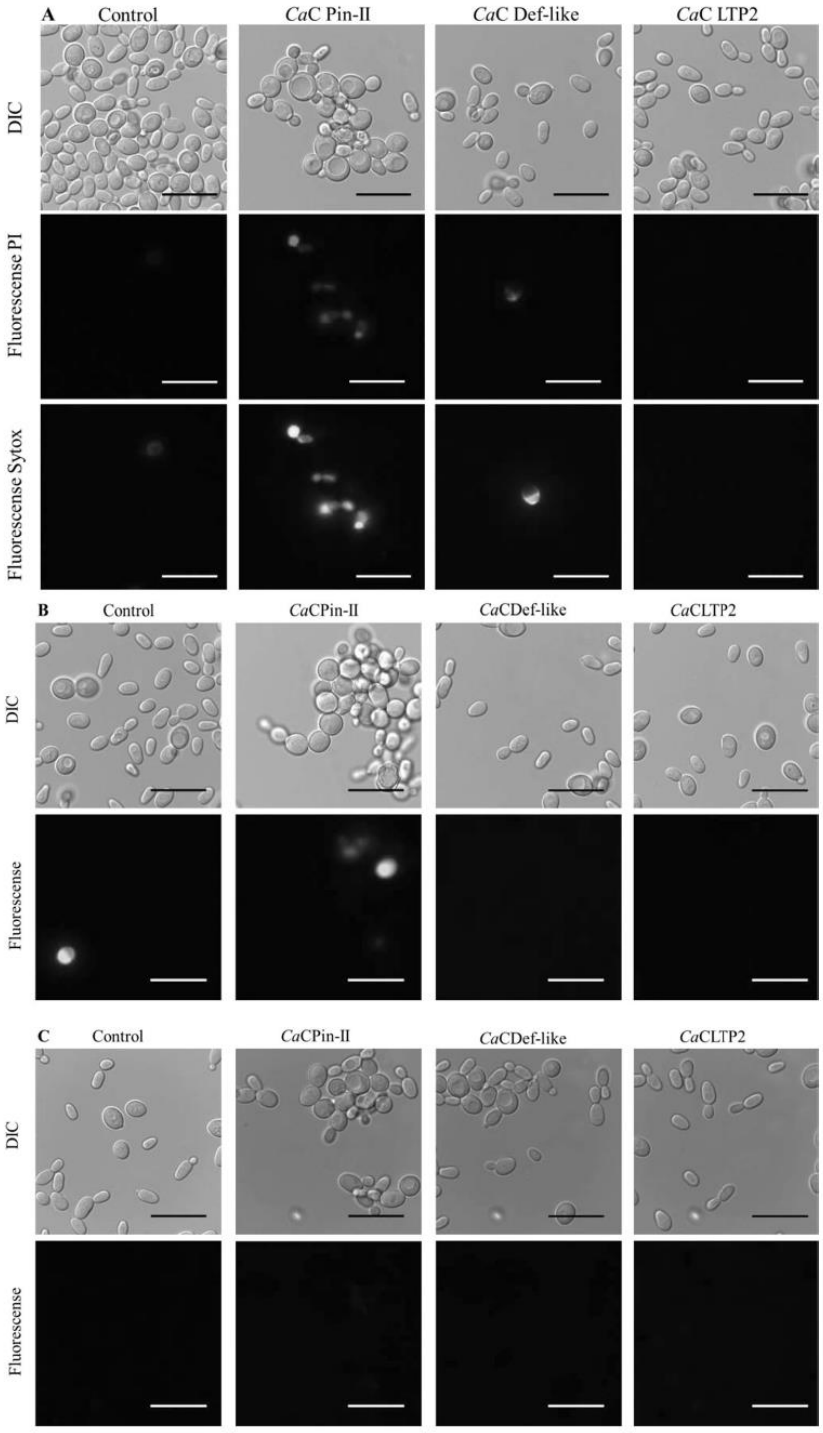


Fig. 6 Effect of the peptides in *C. buinensis*. Optical and fluorescence microscopy images of *C. buinensis* cells after treatment with peptides (200 µg mL^{-1}) for 24 h. **A** Images of cell death monitoring and membrane permeabilization assays. Control cells were treated only with propidium iodide (PI) stain or the SYTOX® Green probe. **B** Images of reactive oxygen species assay detection. Control cells were treated only with the 2′,7′-dichlorofluoresceindiacetate probe. **C** Images of metacaspase activity detection. Control cells and cells treated with peptides were incubated with the FITC-VAD-FMK probe and analyzed by fluorescence microscopy. Green fluorescence indicates positive staining for caspase activity. Bars = 20 µm. *DIC* differential interference contrast

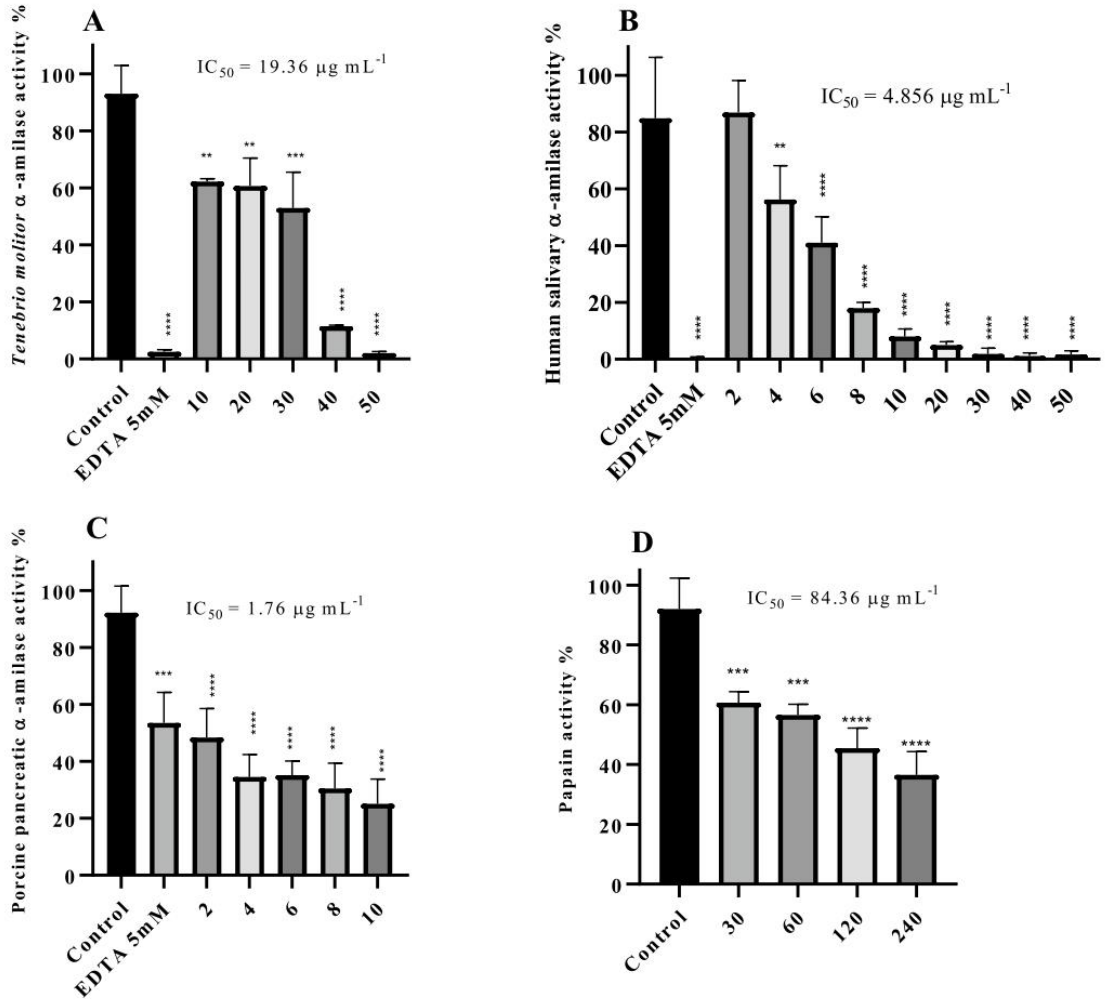


Fig. 7 Effect of *Ca*CPin-II on α-amylase and protease activities. Test with α-amylase from the intestine of *Tenebrio molitor* insects (**A**), from human saliva (**B**), and from pig pancreas (**C**); test with papain (**D**). Numbers below each bar indicate the peptide concentrations (µg mL^{-1}). In the positive control of α-amylase inhibition, 5 mM EDTA was used. Asterisks indicate a significant difference when compared to the control by Tukey test, $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$

infections in humans [55] and investigated their potential for the preparation of new drugs against four *Candida* strains of clinical interest: *C. albicans*, *C. buinensis*, *C. tropicalis*, and *C. parapsilosis*.

The three peptides from the leaf extracts obtained by C18 reversed-phase HPLC were named *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 (*Ca*C = *Capsicum annuum* cv. Carioquinha) because of their amino acid sequence similarity to the inhibitory repeat domains (IRDs) of potato type inhibitor-II protease inhibitors, defensin-like protein, and non-specific lipid transfer protein type 2, respectively (Fig. 2). The spatial conformations determined by homology structure (Fig. 3A–C) were similar to those previously described in the literature [51, 56].

Pin-II-type proteinase inhibitors are serine proteinase inhibitors found mainly in solanaceous plants, with *C. annuum* having the highest number of sequences in the PINIR database [33]. This class of proteinase inhibitors plays a vital role in protecting plants against biotic stress [57], and has been used to develop antifungal agents [58, 59]. A single Pin-II-type proteinase inhibitor comprises multiple IRDs interspersed by 5 to 6 amino acid linker regions [56, 60]. The number of IRDs in a proteinase inhibitor varies, and the

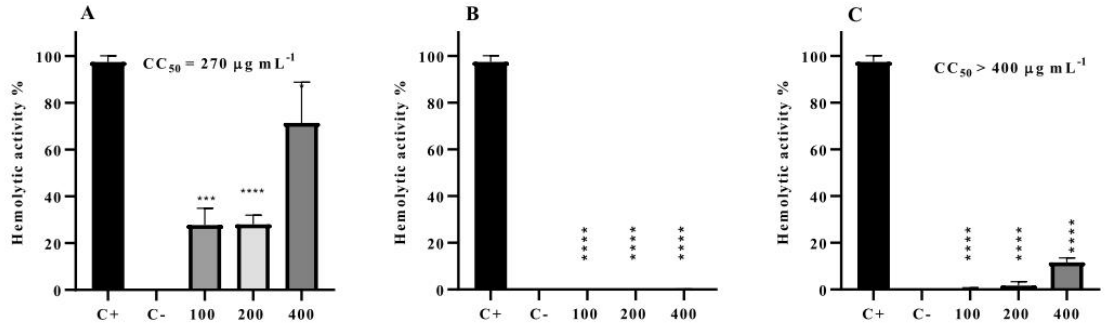


Fig. 8 Hemolytic activity of *Ca*CPin-II (**A**), *Ca*CDef-like (**B**), and *Ca*CLTP2 (**C**) from *C. annuum* leaves (400, 200, and 100 µg mL^{-1}). Test with defibrinated sheep red blood cells (sRBC). A solution containing 1% Triton X-100 was used as a positive hemolysis control (C^+) and erythrocytes in saline were used as a negative control (C^-). Asterisks indicate a significant difference when compared to the control by Tukey test, $^*p<0.05$; $^{***}p<0.001$; and $^{****}p<0.0001$

amino acid sequences of IRDs show subtle differences [61]. IRDs are 50–55 amino acid-long proteins, and proteolytic processing at linker regions releases IRDs from the parent Pin-II type proteinase inhibitor molecule [62, 63]. Each IRD is composed of a heavy and a light chain linked by a linker region. The light chain contains a tripeptide loop called the reactive center loop (RCL), which is the primary interaction site for the target serine protease and functions independently of the native IRD scaffold [33]. Due to this, the alignment of the P_0 amino acid sequence (Fig. 2A) was performed using a specific database for Pin-II type proteinase inhibitor, which contains amino acid sequences of the IRDs, since the BLASTp database contains only the parents Pin-II type proteinase inhibitor amino acid sequences. Secondary structure analysis of the *Ca*CPin-II peptide by circular dichroism spectroscopy was not compatible with the structure determined by homology modeling but was compatible with the modeling of the fragment of 35 amino acid residues (Fig. 3D, E). Therefore, we suggest that the *Ca*CPin-II peptide is smaller than Pin-II-type proteinase inhibitors described in the literature. This 35-residue fragment, despite having 100% similarity to Pin-II-type proteinase inhibitors, does not have all the necessary cysteines to form the four disulfide bridges found in this class of peptides, which explains its structure being different from that predicted. In addition, it lacks a large part of the light chain and the trypsin and chymotrypsin (RCL)-binding site, which explains why *Ca*CPin-II does not inhibit the activity of these enzymes.

Plant defensins play a role in both the response against pathogens and the control of plant growth and development [64], and their antifungal activity has been extensively described [19, 65]. The defensin signatures are a cysteine-stabilized αβ motif (CSαβ) and a γ-core motif GXC(X3–9)C [66], and peptides that contain variations in this typical structure are called defensin-like [67, 68], such as *Ca*CDef-like peptide. Nonspecific LTPs (nsLTPs) are a class of AMPs found in all land plants. LTPs have a tunnel-like hydrophobic cavity that enables them to bind and transport various types of lipids [69, 70]. In 2011, Edstam et al. classified plant LTPs into ten types based on the spacing between Cys residues in the eight-Cys motif (8CM), intron position, and polypeptide sequence identity. LTP2 is one of the most well-studied type [71, 72]. Several studies have demonstrated the toxicity of LTPs against fungal pathogens including *Candida* [22, 73–76]. Alterations in the fungal membrane permeability may be involved in the in vitro toxicity of LTPs [77].

Except for *C. albicans* and *C. buinensis* treated with *Ca*CPin-II, the other *Candida* species tested here did not show major growth alterations when treated with the *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 peptides at concentrations up to 200 µg mL^{-1} (Fig. 4). These peptides caused morphological changes in the treated cells (Fig. 6), which might be related to the optical density values observed in the growth inhibition assays (Fig. 4). It has already been shown that a fraction rich in IRD-containing peptides from *C. chinensis* seeds caused morphological changes in *C. tropicalis*, including cellular agglomeration and formation of pseudohyphae [78]. We performed other assays to evaluate the effects of the peptides on the tested *Candida* species. *Candida albicans* and *C. buinensis* were the species with the greatest reduction in cell viability after treatment with 200 µg mL^{-1} peptides for 24 h, compared to *C. tropicalis* and *C. parapsilosis* (Fig. 5). The morphological changes and reduction in the density of *C. buinensis* cells treated with 200 µg mL^{-1} of *Ca*CPin-II seen in the microscopy images suggest that this peptide has a strong fungistatic effect that led to a 97% reduction in the viability of treated cells.

The membrane permeabilization assay with the SYTOX Green probe and the assay for labeling dead and damaged

cells with propidium iodide dye showed that, in some cases, the peptides permeabilized the yeast membranes, which may be related to the reduction in viability and cell death (Fig. 6A). Yeast membranes can be structurally compromised, leading to permeabilization. Labeling with propidium iodide is not specific for dead cells. This probe has been shown to label the membranes of stressed *S. cerevisiae* cells that have transient permeability, but are still viable [79]. The same cells stained with the SYTOX Green probe were also stained with propidium iodide, indicating that the damage was caused by membrane permeabilization (Fig. 6A). Plant defensins display several possible antifungal mechanisms such as cell membrane disruption and intracellular targets [80–82]. Other studies have shown that plant defensins can permeabilize cell membranes of microorganisms [19, 44, 46, 81]. AMPs may also have targets other than the cell membrane. *Ca*CLTP2 reduced the viability of *C. albicans* and *C. buinensis* (Fig. 5) without affecting membrane permeabilization (Fig. 6). The antimicrobial activity of some plant LTPs is found not to be related to their ability to interact with lipids, and it has been shown that this class of proteins may possess antimicrobial activity without binding lipid molecules [83, 84]. The mechanism of antimicrobial action of representatives of the LTP class remains unclear. Nevertheless, the cell membrane is considered a potential target for LTP antimicrobial action via electrostatic interactions that cause the destabilization and permeabilization of the membrane [85], as shown by the action of *Ca*CLTP2, which permeabilizes *C. tropicalis* membranes (Table 1).

Membrane permeabilization and increased ROS production are among the common modes of action of defensins and other AMPs, which might lead to PCD pathway activation [86, 87] and may be involved in the damage caused by peptides in these cells. Increased oxidative stress appears in the early stages of the apoptotic process [88], and the increased production of ROS molecules in target organisms is a recurrent mode of action employed by plant defensins and several other AMPs [46, 81]. Some *C. tropicalis* cells treated with *Ca*CPin-II and *Ca*CDef-like showed an increase in reactive oxygen species and the activation of metacaspases (Table 1), suggesting that the initial stages of apoptosis were in place.

To better understand the mechanism of action of the identified AMPs, we performed inhibition assays on several important enzymes such as proteases and amylases. Protease inhibitors, including fungal cells, affect gene expression, cell proliferation, and cell death [89], including fungal cells [90]. Several protease inhibitors affect fungal cells, leading to normal nutritional and/or growth functions. The mechanisms of action include membrane pore formation and/or cell wall disruption. These events disrupt ion flow and/or cause the leakage of internal cellular components, thereby affecting cell viability [91, 92]. Although the Pin-II type PIs are serine protease inhibitors, *Ca*CPin-II at concentrations up to 300 μg mL^{-1} and leaves extract at concentrations up to 1.200 μg mL^{-1} did not inhibit the tested serine proteases (data not shown). This fact, together with data from the secondary structure analysis of *Ca*CPin-II, suggests that this peptide lacks the binding site to cause the inhibition of serine proteases. Because *Ca*CPin-II was isolated from a *C. annuum* cultivar by genetic breeding [93], we suggest that the IRD precursor protein in this plant might be undergoing alternative proteolytic processing. However, the *Ca*CPin-II peptide showed cysteine protease activity and strong amylase inhibitory activity (Fig. 7). We suggest that *Ca*CPin-II is capable of inhibiting other serine proteases such as subtilisin, and/or that it is a new bifunctional inhibitor with activity against cysteine proteases and α-amylases. The combination of protease and α-amylase inhibition is potentially useful in plant defense and physiology [94, 95].

According to in silico tests, peptides are incapable of permeabilizing membranes (data not show). However, membrane permeabilization tests revealed positive labeling in some cells. This may be due to two hypotheses. First, the in silico test is not specific for fungal membranes, which have physicochemical characteristics different from those of mammalian cells, such as lipid composition and electrostatic charge. The second hypothesis is that permeabilization is a secondary consequence of oxidative stress. Recent studies have suggested that other intracellular targets of AMPs and the capacity for membrane permeabilization could be secondary events caused by the endogenous increase in ROS production [80–82]. AMPs tend to exhibit selective toxicity because of their biochemical characteristics, and are considered promising sources of new molecules with therapeutic potential [16]. Nevertheless, it is important to evaluate the toxicity of any AMP-derived drug candidate in mammalian cells for approval by regulatory agencies. Hemolytic activity is a test system used in quantitative cytotoxicity assays. We observed that *Ca*CDef-like were not toxic to sheep erythrocytes at all the concentrations tested ($CC_{50} > 400$ μg L^{-1}) and that *Ca*CLTP2 had low toxicity at the concentrations used in antifungal tests (200 μg L^{-1}). Thus, the effects of these peptides on yeast cells did not match their cytotoxicity toward erythrocytes, thereby showing selectivity.

In conclusion, our study demonstrated that three novel AMPs isolated from *C. annuum* leaves. These AMPs displayed in vitro antifungal activity against some pathogenic *Candida* species, and *Ca*CPin-II was toxic to mammalian cells under the tested conditions. Thus, we suggest that *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2 are promising peptides for the development of novel antifungal treatments.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-023-10112-3.

Acknowledgements This work was performed at the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). We wish to thank L.C.D. Souza and V.M. Kokis for technical assistance.

Author Contribution The study was conceived by MBC and VMG. Experimental procedures were carried out by MBC, GBT, F A-S, MSS, MCC, ATSF, and RR. Data analyses were performed by MBC, JAAP, TMV, AOC, RR, OVM, and MARBC. The paper was written by MBC, GBT, FA-S, and VMG. All authors reviewed the manuscript.

Funding We acknowledge the financial support of the Brazilian agencies CNPq (307590/2021–6), FAPERJ (E-26/200567/2023; E-26/210353/2022). This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES), finance code 001.

Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare no conflict of interest.

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Supplementary Information

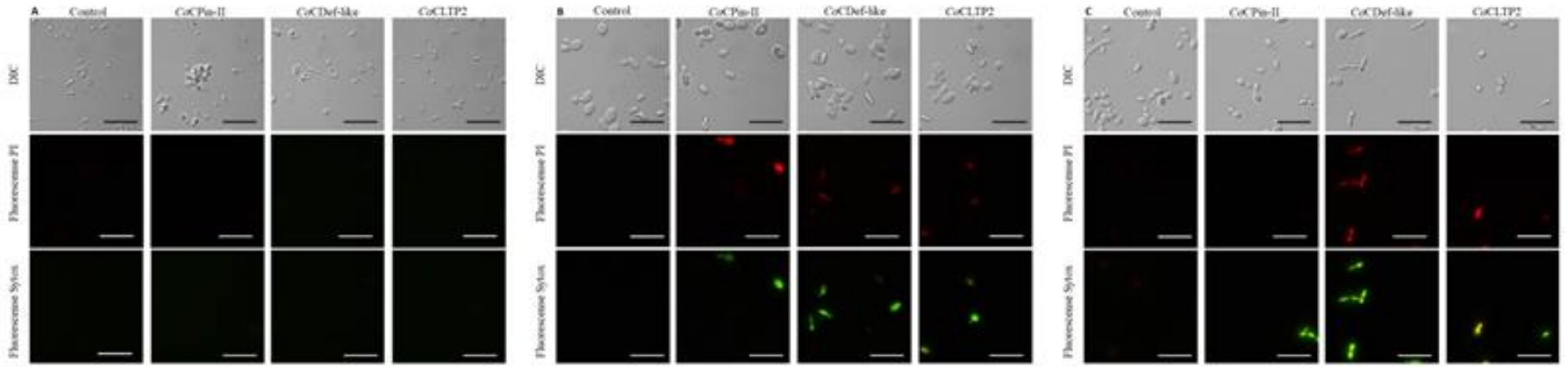

Figure Images of cell death monitoring and membrane permeabilization assays of *Candida albicans* (A), *Candida tropicalis* (B) and *Candida parapsilosis* (C) cells after treatment with peptides (200 µg mL^{-1}) for 24 hours. Control cells were treated only with propidium iodide (PI) stain or the SYTOX® Green probe. Bars =20 µm. Abbreviation: DIC, differential interference contrast.

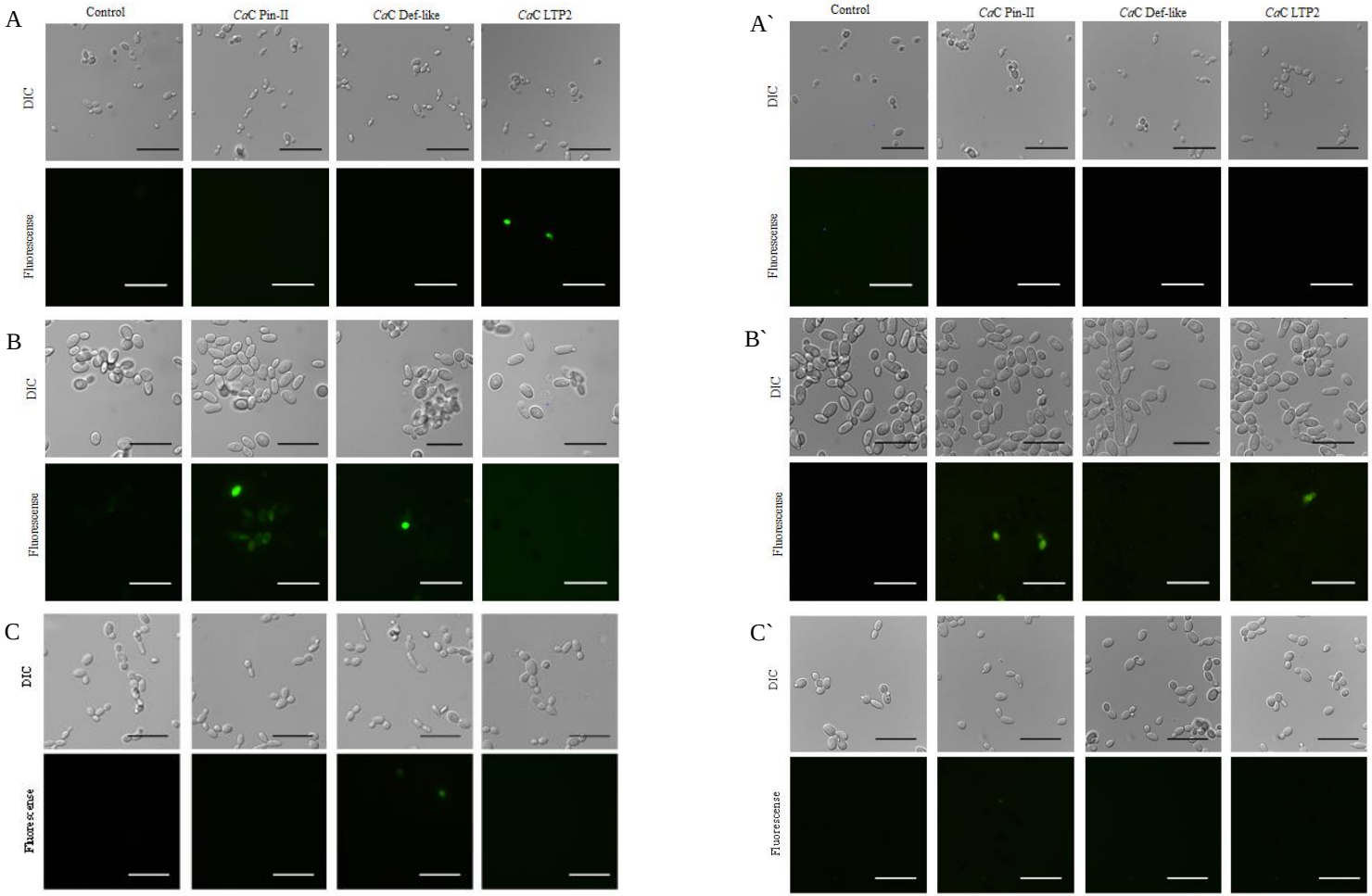
A
Control
CaC Pin-II
CaC Def-like
CaC LTP2
DIC
Fluorescense
B
DIC
Fluorescense
C
DIC
Fluorescense
A`
Control
CaC Pin-II
CaC Def-like
CaC LTP2
DIC
Fluorescense
B`
DIC
Fluorescense
C`
DIC
Fluorescense

Figure Images of reactive oxygen species assay detection in *Candida albicans* (A), *Candida tropicalis* (B) and *Candida parapsilosis* (C) cells after treatment with peptides (200 μg mL^{-1}) for 24 hours. Control cells were treated only with the 2′,7′-dichlorofluoresceindiacetate probe. Images of metacaspase activity detection in *Candida albicans* (A`), *Candida tropicalis* (B`) and *Candida parapsilosis* (C`) cells after treatment with peptides (200 μg mL^{-1}) for 24 hours. Control cells and cells treated with peptides were incubated with the FITC-VAD-FMK probe and analyzed by fluorescence microscopy. Green fluorescence indicates positive staining for caspase activity. Bars = 20 μm. Abbreviation: DIC, differential interference contrast.

4 - CAPÍTULO 2

Insecticidal activity of *Capsicum annuum* L. leaf proteins on cowpea weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae) development

Chapter 2: Manuscript submitted

Insecticidal activity of *Capsicum annuum* L. leaf proteins on cowpea weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae) development

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Type of article: Research article

Situation: Published

Journal: Journal of Asia-Pacific Entomology

Impact factor: 1.5

Resumo

Vários peptídeos antimicrobianos (AMPs) de plantas do gênero *Capsicum* foram isolados e caracterizados, apresentando atividade biocida. Alguns desses compostos exibem atividade inseticida. Neste estudo, obtivemos uma fração contendo um inibidor de protease do tipo Pin-II do sobrenadante do extrato da folha de *C. annuum* e comparamos a toxicidade deste sobrenadante do extrato (ExS) com o extrato total, denominado (Ex) contra o desenvolvimento de *Callosobruchus maculatus*. Ex e ExS foram incorporados a sementes artificiais contendo farinha de cotilédones de *Vigna unguiculata*, o que reduziu a oviposição e interferiu no desenvolvimento larval pós-eclosão de *C. maculatus*, causando redução na massa larval. Uma diminuição na oviposição de até 72,6% foi observada em sementes artificiais contendo 15% (m/m) de Ex e 2% (m/m) de ExS. Diminuições de até 60% e 55% foram observadas na massa larval aos 20 dias após a oviposição (DAO) para larvas criadas com dietas contendo Ex e ExS, respectivamente. Alterações nos níveis de glicose, proteínas, triglicerídeos e colesterol e nas atividades de α-amilases, cisteína proteases e lipases também foram detectadas nessas larvas. Nossos dados mostram que Ex e ExS têm efeitos deletérios sobre *C. maculatus*, com potencial para o desenvolvimento de métodos de controle desse inseto.

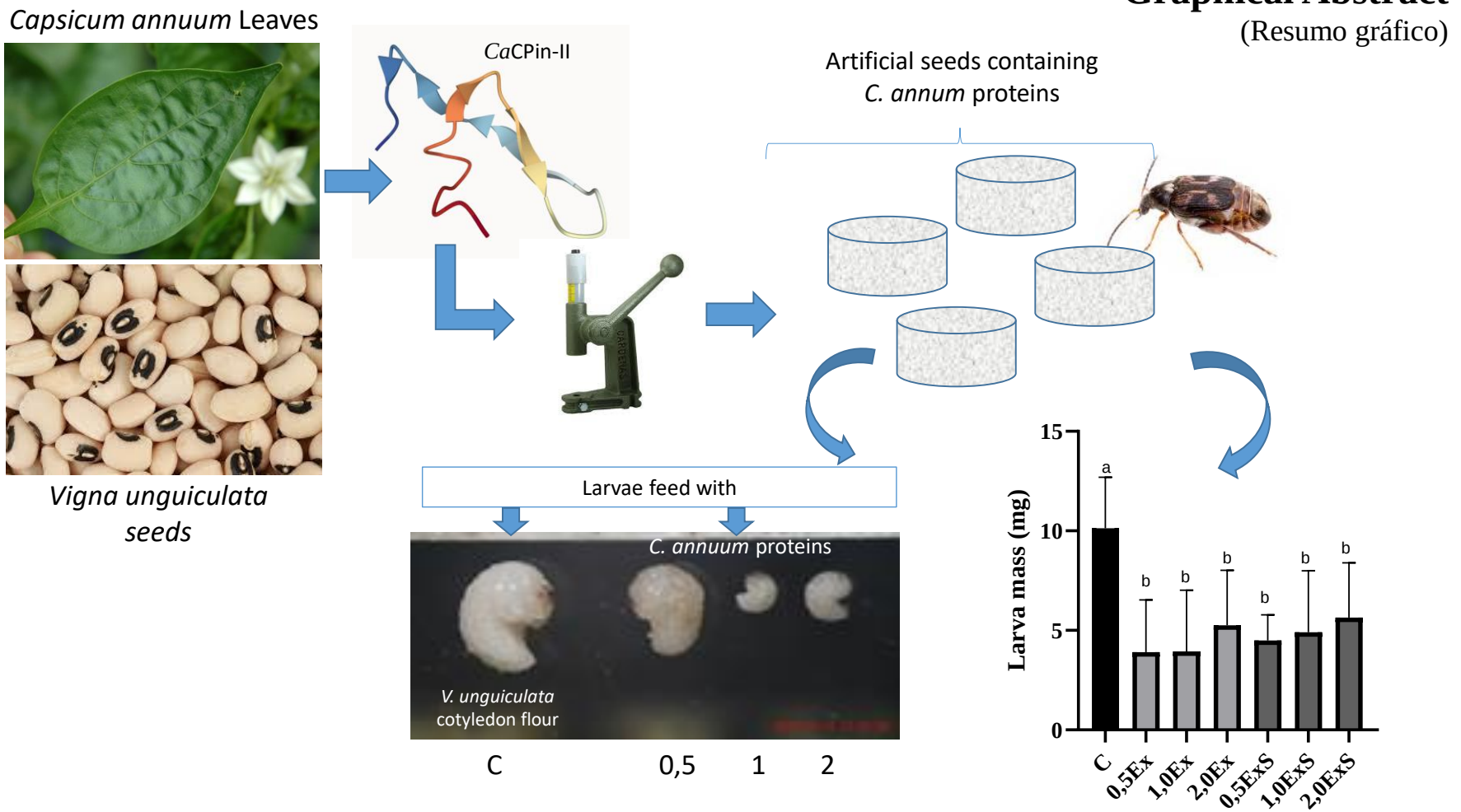
Palavras-chave: *Callosobruchus maculatus*; controle de pragas; *Capsicum annuum*; atividade inseticida; inibidores de protease

Highlights

- *Callosobruchus maculatus* is the main pest of *Vigna unguiculata* seeds;
- Proteins of *Capsicum annuum* leaf interfered with the *C. maculatus* post-hatching development;
- *Capsicum annuum* proteins decreased insect oviposition and larval mass;
- *C. annuum* protease inhibitor was toxic to *C. maculatus* larvae;
- Larvae fed *Capsicum annuum* proteins showed changes in energy reserves and digestive enzymes activities.

Graphical Abstract

(Resumo gráfico)



Journal of Asia-Pacific Entomology 26 (2023) 102158

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full length article

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ARTICLE INFO

Keywords:
Callosobruchus maculatus
Pest control
Capsicum annuum
Insecticidal activity
Protease inhibitors

ABSTRACT

Several antimicrobial peptides (AMPs) from plants of the genus *Capsicum* have been isolated and characterized, showing biocidal activity. Some of these compounds exhibit insecticidal activity. In this study, we obtained a fraction containing a Pin-II protease inhibitor from the supernatant of the leaf extract of *C. annuum* and compared the toxicity of this extract supernatant (ExS) with that of the total extract, named Ex, against *Callosobruchus maculatus* development. Ex and ExS were incorporated into artificial seeds containing cotyledon flour from *Vigna unguiculata*, which reduced oviposition and interfered with the post-hatching larval development of *C. maculatus*, causing a reduction in larval mass. A decrease in oviposition of up to 72.6% was observed in artificial seeds containing 15% (m/m) Ex and 2% (m/m) ExS. Decreases up to 60% and 55% were observed in the larval mass at 20 days after oviposition (DAO) for larvae raised on diets containing Ex and ExS, respectively. Alterations in glucose, protein, triglyceride, and cholesterol levels and the activities of α-amylases, cysteine proteases, and lipases were also detected in these larvae. Our data show that Ex and ExS have deleterious effects on *C. maculatus*, with the potential for developing methods to control this insect, such as products for fumigating grain during postharvest management.

Introduction

Globally, grain productivity and consumption are increasing annually to meet growing demands for food and other inputs (Saath and Fachinello, 2018). However, pest and pathogen control continues to be a challenge for agricultural production, and the damage caused by herbivorous insects results in significant losses in the fields and the storage and transport of grains. Several pests feed on grains during postharvest storage because of their high nutrient concentrations (Mishra et al., 2018). In Brazil, annual losses caused by insect attacks on major crops range from 2 to 43% (Oliveira et al., 2014) and represent a reduction of approximately 25 million tons of food, fiber and biofuels. Grain losses correspond to approximately 12.5 million tons (Pozebon and Arnemann, 2021). Grains serve as nutritional bases in developed and underdeveloped countries (Poutanen et al., 2021) because they store large amounts of nutrients. Among the grains used in food, legumes stand out for their high nutritional value and generally contain more protein than cereals (Çakir et al., 2018), thus representing an important source of protein in places where animal protein is not accessible to all people (FAO, 2016). Cowpea (*Vigna unguiculata* (L.) Walp.) is an important legume found in the semiarid tropics of Asia, Africa, and Central and South America. Cowpea seeds are mainly grown in Nigeria, Niger, and Brazil (Boukar et al., 2019). These grains are protein sources relevant to populations in developing countries (Abebe and Alemayehu, 2022).

The cowpea weevil *Callosobruchus maculatus* (Coleoptera: Chrysomelidae: Bruchinae) is the main pest affecting *V. unguiculata* in the postharvest period (Cardoso et al., 2017) and may affect other species of

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https://doi.org/10.1016/j.aspen.2023.102158
Received 17 July 2023; Received in revised form 19 September 2023; Accepted 24 September 2023
Available online 26 September 2023

the genus *Vigna* and other legumes, such as soybean (*Glycine* max) and pea (*Lens culinaris*) (Tuda et al., 2014). This insect causes significant damage to seeds and is difficult to control, as its life cycle is fast and females lay many eggs. The larvae that develop inside the eggs oviposit on the seed coat, hatch, penetrate the seeds, and develop by consuming the seed cotyledons (Beck and Blumer, 2011). Adults emerge approximately 30 days after oviposition (DAO), and the adult stage lasts approximately 10–14 days, during which females lay approximately 80 eggs throughout their lifetime (Beck and Blumer, 2011; Cardoso et al., 2017).

The use of plants and their products to control insects and microorganisms has historically been related to the advent of agriculture (Ngegba et al., 2022). However, if the use of botanical compounds is initially restricted to procedures and empirical knowledge, then these compounds' chemical nature and mechanisms of action are known. Among the various products, protein compounds and secondary metabolites are noteworthy for their roles in adaptive processes and defense mechanisms. Plants can produce both for different purposes: as constituent substances, reducing the digestibility of plant tissues and causing direct toxicity; or as induced substances synthesized in response to tissue damage (Garcia et al., 2021). Several compounds of a protein nature with insecticidal activities have been previously described (Velasques et al., 2017). Antimicrobial peptides (AMPs) are part of the biochemical defense arsenal that participates in the innate immunity of plants (Campos et al., 2018). Several AMPs with insecticidal activities have already been isolated from plants. They are the families of cyclotides (Huang et al., 2019), lipid transfer proteins (LTPs) (Maximiano and Franco, 2021), defensins (Carvalho and Gomes, 2012) and protease inhibitors (Velasques et al., 2017). Many of these AMPs have been isolated from plants of the genus *Capsicum* (Afroz et al., 2020; Yadav et al., 2021; Oliveira et al., 2022a).

Cherene et al. (2023) isolated and characterized three AMPs from *Capsicum annuum* leaf extract: *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2. In this study, we aimed to obtain an enriched fraction of the protease inhibitor, named *Ca*CPin-II, and investigate the toxicity of the *C. annuum* leaf total extract (Ex) and this *Ca*CPin-II inhibitor-enriched fraction (ExS) to *C. maculatus* larval development.

Materials and methods

Biological materials

Insects

Callosobruchus maculatus were obtained from the colony maintained by the Laboratório de Química e Função de Proteínas e Peptídeos do Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense in Campos dos Goytacazes - RJ. Insects that developed on the seeds of *V. unguiculata* (cv. Fradinho) inside glass flasks with holes in the lid for air entry in the dark in a B.O.D. incubator with controlled temperature (28 °C) and humidity (60%). Larvae were collected 20 days after oviposition (DAO), photographed, weighed, and subjected to biochemical dosages.

Vigna unguiculata seeds

Vigna unguiculata (cv. Fradinho) seeds were commercially obtained from local markets in Campos dos Goytacazes and kept at −70 °C until use.

Capsicum annuum leaves

Capsicum annuum L. var. *annuum* (cv. Carioquinha) seeds were provided by the Laboratório de Melhoramento Genético Vegetal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil. Seeding was carried out in a 72-cell polystyrene foam with commercial substrate and kept in a growth chamber at a controlled temperature of 28 °C and a photoperiod of 12 h. When the seedlings exceeded 10 cm in height, they were transplanted into 5 L pots and transferred to the greenhouse. After 60 days, the leaves were cut close to the petiole and used for extraction.

Extraction and biochemical characterization of leaf extracts

Obtaining Capsicum annuum leaf extracts

Capsicum annuum leaves (10 g) were macerated and extracted with 30 mL of 60% methanol (v/v) at room temperature for 24 h. Then, this material was filtered to remove debris and partitioned with dichloromethane (1:1, v/v) at room temperature for 24 h, as described by Cherene et al. (2023). The aqueous phase was separated and concentrated by lyophilization. This extract was named Ex. The extract (0.1 g) was resuspended in 10 mL of 20% acetonitrile (v/v) and centrifuged at 6000 rpm for 3 min at room temperature to obtain an enriched fraction of one of its peptides. The supernatant was separated and concentrated by lyophilization. The extract supernatant is referred to as ExS.

Ex and ExS electrophoretic profile

Protein quantification was performed by the bicinchoninic acid (BCA) method described by Smith et al. (1985) with modifications, using ovalbumin as the protein standard. SDS-tricine-gel electrophoresis was performed according to the method described by Schägger and von Jagow (1987).

Biochemical characterization of ExS peptides

The extracted supernatant (ExS) was analyzed using reversed-phase chromatography (HPLC) to assess whether centrifugation could enrich this fraction. The extract supernatant (ExS) of *C. annuum* leaves (0.1 g) was dissolved in 10 mL of 20% acetonitrile and injected onto an HPLC VP-ODS (250 × 4.6 mm i.d.) C_{18} reversed-phase column (Shim-pack, Shimadzu) with a C_8 guard column (*Pelliguard*, Sigma). The column was equilibrated and run with 100% solvent A (0.1% trifluoroacetic acid (TFA)) for the first 2 min, followed by a gradient of solvent B (90% acetonitrile containing 0.1% TFA). The concentration of solvent B increased to 10%, 32%, and 50% from 2 to 10 min, 10 to 60 min, and 60 to 74 min, respectively, and it was maintained at 50% for 1 min. Then, the concentration of solvent B was reduced to 0% and was kept at 0% until the end of the run at 85 min. The chromatography was developed at a flow rate of 0.5 mL min^{-1} at 37 °C. The presence of proteins was determined using online absorbance measurements at 220 and 280 nm.

Toxicity of Capsicum annuum leaf extracts to Callosobruchus maculatus

The extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves were incorporated into an artificial seed system, according to Macedo et al. (1993). The extract (Ex) at concentrations of 0.5 to 15% and extract supernatant (ExS) at concentrations of 0.5%, 1.0%, and 2.0% were added to *V. unguiculata* (cv. Fradinho) cotyledon flours to produce 400 mg/per artificial seed. For more efficient homogenization, the mixture of flour and extracts was diluted in approximately 1.5 mL of distilled water, homogenized, and recovered by lyophilization. After lyophilization, each mixture was placed into a cylindrical brass mold (2.7 × 1.3 cm) and pressed using a hand press to obtain the artificial seed. Three artificial seeds were prepared for each concentration. Control artificial seeds were prepared using 400 mg of *V. unguiculata* (cv. Fradinho) cotyledon flour. The artificial seeds were infested with 2-day-old females (three females per seed) for 24 h, and after this period, the females were removed. Excess eggs were removed with a fine needle, leaving only three eggs per seed, to decrease larval competition. The seeds were incubated at controlled temperature (28 °C) and humidity (60%). Larval neonate development and hatching on artificial seeds were monitored by observing the larvae inside the eggs at 1, 4, 6, 7, and 8 days after oviposition (DAO). At the 20th DAO, all the seeds were broken to remove the larvae. The larval mass was determined and compared with that of larvae that developed in the control seeds.

Furthermore, the larvae were photographed to analyze their morphology and subjected to biochemical dosages. Experiments were performed in triplicate, with 3 seeds per experiment (total of nine seeds and 27 eggs).

Quantification of macromolecules and enzyme activities in Callosobruchus maculatus larvae

Larval extraction

Fourth instar larvae developed in artificial seeds (20 DAO) were subjected to biochemical quantification. The larvae were macerated in 0.15 M sodium chloride solution (1 mg/15 μL) for extraction. The extract was stirred for 30 min at 4 °C and centrifuged, and the supernatant was used for glucose, protein, lipase and α-amylase measurements. The precipitate was extracted with a 0.5% Tween 20 solution (10 mg/15 μL) under stirring at 4 °C for 30 min. The supernatant obtained after centrifugation was used to determine the triacylglycerol and cholesterol levels. For assays of cysteine protease activities, the larvae were macerated in citrate phosphate buffer pH 5.0 (100 mM sodium citrate, 100 mM sodium phosphate, 0.1% Triton x-100, and 1.5 mM DTT). The larval extract was agitated at 4 °C for 1 h and centrifuged at 2,000 × g for 10 min.

Protein quantification

For protein quantification in the larvae, the BCA method was used, as described by Smith et al. (1985). The larval extract (10 μL) was mixed with 200 μL of BCA solution (2 mL of BCA to 0.04 mL of copper sulfate) in a 96-well plate. The plate was incubated at 37 °C for 30 min, and absorbance was read at 540 nm. An ovalbumin curve (Sigma Aldrich), with concentrations between 0.2 and 1 μg μL^{-1}, was used as a standard to calculate the protein concentration of the samples. The protein concentration in test larvae was expressed as a percentage as a function of the protein levels in control larvae, which were considered 100%.

Glucose quantification

The Monoreagent Glucose Kit was used according to the manufacturer's instructions (BioClin). This kit consists of two reagents: Reagent N1 (enzymatic reagent) containing buffer, phenol, 4-aminoantipyrine, glucose oxidase, peroxidase, stabilizer, surfactant, and preservative and Reagent N2 (standard) consisting of glucose (100 mg/dL) and preservative. The assay was performed as described by Oliveira et al. (2022b). Next, 200 μL of the enzymatic reagent was added to the larval extract (2 μL). Next, 200 μL of the enzymatic reagent was added to glucose (2 μL). Samples were homogenized and incubated at 37 °C for 10 min. The absorbance was measured at 492 nm. The glucose concentration in test larvae was expressed as a percentage as a function of the glucose in control larvae, which was considered 100%.

Triglyceride quantification

This assay was performed using the Monoreagent Triglyceride Kit following the manufacturer's instructions (BioClin). This kit consists of two reagents: Reagent N1 (enzymatic reagent), which contains 4-chlorophenol, lipoprotein lipase, glycerol kinase, peroxidase, glycerol-3-phosphate oxidase, 4-aminoantipyrine, ATP, activator, stabilizer, surfactant, and preservative, and Reagent N2 (standard), which contains triglycerides (100 mg/dL) and a diluent. The assay was performed as described by Oliveira et al. (2022b). Next, 200 μL of the enzymatic reagent was added to the larval extract (2 μL). The triglyceride (2 μL) was also added to 200 μL of the enzymatic reagent. Samples were homogenized and incubated at 37 °C for 10 min. The absorbance was measured at 500 nm. The triglyceride concentration in test larvae was expressed as a percentage as a function of the triglyceride in control larvae, which was considered 100%.

Cholesterol quantification

The Monoreagent Cholesterol Kit (Bioclin) was used according to the manufacturer's instructions. This kit consists of two reagents: Reagent N1 (enzymatic reagent), which contains buffer, phenol, 4-aminoantipyrine, cholesterol oxidase, lipoprotein lipase, peroxidase, chelator, surfactants, stabilizers, and a preservative; and Reagent N2 (standard), which contains cholesterol (200 mg/dL), solubilizers, surfactants, and stabilizers. Next, 200 μL of the enzymatic reagent was added to the larval extract (2 μL). The assay was performed as described by Oliveira et al. (2022b). Next, 200 μL of the enzymatic reagent was added to the cholesterol (2 μL). Samples were homogenized and incubated at 37 °C for 10 min. The absorbance was measured at 500 nm. The cholesterol concentration in test larvae was expressed as a percentage as a function of the cholesterol in control larvae, which was considered 100%.

Determination of α-amylase activity

Following the manufacturer's instructions, this assay was carried out using the Monoreagent Amylase kit (Bioclin). This kit consists of two reagents: Reagent N1 (substrate), which contains starch, a phosphate buffer (pH 7.0), a stabilizer, and a preservative. Reagent N2 (color reagent; iodine stock solution): Reagent N2 was used to prepare the working reagent, which consisted of 5 mL of reagent N2 and 45 mL of distilled water. The assay was performed as described by Oliveira et al. (2022b). A 100 μL aliquot of substrate for each sample point was incubated at 37 °C for 2 min. Subsequently, this substrate was added to the larval extract (2 μL) and the negative control (0.5% Tween-20 solution) (2 μL). The solutions were incubated for 7.5 min at 37 °C. Then, 100 μL of the working reagent was added to the samples along with 800 μL of water. The absorbance was measured at 630 nm. α-Amylase activity in test larvae was expressed as a percentage as a function of the α-amylase activity in control larvae, which was considered 100%.

Determination of lipase activity

Following the manufacturer's instructions, this assay was carried out using a Monoreagent Lipase Kit (Bioclin). This kit consisted of five reagents: Reagent N1 (buffer) Tris 100 mM, pH 8.5; Reagent N2 (enzymatic inhibitor – 8 mM), which contains phenylmethylsulfonyl fluoride and solubilizer; Reagent N3 (color reagent – 3 mM) – DTNB, sodium acetate, and stabilizer; Reagent N4 (substrate – 20 mM) – dithiopropanol thiobutyrate and surfactant; and Reagent N5 (acetone). The assay was performed as described by Oliveira et al. (2022b). The larval extract (5 μL) was mixed with 100 μL of buffer, 5 μL of enzyme inhibitor, and 10 μL of color reagent. For each sample, there was a control containing no enzyme inhibitors. Samples were incubated at 37 °C for 2 min. A 10 μL volume of substrate was added to the samples, and 10 μL of saline buffer (0.15 M sodium chloride solution) was added to the controls. The samples were incubated at 37 °C for 30 min. The reaction was stopped using 200 μL of acetone. The samples were homogenized, kept at rest for 5 min, and centrifuged at 2,000 × g for 5 min. The absorbance of the supernatant was measured at 405 nm. The tests were performed in triplicate. Lipase activity in test larvae was expressed as a percentage as a function of the lipase activity levels in control larvae, which were considered 100%.

Quantification of cysteine proteases

The test was performed according to Michaud et al. (1994), using azocasein as a substrate. A 1% azocasein solution was prepared in citrate–phosphate buffer (100 mM sodium citrate, pH 5.6, 100 mM sodium phosphate, 0.1% Triton X-100 and 1.5 mM DTT). A total of 40 μL of larval extract (previously diluted in a 1:2 ratio) was added to the azocasein solution (80 μL). Samples were incubated at 37 °C for 1 h. The reaction was stopped using 10% trichloroacetic acid (TCA) (300 μL). The samples were centrifuged at 2000 × g for 5 min, and 300 μL of 1 M sodium hydroxide was added to the supernatant. Absorbance was measured at 440 nm. A papain (Fluka) curve was used as a standard to calculate the cysteine protease concentration. Cysteine protease activity in test larvae was expressed as a percentage as a function of the cysteine protease activity levels in control larvae, which were considered 100%.

Enzyme inhibition assays

The ability of Ex and Exs to inhibit enzymatic activity was also evaluated. An amylase activity assay for *Tenebrio molitor* intestine α-amylase was performed as previously described by Bernfeld (1955). Ex and ExS at 10 to 50 μg mL^{-1} were incubated with 10 U of α-amylase, and each enzyme mixture was equilibrated in a water bath at 37 °C for 15 min. After that, 25 μL of 1% starch solution (Sigma-Aldrich Co.) was added, and the samples were incubated in a water bath at 37 °C for 30 min. The samples were removed from the water bath and mixed with 400 μL of 3.5 dinitrosalicylic acid (DNS). Each sample was boiled for 5 min and, after cooling, mixed with 400 μL of water. Substrate hydrolysis was determined by measuring absorbance at 540 nm (Chameleon V, Hidex). For the positive control of α-amylase inhibition, 5 mM EDTA was used. All inhibition assays were performed in triplicate. Trypsin and chymotrypsin inhibitory activities were quantified by measuring the hydrolytic activity of porcine trypsin and bovine chymotrypsin toward the substrates BapNa (N-benzoyl-Dl-arginyl-*p*-nitroanilide) and BatNa (N-benzoyl-L-tirosil-*p*-nitroanilide), respectively. Inhibitory activity was determined by incubating Ex and ExS at increasing concentrations from 25 to 300 μg mL^{-1} with enzymes and their respective substrates in 50 mM Tris-HCl buffer (pH 8.0) at 37 °C in a final volume of 200 μL. A control sample without the peptides was used for each assay. The reaction was stopped with 100 μL of 30% (v/v) acetic acid. Substrate hydrolysis was quantified by measuring the absorbance of *p*-nitroaniline at 405 nm (Macedo et al., 2007). The half-maximal inhibitory concentration (IC_{50}) values were calculated using nonlinear regression of log (inhibitor) vs. the normalized response equation to the experimental data.

Statistical data analysis

The assays were performed in triplicate. Means, standard deviations, and statistical analyses were performed using GraphPad Prism software (version 8.0). Data have not been transformed. Analysis of variance (ANOVA) and Tukey's test (T test) were performed as described by Bridge and Sawilowsky (1999). The results were considered significantly different from the control at $p < 0.05$.

Results

Biochemical characterization of Ex and ExS

The chromatogram of the extract supernatant (ExS) of *C. annuum* leaves in reversed-phase chromatography on HPLC showed a single peak eluted with 8.0% acetonitrile (Fig. 1). The electrophoretic profile of the extract (Ex) showed a single band corresponding to the three previously isolated peptides with molecular masses between 3.5 and 6.5 kDa (Cherene et al., 2023). The electrophoretic profiles of the extract supernatant (ExS) and a single chromatographic peak (*Ca*CPin-II) showed a single band at approximately 6.5 kDa (Fig. 1, insert). The protein concentrations of Ex and ExS were 1.4 and 2.5 mg/g, respectively.

Toxicity of Ex and ExS to C.maculatus larval development

The addition of 5%, 10%, and 15% of Ex extract to artificial seeds caused a significant decrease in oviposition by 31.5, 58.9 and 72.6%, respectively (Fig. 2A). Larval development was monitored at 7 DAO when it was observed that the control larvae had already hatched. In contrast, the larvae that developed in the seeds treated with Ex at these concentrations remained inside the egg (data not shown). Fig. 2B-F shows the dead larvae at 20 DAO inside the eggs, and in some cases, the mark of the attempt to penetrate the seed (red arrows); therefore, the addition of Ex at these concentrations in the artificial seeds caused the death of the larvae before hatching, so that there were no live larvae at 20 DAO.

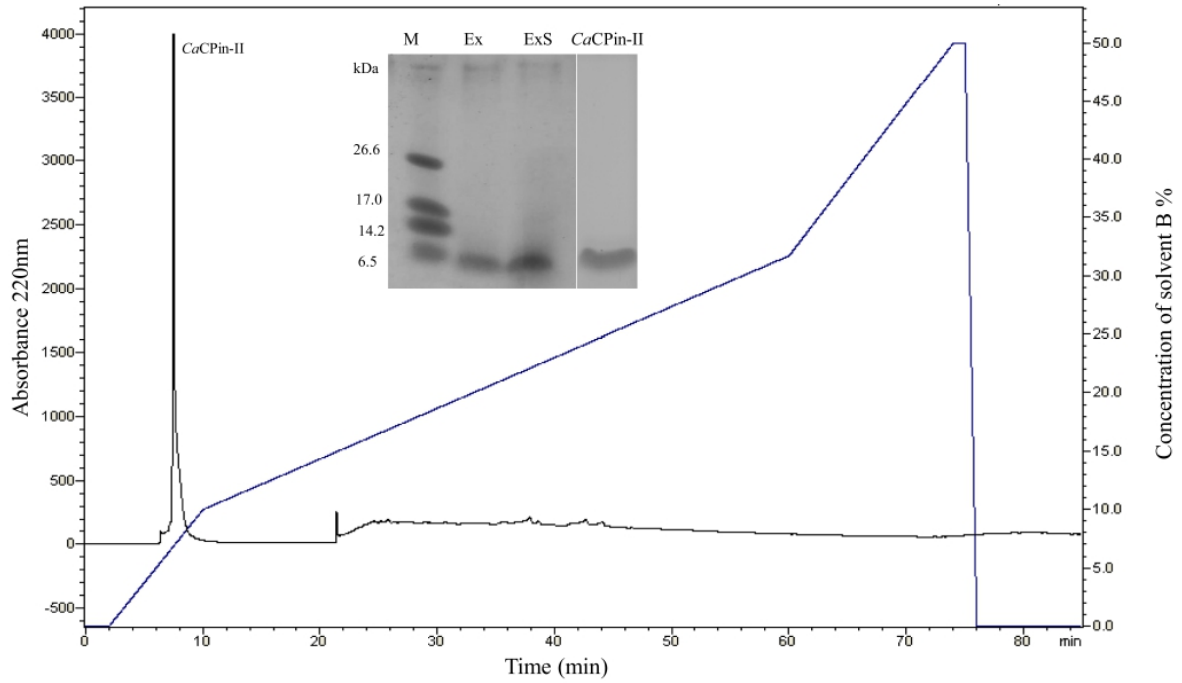

Fig. 1. Chromatogram of the extract supernatant (ExS) of *C. annuum* leaves in a reversed-phase C_{18} column. The column was equilibrated and run with 0.1% trifluoroacetic acid (TFA) (solvent A) and eluted using a gradient of 90% acetonitrile in 0.1% (solvent B). The flow rate used was 0.5 mL min^{-1}. (Insert) Electrophoretic visualization in an SDS-tricine-gel of extract (Ex), extract supernatant (ExS) and single chromatographic peak (*Ca*CPin-II) of *C. annuum* leaves (cv. Carioquinha). M - Molecular mass (kDa) markers.

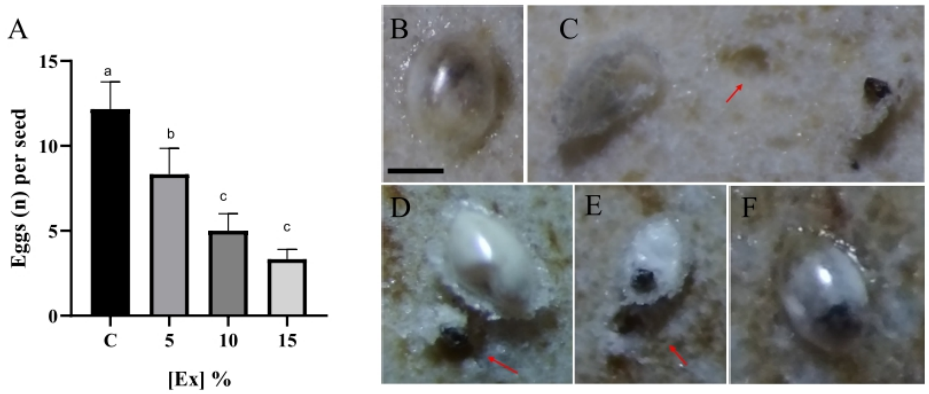

Fig. 2. (A) Oviposition of *C. maculatus* in artificial seeds containing extract (Ex) of *C. annuum* leaves. Numbers below each bar indicate the Ex concentrations (% m/m) in artificial seeds. Images of dead larvae at 20 DAO (days after oviposition) developed in artificial seeds containing extract (Ex) of *C. annuum* leaves at concentrations of (B and C) 5.0%, (D) 10.0% and (E, F) 15.0% m/m. The red arrow indicates the hole in the seed made by the larva after hatching. Values represent means (±SDM), and statistical treatments were performed using one-way ANOVA (Tukey's test). df = 3; 11, F = 35.7, P < 0.0001. Values followed by different letters were significantly different according to Tukey's test. Bar = 0.1 mm.

Because of this, the extract (Ex) and extract supernatant (ExS) were incorporated into artificial seeds at lower concentrations: 0.5%, 1.0%, and 2.0%, and larval development and hatching were monitored until 8 DAO. There were no apparent morphological differences in larval development up to 8 DAO between the control and larvae grown in artificial seeds containing 0.5%, 1.0% (data not shown), or 2.0% Ex and ExS (Fig. 3). At 1 and 4 DAO, the egg contents were clear and transparent on both seeds (control and experimental seeds), and at 6 DAO, the larvae were fully formed inside the egg; at 8 DAO, the eggs had a whitish appearance due to the presence of flour inside them. The larvae eclosed and penetrated the artificial seeds (Fig. 3).

To compare the toxic effects of Ex and ExS on larval development, ExS was also incorporated at 0.5%, 1.0%, and 2.0% into the insect diet. The addition of ExS to artificial seeds had a repellent effect, as it reduced oviposition up to 72.6% when compared to the control seeds (Fig. 4A). There were no significant differences in larval survival at 20 DAO (Fig. 4B).

The ingestion of Ex and ExS at concentrations of up to 2.0% interfered with the post-hatching development of the larvae (Fig. 5A-C). At 20 DAO, larvae developed in seeds containing 0.5, 1, and 2% Ex showed decreases in their body mass of 60.9, 60.5, and 47.4%, respectively. In contrast, larvae developed in seeds containing 0.5, 1 and 2% ExS showed decreases in their body mass of 55.1, 50.9 and 43.6%, respectively, when compared to the larvae developed in the control seeds (Fig. 5A). At 20 DAO, only larvae developed in seeds containing 0.5 and 1% Ex and 0.5 and 2% ExS showed significant decreases in length of 42.6, 26.4, 30.8 and 23.6%, respectively (Fig. 5B).

There was no significant difference in body mass (Fig. 5A) or length (Fig. 5B) between the larvae that developed in artificial seeds containing Ex and ExS.

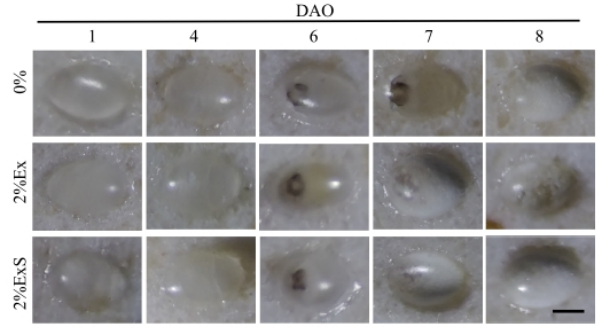

Fig. 3. *C. maculatus* larval neonate development on artificial seeds containing 0 or 2% extract (Ex) and extract supernatant (ExS) of *C.* annuum leaves. The development and hatching were monitored at 1, 4, 6, 7 and 8 DAO (days after oviposition). Bar = 0.1 mm.

Biochemical parameters of 20 DAO larvae developed in seeds containing Ex and ExS

The patterns of glucose levels and amylase activity of larvae fed Ex and ExS were similar (Fig. 6A), except for the larvae fed with a diet increased by 2.0% ExS, which showed a reduction in glucose levels and amylase activity of 53.0 and 60.0%, respectively. Some treatments increased these rates. These increases were significant in larvae fed a diet increased by 1% Ex (94.5% for amylase), 2% Ex (69.2% for glucose and 101.8% for amylase), and 1% ExS (80.7% for amylase). There was an increase in the protein content of the larvae subjected to all diets, except for the larvae developed in the diet increased by 2% ExS, where a reduction of 50.4% was observed (Fig. 6B). The increase in protein levels was significant for larvae fed Ex 2% (77.8%) and ExS 1% (71.6%). Cysteine protease levels were greatly increased relative to control larvae, with the smallest increase for larvae fed 2% ExS (658.8%) and the greatest increase for larvae fed 2% ExS (1576.3%). To evaluate the parameters of lipid metabolism in the larvae, cholesterol, triglyceride, and lipase levels were measured (Fig. 6C). The larvae that developed in artificial seeds supplemented with Ex and ExS showed a significant decrease in cholesterol levels compared to control larvae. Larvae fed ExS 2% had the lowest reduction in cholesterol levels (84.5%); however, without significant difference related to the larvae fed with Ex at the same concentration. However, triglyceride levels and lipase activity in larvae fed diets with Ex and ExS were increased for all treatments, except for larvae fed ExS 2%, which showed a reduction in triglyceride levels (47.0%) and lipases (52.8%).

Inhibitory effect of Ex and ExS on Tenebrio molitor amylase

To understand the possible toxicity mechanism of Ex and ExS on the larval development of *C. maculatus*, their ability to inhibit two important classes of enzymes, amylases and proteases, was evaluated. Both Ex and ExS were able to inhibit the action of *T. molitor* intestinal α-amylase, and the IC_{50} values were calculated to be 23.7 and 20.0 μg mL^{-1}, respectively (Fig. 7). Nevertheless, Ex and ExS were not able to inhibit the activity of the serine proteases trypsin and chymotrypsin at concentrations of up to

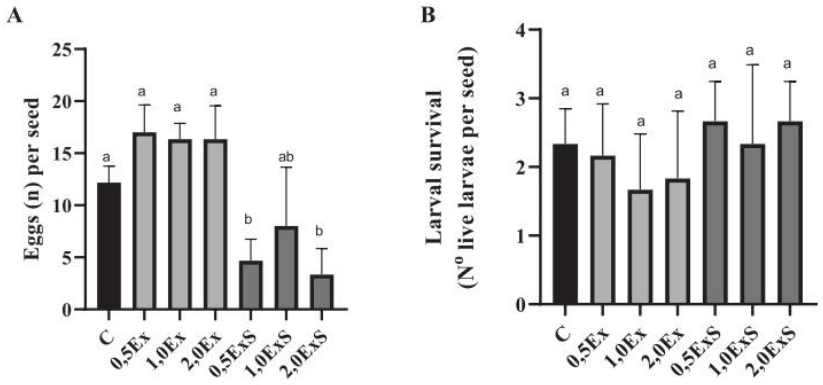

Fig. 4. (A) Oviposition and (B) survival at 20 DAO (days after oviposition) of *C. maculatus* larvae developed in artificial seeds containing extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves at concentrations of 0.5 to 2.0% m/m. Values represent means (±SDM), and statistical treatments were performed using one-way ANOVA (Tukey's test). Oviposition (df = 6; 16, F = 15.1, P < 0.0001), survival (df = 6; 26, F = 1.0, P = 0.42). Values followed by different letters were significantly different according to Tukey's test.

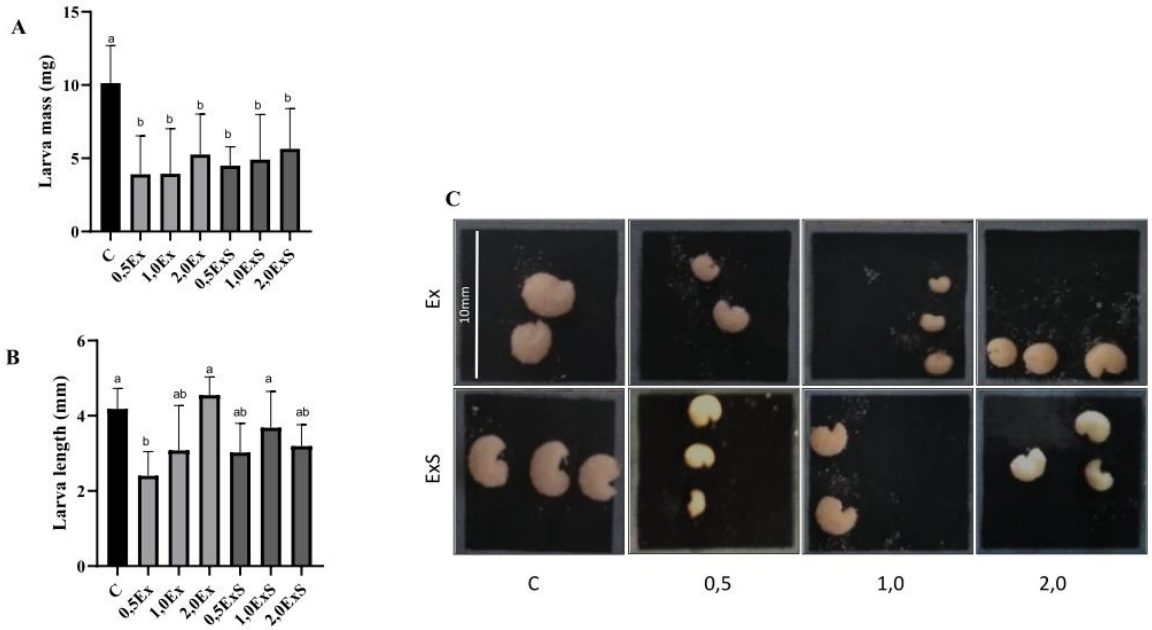

Fig. 5. Toxicity of extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves to *C. maculatus* larvae. (A) Mass, (B) length and (C) images of larvae at 20 DAO (days after oviposition) developed in artificial seeds containing extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves at concentrations of 0.5 to 2.0% m/m. Values represent means (±SDM), and statistical treatments were performed using one-way ANOVA (Tukey's test). Mass/larva (df = 6; 69, F = 8.4, P < 0.0001) and length (df = 6; 47, F = 5.7, P = 0.0002) values followed by different lowercase letters were significantly different according to Tukey's test. Bar (B) = 2 mm, Bar (C) = 10 mm.

300 μg mL^{-1} (data not shown).

Fig. 7 Effect of extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves on the activity of intestinal α-amylase in *Tenebrio molitor* insects. Numbers below each bar indicate the Ex or ExS concentrations (μg mL^{-1}). In the positive control of α-amylase inhibition, 5 mM EDTA was used. Values represent means (±SDM), and statistical treatments were performed using one-way ANOVA (Tukey's test). df = 11; 23, F = 130.2, P < 0.0001. Values followed by different letters were significantly different according to Tukey's test.

Discussion

Several antimicrobial peptides with antifungal, antibacterial (Afroz et al., 2020; Oliveira et al., 2022a) and insecticidal activities (Jadhav et al., 2016; Mishra et al., 2012; Mulla et al., 2021) have been isolated and identified from *C. annuum*. The insecticidal activity of extracts from leaves of *C. annuum* cv. Carioquinha were evaluated in the present study. Three AMPs from *C. annuum* leaf extract (Ex) were isolated and characterized by Cherene et al., 2023: a protease inhibitor, a defensin-like protein, and an LTP2, denominated *Ca*CPin-II, *Ca*CDef-like, and *Ca*CLTP2, respectively. In this study, we obtained the extracted supernatant (ExS) by centrifugation. The ExS chromatographic profile was

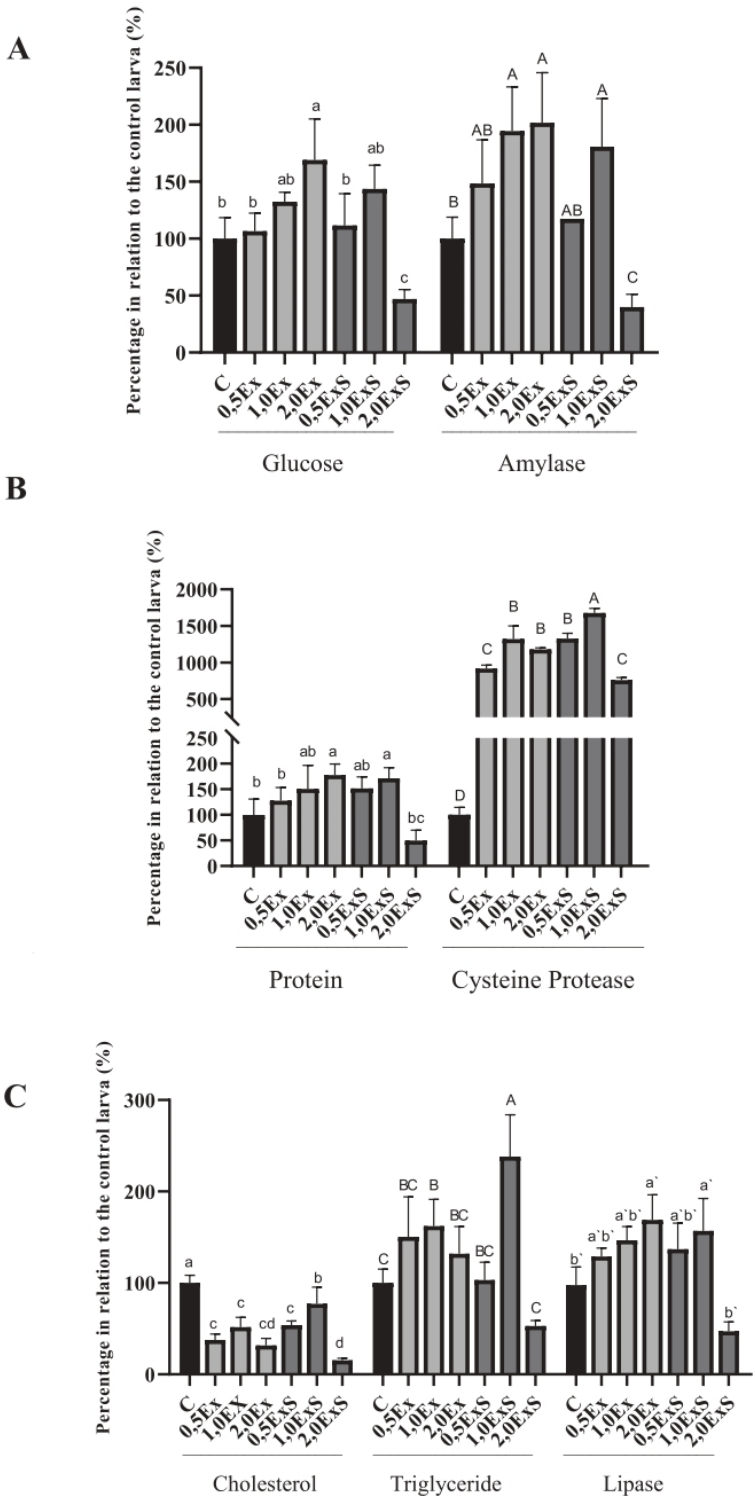

Fig. 6. (A) Glucose and amylase, (B) protein and cysteine protease, (C) cholesterol, triglyceride and lipase levels (% in relation to the control larvae) in 20 DAO (days after oviposition) larvae of *C. maculatus* developed in artificial seeds containing extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves in concentrations of 0.5 to 2.0% m/m. Values represent means (±SDM), and statistical treatments were performed using one-way ANOVA (Tukey's test). Glucose ($df = 6; 26$, $F = 12.9$, $P < 0.0001$), amylase ($df = 6; 22$, $F = 10.8$, $P < 0.0001$), protein ($df = 6; 30$, $F = 10.9$, $P < 0.0001$), cysteine protease ($df = 6; 14$, $F = 117.5$, $P < 0.0001$), cholesterol

(df = 6; 35, F = 52.9, P < 0.0001), triglycerides (df = 6; 24, F = 12.0, P < 0.0001), and lipase (df = 6; 17, F = 11.5, P < 0.0001). Values followed by different letters were significantly different according to Tukey's test.

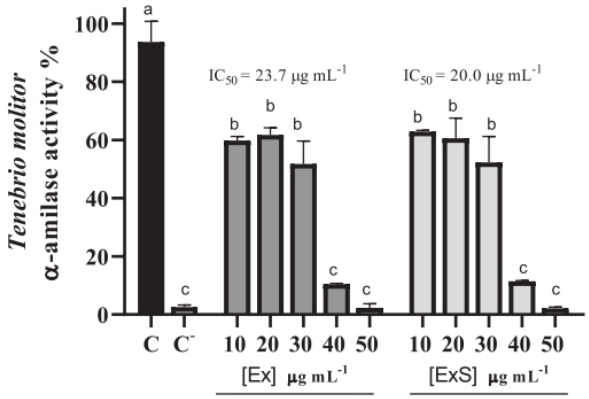

Fig. 7. Effect of extract (Ex) and extract supernatant (ExS) of *C. annuum* leaves on the activity of intestinal α-amylase in *Tenebrio molitor* insects. Numbers below each bar indicate the Ex or ExS concentrations (µg mL^{-1}). In the positive control of α-amylase inhibition, 5 mM EDTA was used. Values represent means (±SDM), and statistical treatments were performed using one-way ANOVA (Tukey's test). df = 11; 23, F = 130.2, P < 0.0001. Values followed by different letters were significantly different according to Tukey's test.

analyzed under the same conditions as Ex (Cherene et al., 2023). It showed only one peak eluted at the same elution time as the protease inhibitor, *Ca*CPin-II. The Ex and ExS electrophoretic profiles were similar, although Ex is composed of three peptides with similar molecular masses, between 3.5 and 6.5 kDa (Cherene et al., 2023) and ExS is composed of only one of these peptides, the protease inhibitor named *Ca*CPin-II. These data suggested that ExS is an enriched fraction of *Ca*CPin-II. Several Pin-II protease inhibitors have been isolated and have shown insecticidal activity (Mishra et al., 2010; Yadav et al., 2021). The presence of defensins and LTP in Ex may also contribute to its deleterious effects on *C. maculatus* larvae. Plant defensins (Kovaleva et al., 2020) and LTPs (Maximiano and Franco, 2021) with insecticidal activity have been isolated and characterized.

One of the causes of plant extracts' toxic and biocidal activities is their ability to inhibit important enzymes involved in the physiology and metabolism of organisms (Velasques et al., 2017). Many plant proteins and AMPs exhibit inhibitory activity. Pereira et al. (2018), including defensins and LTPs (Carvalho and Gomes, 2012; Diz et al., 2011; Zottich et al., 2011). Insect development is compromised after contact with plant defensins, which is related to the inhibition of insect digestive tract enzymes, including proteases and amylases, as they deprive animals of energy from starch and protein degradation (Bloch and Richardson, 1991; Osborn et al., 1995). Ex and ExS were able to inhibit amylases in the intestine of *T. molitor* but were not able to inhibit serine proteases at the concentrations tested. However, *Ca*CPin-II is capable of inhibiting papain (Cherene et al., 2023), an enzyme of the cysteine protease class, the most abundant class of proteases in the digestive tract of *C. maculatus* (de Sá et al., 2017; Moon et al., 2004). The most important enzymes in the *C. maculatus* digestive tract are amylases and proteases, which feed on bean seeds, a dietary source rich in protein and starch (Abebe and Alemayehu, 2022).

Insects cause great economic losses in the grain production chain, from cultivation to storage and marketing, and are usually controlled using chemical pesticides. The constant use of such pesticides damages human health and the environment, in addition to promoting the emergence of resistant insects, which encourages the search for alternatives for pest control. Global concern for health and the environment has increased political action in the search for new substances of biotechnological interest for use in agriculture that provide sustainable development and positive impacts on the world economy (Velasques et al., 2017). A bioinsecticide produced from *Clitoria ternatea* extract containing an AMP from the cyclotide family is already available on the market (Grover et al., 2021), opening the market for the creation of new bioinsecticides based on plant extracts. Both Ex and ExS interfered with *C. maculatus* larval development. Adding Ex at concentrations of 5, 10, and 15% and ExS at concentrations of 0.5, 1, and 2% to artificial seeds caused an antixenosis effect, as it decreased oviposition. Antixenosis is a phenomenon in the insect-plant relationship that occurs when an insect species is repelled or not attracted by the plant (Kogan and Ortman, 1978; Peterson et al., 2017). It is related to plant characteristics that directly affect insect survival, such as the presence of chemical compounds that make the plant less favorable for use as a food source and/or site for oviposition (Boiça Júnior et al., 2015; Stout, 2013). Females choose sites for oviposition (Craig and Itami, 2008) so that their offspring can find an adequate food supply and develop, ensuring the species' survival. (Jaenike, 1978; Schoonhoven et al., 2005). The results suggest that the reduction in oviposition was due to the addition of Ex and ExS to the seeds, as all other structural and physical characteristics of the artificial seeds were the same. Therefore, Ex and ExS have the potential to be developed as alternative methods for controlling *C. maculatus* infestation in grains. In addition to reducing oviposition, Ex at concentrations of 5, 10, and 15% caused the death of the larvae before they began to penetrate the seed, so at 20 DAO, there were no live larvae to be analyzed. Seeds containing lower concentrations of Ex and ExS were used to evaluate neonatal larval development, toxicity, and biochemical and metabolic parameters of the larvae after 20 DAO.

The addition of Ex and ExS at concentrations between 0.5 and 2% to the diet of *C. maculatus* did not cause a significant change in the survival and length of the 20 DAO larvae but significantly decreased their mass and altered their biochemical and metabolic parameters. This phenomenon, in which a plant has a deleterious effect on an insect species' growth, development, fecundity, and survival, is known as antibiosis and is part of the insect-plant relationship. (Kogan and Ortman, 1978; Peterson et al., 2017). This may be related to toxic compounds in Ex and ExS. This hypothesis was confirmed by the fact that until hatching, the neonate larval development of *C. maculatus* was similar both in control artificial seeds (cotyledon flour only) and in those containing Ex and ExS. The alterations occurred from the moment the larvae began to penetrate and feed on the artificial seed containing Ex and ExS. Seeds of non-host species of *C. maculatus*, such as *Canavalia ensiformis*, *Albizzia lebeck*, *Dioclea maxima*, and *Phaseolus vulgaris*, are toxic to *C. maculatus*, preventing larval penetration by 100% (de Sá et al., 2018, 2014; Silva et al., 2018; Souza et al., 2011).

The analysis of 20 DAO larvae suggests that larvae grown in artificial seeds containing Ex and Exs present a delay in development compared to control larvae. There was no significant difference in the length of the 20 DAO larvae fed Ex and ExS compared to the control. Values between 2.43 and 3.64 mm for third- and fourth-instar larvae are described in the literature for larvae grown in natural seeds of *V. unguiculata* susceptible to infestation (Devi and Devi, 2014). During the larval development of *C. maculatus*, significant mass gain occurs between the 17th and 20th DAO (fourth instar). There was a significant reduction in the mass of larvae fed Ex and ExS, which corroborates the hypothesis of a delay in development caused by Ex and ExS.

The levels of glucose, cholesterol, triglycerides, and proteins, as well as the levels of amylase, lipases, and cysteine protease enzymes, were also altered in larvae fed Ex and ExS, indicating effects on physiological and metabolic patterns, such as the digestive process. Changes in macromolecule content and digestive enzyme activity may occur

because of two factors: *C. maculatus* larvae can modulate digestive enzyme levels in response to changes in diet (Oliveira et al., 2022b). The second factor is that the toxic effects of the altered diet delay the development of *C. maculatus* larvae, generating a biochemical pattern incompatible with the chronological age of the larva. Failures in the processes of digestion and absorption of nutrients can alter growth, development, and energy storage (Holtof et al., 2019; Kanost et al., 1990). Some works have already demonstrated that the levels of hydrolases decreased in *C. maculatus* larvae in response to the presence of toxic compounds in the diet. Reductions in the levels of amylase, glycosides, cysteine proteases, and aspartic proteases were found in larvae fed a diet containing *Canavalia ensiformis* flour (de Sá et al., 2017) and *Phaseolus vulgaris* seed coat flour (de Sá et al., 2014). Reduction in the levels of cysteine proteases was found in larvae fed a diet containing chitin-binding protein isolated from seeds of a resistant cultivar of *V. unguiculata* (Ferreira et al., 2021). Larvae developed in the natural seeds of resistant cultivars of *V. unguiculata* also demonstrated reduced activities of amylases, cysteine proteases, and glycosidases (Cruz et al., 2016). However, our study found increased hydrolase activity in most larvae that developed in Ex and ExS seeds. Increases in enzyme activity and expression in *C. maculatus* larvae in response to changes in diet have been observed. Digestive enzyme genes, such as cysteine and cathepsin D-like aspartic proteases, were up-regulated in the gut of larvae fed cystatin (a cysteine protease inhibitor) (Ahn and Zhu-Salzman, 2009; Chi et al., 2009). The activities of amylases and cysteine proteases were increased in larvae developed with a diet containing cystatins (Nogueira et al., 2012). Cysteine protease activity was increased in larvae grown on a diet containing a fraction of *Schinus terebinthifolius* seed extract (Oliveira et al., 2022b). The overproduction of digestive proteases is an insect strategy to overcome inhibitors by increasing the production of inhibitor-insensitive protease isoforms and/or activating proteases that can hydrolyze the inhibitor (Guo et al., 2012). Another factor that may explain the increased activity of cysteine proteases is their direct cytotoxic effect on the structure of the peritrophic membrane of insects, which interferes with the digestion, absorption, and recycling of digestive enzymes. An increase in the activity of cysteine proteases could be a way for larvae to compensate for this interference (Oliveira et al., 2022b). The increased protein and cysteine protease activity levels observed in larvae grown on diets containing Ex and ExS may also indicate delayed larval development. The activity of cysteine proteases and the level of proteins increase during larval development, while amino acids from the digestion of food proteins are stored in the form of proteins in the fat body of insects at this stage. Therefore, stored proteins are used as a source of amino acids for the synthesis of protein structures in the adult stage (Arrese and Soulages, 2010; Holtof et al., 2019; Kanost et al., 1990).

In this work, we found increased glucose levels and α-amylase activities in larvae fed diets with Ex and ExS at almost all tested concentrations. During the larval stage, insects accumulate reserves without feeding (Arrese and Soulages, 2010), which in *C. maculatus* corresponds to the metamorphosis and adult stages (Beck and Blumer, 2011). Glycogen stored in the insects fat body is an important energy reserve, and its metabolism relies mainly on amylases, the enzymes responsible for the digestion of dietary starch (Holtof et al., 2019) and the hydrolysis of glycogen to release glucose during periods of energy demand (Arrese and Soulages, 2010). During early pupation (21–22 DAO), the larvae no longer feed, and glucose levels and α-amylase activities decrease (Arrese and Soulages, 2010). These data also corroborate the hypothesis that the 20 DAO larvae fed a diet supplemented with Ex and ExS had a delay in development. Although *C. maculatus* larvae do not contain large amounts of triglycerides, they are the main reserve lipids of insects and are stored in adipocytes, the main reserve cells of the fat body; lipases are the main enzymes responsible for its hydrolysis (Arrese and Soulages, 2010). As the larvae develop, the triglyceride stores increase, and the highest levels of lipase activity are found in the pupal stage, after 20 DAO, when the triglyceride stores are consumed. Therefore, the increase in triglyceride and lipase levels and the decrease in cholesterol levels found in larvae fed Ex and ExS do not seem to be related to a delay in development but may be related to a change in the digestive, absorptive, and metabolic patterns. Ex and ExS have inhibitory activities on the enzymes α-amylase and cysteine proteases, which are responsible for the hydrolysis of the main components of the diet of *C. maculatus*.

Our results show that the leaf extract of *C. annuum* cv. Carioquinha contains a protease inhibitor that is potentially toxic to *C. maculatus* larvae. The leaf extract and the protease inhibitor *Ca*CPin-II have the potential to be used to develop healthier and more sustainable biotech agricultural inputs to control this pest, such as products for fumigating grain during post-harvest management.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We acknowledge the financial support of the Brazilian agencies CNPq (307590/2021-6) and FAPERJ (E-26/200567/2023).

Author Contribution

The study was conceived by MBC, AEAO, and VMG. Experimental procedures were carried out by MBC, SRF, LAS, and RR. Data analyses were performed by MBC, SRF, AEAO, VMG, and AOC. The paper was written by MBC and SRF. The paper was reviewed by VMG, AEAO.

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5 - CAPÍTULO 3

Evaluation of *in vitro* and *in vivo* toxicity of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 antimicrobial peptides

Chapter 3: Manuscript in preparation

Evaluation of *in vitro* and *in vivo* toxicity of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 antimicrobial peptides

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Type of article: Research article

Situation: in preparation

Journal: Toxicon

Impact factor: 3.035

Resumo

Peptídeos antimicrobianos (AMPs) são candidatos promissores para o desenvolvimento de novos fármacos. No entanto, é fundamental avaliar se novas moléculas com potencial terapêutico são tóxicas para células de mamíferos, visto que a toxicidade para o hospedeiro é um dos principais motivos da não aprovação do medicamento pelos órgãos reguladores. Este trabalho teve como objetivo avaliar o perfil toxicológico de três AMPs, *Ca*CPin-II, *Ca*CDef-like e *Ca*CLTP2, com potencial antifúngico isolados de folhas de *Capsicum annuum* por citotoxicidade *in vitro* contra células de mamíferos e a toxicidade sistêmica *in vivo* utilizando larvas de *Galleria mellonella* como estudo modelo. A citotoxicidade dos AMPs foi avaliada em algumas linhagens de células humanas, a saber: endotélio vascular, adenocarcinoma cervical, epitélio prostático, epitélio mamário e fibroblastos, e em macrófagos murinos. A viabilidade celular foi avaliada medindo a atividade metabólica. Para elucidar o mecanismo de toxicidade do peptídeo, sua capacidade de se ligar à superfície celular e permeabilizar as membranas foi avaliada medindo o potencial zeta e a absorção da sonda fluorescente SYTOX® Green, respectivamente. A toxicidade sistêmica in vivo de foi avaliada pela taxa de sobrevivência das larvas de *G. mellonella* inoculadas com peptídeos. Os AMPs não diminuíram a viabilidade celular e nem permeabilizaram as membranas das linhagens celulares nas concentrações testadas. Apenas *Ca*CLTP2 teve a capacidade de interagir com a superfície celular, mas não foi capaz de permeabilizá-las. *Ca*CPin-II apresentou toxicidade *in vivo*, pois a taxa de sobrevivência das larvas após o teste foi 60% menor que os controles. Os resultados sugerem que esses peptídeos têm potencial como agentes antimicrobianos, pois apresentam baixa ou nenhuma toxicidade para células de mamíferos e podem servir como estrutura para o desenvolvimento de fármacos.

Palavras-chave: Peptídeos bioativos; citotoxicidade; desenvolimento de fármacos; toxicidade *in vivo*; testes pré-clínicos

Abstract

Antimicrobial peptides (AMPs) are promising candidates for the development of new drugs. However, thorough studies on the toxicity of these molecules are scarce, which is a gap, as host toxicity is one of the main reasons for non-approval of the drug by regulatory agencies. This work aimed to evaluate the toxicological profile of three AMPs, *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2, with antifungal potential isolated from leaves of *Capsicum annuum* by *in vitro* cytotoxicity against mammalian cells and the systemic *in vivo* toxicity using *Galleria mellonella* larvae as study model. The AMPs cytotoxicity was evaluated in a panel of human cell lines, namely: vascular endothelium, cervical adenocarcinoma, prostatic epithelium, mammary epithelium and fibroblasts, and in murine macrophages. Cell viability was evaluated through metabolic activity. To elucidate the peptides' toxicity mechanism, their ability to bind to the cell surface and to permeabilize membranes was evaluated by measuring the zeta potential and the absorption of the SYTOX® Green fluorescent probe, respectively. The *in vivo* systemic toxicity was evaluated by the survival rate of the *G. mellonella* larvae inoculated with peptides. The AMPs did not decrease cell viability and nor permeabilized the membranes of the cell lines at the tested concentrations. Only *Ca*CLTP2 had the ability to interact with the cell surface, but it was not able to permeabilize them. *Ca*CPin-II showed *in vivo* toxicity, as the larvae survival rate after the test was 60% lower than the controls. The results suggest that these peptides have potential as antimicrobial agents because they have low or no toxicity to mammalian cells, and can serve as a framework for drug development.

Keywords Bioactive peptides - cytotoxicity - drug development - *in vivo* toxicity – preclinical trials

1. Introduction

Antimicrobial peptides (AMPs) are part of innate defense or produced as a microorganisms competition strategy to limit the growth of others one (Moretta et al., 2021) and are ubiquitous in nature, existing in various organisms, including bacteria, fungi, animals and plants (Seyfi et al., 2020). AMPs are expressed by specific genes constitutively or inducible by specific external factors (Lei et al., 2019), forming short ribosomally synthesized peptides of L-amino acids (Carvalho and Gomes, 2012). Most of them are small cationic peptides that have low molecular mass, commonly in the range of 6 to 100 amino acids, amphipatic design, and are collected according to their sequence homology, functional similarities and three-dimensional structure (Huan et al., 2020; Mookherjee et al., 2020; Seyfi et al., 2020). Structural classes of plant AMPs are mainly represented by the groups of defensins, thionins, lipid transfer proteins (LTPs) and cyclotides which had their N- and C-terminals bound and, therefore are cyclic peptides (Ojeda et al., 2019; Zasloff, 2019). As part of the first line of host defense against pathogen attack, AMPs have biocidal activities against bacteria, fungi, viruses, parasites and insects, but also have other biological activities, such as antitumor and modulators of the immune system, representing one of the oldest innate defense components in evolutionary history (Wei and Zhang, 2022). Therefore, these peptides with spread-spectrum activities emerge as a good candidates for drug development (Koo and Seo, 2019; Lewies et al., 2019).

The interest in AMPs research is due to their multiple mechanism of action and multiple targets, that include direct action in membranes but also action in intracellular targets, in addition to being able to act in synergism with conventional drugs (Lewies et al., 2019). Therefore, these molecules can destroy pathogens by damaging multiple targets, which might greatly reduce the emergence of drug-resistant pathogens (Zhang et

al., 2021). These peptides have selective toxicity and low rate of microbial resistance induction and does not trigger stress response and mutagenesis pathways in bacteria (Lewies et al., 2019; Rodríguez-Rojas et al., 2014). Although naturally occurring and synthetic AMPs have shown promising results for new drugs, some disadvantages have been pinpointed for AMP-based therapies, including chemical and physical instability, low pharmacokinetic characteristics, short half-life *in vivo*, proteolytic degradation and toxicity (Cardoso et al., 2020; Chen and Lu, 2020). Toxicity of AMPs to mammalian cells is still the major obstacle in their development and clinical applications (Khabbaz et al., 2021). To overcome these shortcomings, it is essential to further explore the mechanisms of action of AMPs to understand their activity and biotoxicity (Wei and Zhang, 2022).

Drug development requires several steps with complex and important tests that guarantee the selection and approval of only effective and non-toxic molecules (Pognan et al., 2023; Robles-Loaiza et al., 2022). Selectivity is one of the main requirements for their progression into the clinics. Although many molecules are constantly discovered, only a tiny fraction is converted into secure and effective therapeutical molecules (Mohs and Greig, 2017). The pharmacologic and biochemical characteristics of the drug candidate are established using an extensive range of *in vitro* and *in vivo* test procedures. It is also a regulatory requirement that the drug is administered to animals to assess its safety (Tamimi and Ellis, 2009). Drug toxicity remains a latent problem, and peptides are no exception to this rule (Khan et al., 2018). Several bioactive peptides have shown toxicity, especially hemotoxicity (Greco et al., 2020; Ruiz et al., 2014). Therefore, toxicity to healthy eukaryotic cells remains a major bottleneck in the approval rate of new pharmaceutical peptides (Gupta et al., 2015). *In vitro*, *in vivo* and *ex vivo* toxicity evaluation is an essential step in the development of potential new drugs, including the

half-lethal dose (LD50) and half-hemolytic activity (HC50) (da Cunha et al., 2017; Wei and Zhang, 2022). More than 3000 AMPs have been discovered, but only some non-ribosomally synthesized peptides have been approved by the U.S. Food and Drug Administration (FDA), for example gramicidin, daptomycin, vancomycin, oritavancin, dalbavancin, telavancin, telaprevir and colistin (also known as polymyxin E), all of them mostly used for topical medications. Most of these peptides are usually very limited to be useful for the clinical applications. In fact, many of AMPs failed prior to or during clinical trials (Chen and Lu, 2020; Lei et al., 2019). At the same time, dozens of AMPs are in clinical development around the world, mainly focusing on treatments for skin infections caused by bacterial and fungal pathoges, and in some clinical trials are also addressing AMPs as treatments for cancer and systemic infections (Koo and Seo, 2019). Currently, a defensin derivative is the only example of plant AMP that is in the preclinical trial (Hein et al., 2022).

*Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 are AMPs from *Capsicum annuum* cv. Carioquinha leaves that have been isolated and characterized as a protease inhibitor, a defensin-like and an LTP2, respectively. This AMPs displayed *in vitro* antifungal activity against some opportunistic *Candida* species (Cherene et al., 2023a) and a *Ca*CPin-II enriched fraction has insecticidal activity against *Callosobruchus maculatus* larvae (Cherene et al., 2023b.). At this work we evaluate the *in vitro* cytotoxicity of these AMPs against mammalian cells and the systemic *in vivo* toxicity using *Galleria mellonella* larvae as study model.

2. Materials and methods

2.1. Cell culture

Immortalized cell lines from human breast cancer MDA-MB-231 (ATCC® HTB-26™), SKBR3 (ATCC® HTB-30™) and MCF7 (ATCC® HTB-22™); human fibroblasts Hs68 (ATCC® CRL-1635™); human epithelial cervical cancer HeLa (ATCC® CCL-2™); human prostatic cell lines RWPE-1 (ATCC® CRL-11609D™) and PC-3 (ATCC® CRL-1435™); human cerebral microvascular endothelial cell HBEC-5i (ATCC® CRL-3245™) and murine macrophage RAW 264.7 (ATCC® TIB-61™) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). MDA-MB-231, Hs68, HeLa and RAW 264.7 were cultured as a monolayer in Dulbecco's Modified Eagle Medium (DMEM). SKBR3 and MCF7 were cultured as a monolayer in McCoy′s 5A and Eagle's Minimum Essential Medium (EMEM) media, respectively. RWPE-1 and PC3 were cultured as a monolayer in Defined Keratinocyte SFM and Ham's F-12K (Kaighn's) media, respectively. All the above mentioned media were supplemented with 10% fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific, Waltham, MA, USA). HBEC-5i were cultured in T-flasks pre-coated with attachment factor protein (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) using DMEM:F12 medium with 10% FBS, 1% penicillin–streptomycin and 40.0 µg/mL endothelial cell growth supplement (Sigma-Aldrich, Spain). All cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C (MCO-18AIC (UV), Sanyo, Japan) with the medium changed every other day.

2.2. Obtaining of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides

*Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides were purified from *C. annuum* cv. Carioquinha leaves as described by Cherene et al., 2023a.

2.3. *In vitro* cytotoxicity assay

Peptides *in vitro* citotoxicity against mammalian cell lines were evaluated by measuring the reducing activity in cells using CellTiter-Blue® cell viability assay (Promega, Madrid, Spain), according to the manufacturer's instructions. Briefly, cells were allowed to grow until ~80% confluence in a 75-T-flask under standard conditions as mentioned above. Cells were then carefully detached and seeded at 1.0×10^5 cells.mL^{-1} in 96-well flat-bottomed plates (Corning, USA), of 100 µL per well volume. After 24 h, medium was removed, and adhered cells were incubated with 100 µL of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 dissolved in complete serum-free medium for culturing of the cell line, at concentrations in the range 1.56–200.0 µg.mL^{-1} for an additional 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Then, 20 µL of CellTiter-Blue® Reagent was added to each well and incubated for 3 h under the same conditions. Fluorescence intensity was measured with λexc = 560 nm and λem = 590 nm in an Varioskan Lux microplate reader Thermo Fisher, Spain). Medium without peptides and 1% Triton X-100-containing medium were used as positive controls (100%, normal reducing activity) and negative controls (0%, non-viable cells), respectively. Reducing activity (%) was determined by the following expression:

$$Reducingactivity(\%) = \frac{Fluorescence_{Peptide-treatedcells} - Fluorescence_{Negativecontrol}}{Fluorescence_{Positivecontrol} - Fluorescence_{Negativecontrol}} x100 \quad \text{Eq.1}$$

Experiments were performed in three independent biological replicates performed on separate passages of cells and on separate day and the mean is presented with standard deviation in the graphs.

2.4. ROS induction detection assay

The ability of the peptides to induce intracellular ROS formation was assessed using a DCFDA/H_2DCFDA-Cellular ROS Assay Kit (ab113851, Abcam, USA) and performed according to the manufacturer's instructions. Briefly, cells were allowed to

grow until ~80% confluence in a 75-T-flask under standard conditions, as mentioned above. Cells were then carefully detached and seeded at 1.0×10^5 cells.mL^{-1} in 96-well flat-bottomed plates, with 100 µL of peptides at concentrations in the range 6.25–200.0 µg.mL^{-1}, dissolved in serum-free DMEM without phenol red, for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. 1 hour before the end of the assay, 2x concentrated DCFDA solution (20 µM) was prepared and added 100 µL to each well and incubated for 45 min under the same conditions. Fluorescence intensity was measured with $\lambda exc = 485$ nm and $\lambda em = 535$ nm in an Varioskan Lux microplate reader. Medium without peptides and 50 µM tert-butyl Hydrogen Peroxide (tbHP)-containing medium were used as negative controls (untreated cells) and positive controls, respectively. ROS inductions was determined by the expression:

$$ROSincrease = \frac{Fluorescence_{Peptide-treatedcells}}{Fluorescence_{Negativecontrol}} \quad \text{Eq.2}$$

Experiments were performed on different days using independent cell cultures and the mean is presented with standard deviation in the graphs. Statistical analysis were carried out with GraphPad Prism software (version 8.0 for Windows) and one-way analysis of variance (ANOVA); $p < 0.05$ was considered statistically significant.

2.5. Zeta potential measurements

Zeta potential measurements were performed as described previously (Oliveira et al., 2022). Briefly, cells were harvested from confluent cell cultures by trypsinization, washed, resuspended in 1X PBS buffer, and diluted to final a concentration of 1.0×10^5 cell·mL^{-1} (in 1X PBS). Cell suspensions with peptides at concentrations in the range 25.0 –200.0 µg.mL^{-1} were prepared. Samples with and without peptides were loaded into disposable zeta cells with gold electrodes and allowed to equilibrate for 30 min at 37 °C. Each experiment consisted of a set of 15 measurements with 40 subruns

performed on a Malvern Zetasizer Nano ZS (Malvern, UK), at a constant voltage of 40 V, with a 90 s pause between measurements. The complete experiment was carried out at least two times using independent cellular suspensions and a control (untreated cells) was performed each day and the mean is presented with standard deviation in the graphs. Statistical analysis were carried out with GraphPad Prism software (version 8.0 for Windows) and one-way analysis of variance (ANOVA); $p < 0.05$ was considered statistically significant.

2.6. Plasma membrane permeabilization

Plasma membrane permeabilization was investigated by SYTOX® Green Nucleic Acid Stain uptake according to the methodology described previously (Almeida et al., 2021), with modifications. Briefly, cells were allowed to grow until ~80% confluence in a 75-T-flask under standard conditions. Cells were then carefully detached and seeded at 1.0×10^5 cells.mL^{-1} in 96-well flat-bottomed plates, with 100 µL of peptides at concentrations in the range 6.25–200.0 µg.mL^{-1}, dissolved in serum-free DMEM without phenol red, for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Then, 1 µM Sytox Green was added to each well and incubated for 10 min under the same conditions. Fluorescence intensity was measured with λexc = 485 nm and λem = 520 nm in an Varioskan Lux microplate reader. Medium without peptides and 0.1% Triton X-100-containing medium were used as negative controls (untreated cells) and positive controls, respectively. Sytox uptake was determined by the expression:

$$Sytoxuptake = \frac{Fluorescence_{Peptide-treatedcells}}{Fluorescence_{Negativecontrol}} \quad \text{Eq. 3}$$

Experiments were performed on different days using independent cell cultures and the mean is presented with standard deviation in the graphs. Statistical analysis were carried

out with GraphPad Prism software (version 8.0 for Windows) and one-way analysis of variance (ANOVA); $p < 0.05$ was considered statistically significant.

2.7. *In vivo* toxicity study in *Galleria mellonella*

To evaluate the systemic *in vivo* toxicity of the peptides we used *G. mellonella* larvae as a study model, as described by Mylonakis et al., 2005, with modifications. Thirty last-instar *G. mellonella* larvae of similar weight and size (250 and 350 mg) were used in each of the treatment and control groups. Ten randomly chosen larvae of the required weight were used per group and assays were performed in duplicate (n=20). Peptide solutions (400 μg. mL^{-1}) using PBS as vehicle were prepared and 10 μL were injected with a Hamilton syringe into the hemocoel of each larva through the last left proleg (dose of ~13 μg/g larva). Larvae that received the injection of 10 μL of vehicle (PBS) and larvae that only received the injection needle injury were used as general viability control. Then, larvae were incubated in Petri dishes at 37 °C and the number of dead larvae was counted every 24 h for a period of 7 days. Larvae were considered dead when they showed no movement in response to touch. Percent survival curves were plotted and estimates of differences in survival (log rank Mantel-Cox and Breslow-Wilcoxon tests) were analyzed by the Kaplan-Meier method using software GraphPad (GraphPad Software, Inc., California, CA, USA).

3. Results

3.1. *In vitro* cytotoxicity assay

The *in vitro* cytotoxicity of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 was initially evaluated by measuring the metabolic activity of a panel of nine cell lines after incubation

with the three peptides. For this purpose, the metabolic activity of nine different mammalian cell lines (MDA-MB-231, MCF7, SKBR3, HBEC-5i, HeLa, Hs68, RWPE-1, PC-3 and RAW 264.7) was assessed using the CellTiter-Blue® assay. The results indicate that the three peptides exhibited low toxicity toward the tested cell lines at concentrations up to 200 µg.mL^{-1}, as showed in Fig. 1 for human cell lines MDA-MB-231, MCF7, SKBR3, HBEC-5i, HeLa and Hs68, as well for RWPE-1, PC-3 and RAW 264.7 cell lines (data not shown).

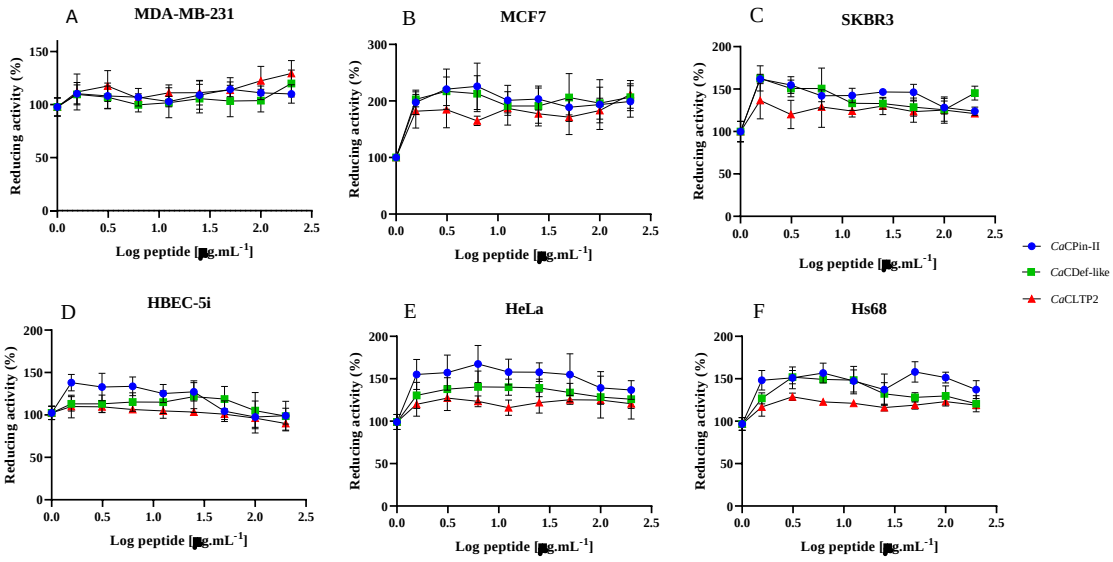

Fig. 1. Effect of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides on the metabolic activity of human cells. Cells from human breast cancer MDA-MB-231 (A), MCF7 (B), SKBR3 (C); human cerebral microvascular endothelial cell HBEC-5i (D); from human epithelial cervical cancer HeLa (E) and human fibroblasts Hs68 (F) were seeded in 96-well plates containing medium (1.0 x 10^5 cells.mL^{-1}) and grown for 24h at 37 °C. After this period, the cells were incubated with the peptides at concentrations ranging from 200.0 to 1.56 µg.mL^{-1} for 1 h and for 24 h. Metabolic activity was measured with the addition of CellTiter-Blue® and fluorescence measured at 590 nm.

3.2. ROS induction

To confirm that *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides do not induce toxicity to the cells, the ROS-inducing activity was investigated *in vitro* using three human cell lines MDA-MB-231, HeLa, and PC-3 as a study model. Cells were incubated at increasing concentrations of peptides for 24 h, and the data showed that peptides at concentrations up to 200 µg.mL^{-1} did not generate oxidative stress in these cell lines, as shown in figure 2 for MDA-MB-231 cell line.

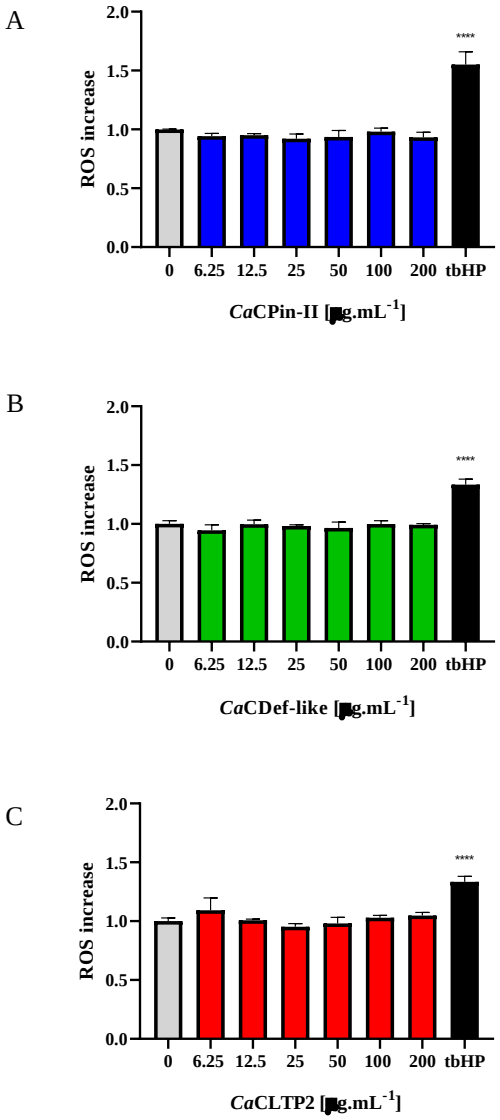

Fig. 2. ROS induction detection assay in human breast cancer MDA-MB-231 cells treated with *Ca*CPin-II (A), *Ca*CDef-like (B) and *Ca*CLTP2 (C) (1 = no increase). Cells were incubated with peptides for 24h (1.0 x 10^5 cells.mL^{-1}) and DCFDA probe was added after this period. Negative control: cells not treated. Positive control: tbHP. Fluorescence intensity was measured at 535 nm. The results presented are mean values obtained over three experiments, each done in triplicate. Dose response graphs were constructed and **** $p < 0.0001$ compared to positive control (0 µg.mL^{-1}) determined by Tukey test.

3.3. Zeta potential

To elucidate *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides interaction with cell plasma membranes, zeta potential of the cells was determined by electrophoretic light scattering using SKBR3, HeLa and RAW 264.7 cell lines as study model. No significant increases were detected in zeta potential values for SKBR3, HeLa and RAW 264.7 cells after 30 min incubation with *Ca*CPin-II (Fig. 3 A) and *Ca*CDef-like (Fig. 3 B), in contrast with *Ca*CLTP2 (Fig. 3 C). Zeta potential of SKBR3 and HeLa cells incubated with 100 µg.mL^{-1} *Ca*CLTP2 was increased by approximately 40% compared to untreated cells. There was no significant difference in the zeta potential of these cells incubated with 100 and 200 µg.mL^{-1} *Ca*CLTP2. The cell line with which *Ca*CLTP2 interacted with the membrane most efficiently was the RAW 264.7 cell. Zeta potencial of RAW 264.7 cells incubated with 25 µg.mL^{-1} *Ca*CLTP2 was increased by 44% compared to untreated cells, and there was no significant difference in zeta potential measures of cells incubated with concentrations of up to 200 µg.mL^{-1} of the *Ca*CLTP2. The data show that *Ca*CLTP2 can interact with cell surface of these cell lines.

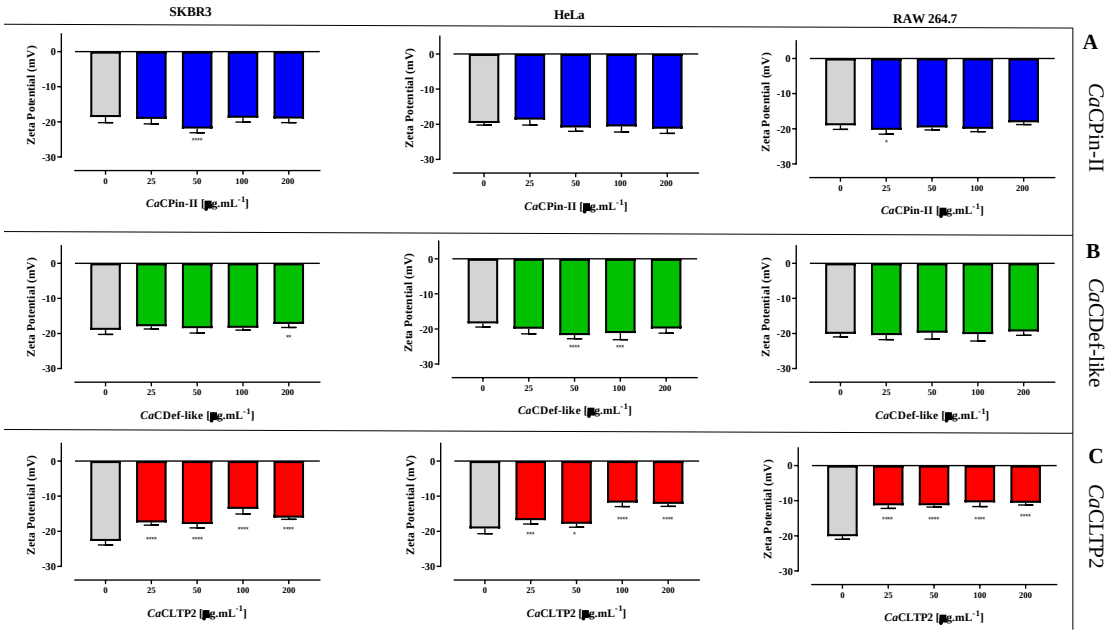

Fig. 3. Peptides interaction with cell surfaces. Zeta potential measurements of human breast cancer cell line SKBR3, human epithelial cervical cancer HeLa and murine macrophage RAW 264.7 were performed in the absence and presence of *Ca*CPin-II (A), *Ca*CDef-like (B) and *Ca*CLTP2 (C), at increasing peptide concentrations (25 to 200 µg.mL^{-1}). Dose response graphs were constructed and * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to negative control (0 µg.mL^{-1}) determined by Tukey test.

3.4. Plasma membrane permeabilization

After zeta potential results, together with the fact that *Ca*CPin-II and *Ca*CLTP2 peptides have hemolytic activity on sheep erythrocytes (Cherene et al., 2023a), we decided to evaluate membrane integrity of SKBR3, HeLa and RAW 264.7 cells after incubations with *Ca*CPin-II and *Ca*CLTP2. To investigate whether this peptides can damage membranes, cells were incubated with peptides. Following a period of 24 h, the permeabilization of the plasma membrane was investigated using Sytox Green. Fig. 4 shows that both untreated and peptide-treated cells did not present Sytox fluorescence, demonstrating that *Ca*CPin-II and *Ca*CLTP2 did not damage membranes.

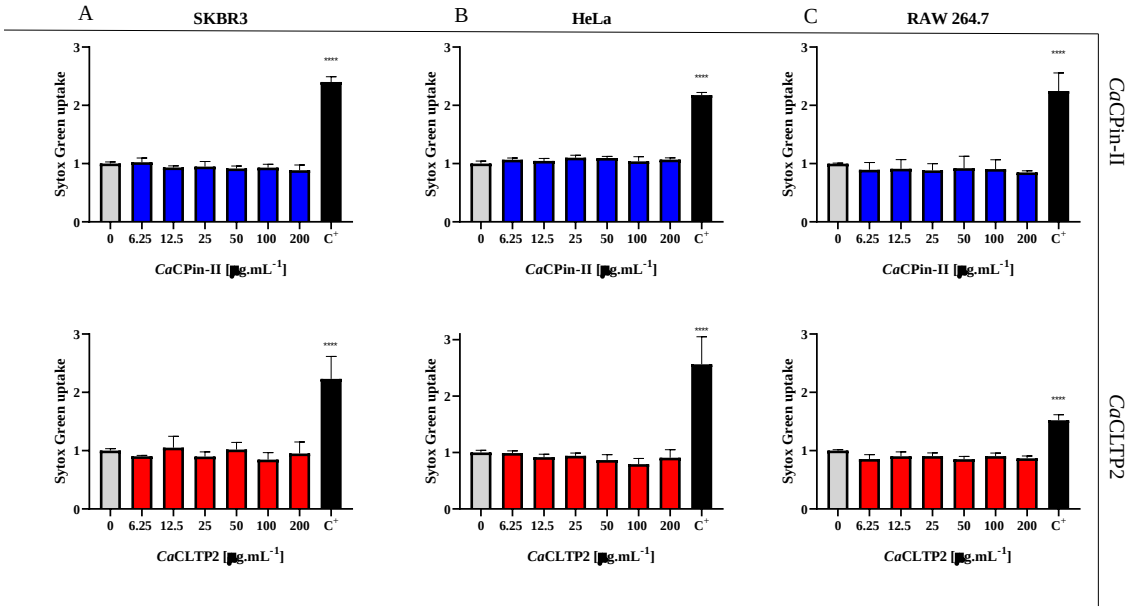

Fig. 4. *Ca*CPin-II and *Ca*CLTP2 membrane permeabilization assay. Sytox Green uptake by human breast cancer cell line SKBR3 (A), human epithelial cervical cancer HeLa (B) and murine macrophage RAW 264.7 (C) (1 = no uptake). Cells were incubated with peptides at increasing concentrations for 24h (6.25 to 200 µg.mL^{-1}) and Sytox Green probe was added after this period. Negative control: cells not treated. Positive control (C^+): 0.1% Triton. Fluorescence intensity was measured at 520 nm. The results presented are mean values obtained over three experiments, each done in triplicate. Dose response graphs were constructed and **** $p < 0.0001$ compared to negative control (0 µg.mL^{-1}) determined by Tukey test.

3.5. *In vivo* toxicity

To assess the correlation of *in vitro* observations with an *in vivo* situation, *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides were used for systemic toxicity studies in animals, with *G. mellonella* larvae as study model. The peptide concentration used in the *in vivo* test was twice as high as the maximum concentration used in *in vitro* cytotoxicity tests. After injecting the peptides into the larvae hemocoel, we observed that *Ca*CDef-like did not cause the death of any larvae over the 7 days of monitoring (Fig. 5). However, *Ca*CPin-II was the peptide that showed the greatest *in vivo* toxicity for *G. mellonella*

larvae, causing a significative (P = 0,0016) and progressive reduction in the survival rate, with 3% of death on day 3 to 40% of death on day 6, and after that day there were no more deaths until the end of the monitoring of the larvae. *Ca*CLTP2 caused the death of 8% of the larvae on the second day after the injection, and after that period the survival rate of the larvae remained the same until the last day of monitoring. There was no death of larvae that received PBS injection during the 7-day monitoring, and 7% of the larvae that received only needle injury (mock inoculation) died on day 6 of monitoring.

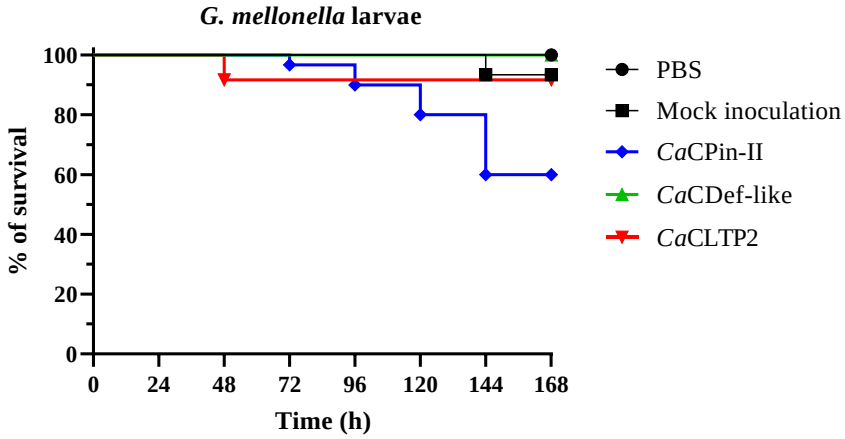

Fig. 5. Peptides *in vivo* systemic toxicity to *G. mellonella*. Kaplan-Meier plots of *G. mellonella* larvae survival after injection of *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 (400 µg.mL^{-1} or ~13 µg peptide/g larvae) using PBS as vehicle. There was no killing of larvae that received PBS. The assays were performed in duplicate (n=20).

4. Discussion

Despite the fact that AMPs are often described as promising candidates for the development of new therapies against drug resistant bacterial and fungal infections, some pharmacological characteristics hinder clinical development (Lei et al., 2019). In particular, toxicity has been poorly addressed despite fact that an AMP-based drug candidates need to show low or no toxicity to mammalian cells to advance in clinical tests (Greco et al., 2020; Mohs and Greig, 2017). Hemolysis is a versatile tool for rapid initial toxicity assessment due to ease associated with isolating erythrocytes (Farag and Alagawany, 2018) and it is not uncommon for erythrocytes from different species of mammals to be used in the initial screening phase (Greco et al., 2020). *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 hemolytic activity on sheep erythrocytes has already been described. *Ca*CPin-II has a HC50 of 270 µg mL^{-1}, *Ca*CDef-like has no hemolytic activity at concentrations up to 400 µg mL^{-1} and *Ca*CLTP2 showed a weak hemolytic effect at a concentration of 200 µg mL^{-1}, causing 1.7% hemolysis in tests using sheep erythrocytes (Cherene et al., 2023a). However, isolated and washed erythrocytes are more vulnerable cells than adhered cells in *in vitro* culture, and different methods of testing hemolytic activity can generate significant variations in results for the same compound (Greco et al., 2020; Helmerhorst et al., 1999). Therefore, it is important to study the *in vitro* cytotoxic activity of a compound using other cell lines and using standardized methods to obtain more accurate results.

We evaluated cell viability quantifying the metabolic conversion of resazurin to resorufin. Virtually all toxicological and pharmacological studies include at some point the assessment of cell viability and/or metabolic activity, and resazurin reduction is probably the most widely used method to assess the metabolic activity of cells (Vieira-da-Silva and Castanho, 2023). The results of the cytotoxic activity of the peptides showed that the cells tested did not have their metabolic activity reduced, even in the presence of

*Ca*CPin-II, which has weak hemolytic activity on sheep erythrocytes already described. Our results align with those of a study comparing the hemotoxicity of some synthetic AMPs using erythrocytes from different mammalian species and the cytotoxicity with immortalised human keratinocyte (HaCaT), human liver cancer cell (HepG2) and human epithelial cervical cancer cell (HeLa). This study showed that all these cell lines have more tolerance for the tested peptides in comparison to human erythrocytes and there was no direct relationship between hemolytic activity and cytotoxicity (Greco et al., 2020).

Membrane permeabilization and increased ROS production are among the most common modes of action of many AMPs which might lead to programmed cell death (PCD) pathway activation (Aerts et al., 2007; Kulkarni et al., 2009). ROS generally play an important role in cellular signaling and are produced in cells by means of normal physiological processes or by enzymatic and non-enzymatic mechanisms associated with pathological processes (Camini et al., 2017). Increased oxidative stress appears in the early stages of the apoptotic process (Kowaltowski et al., 2009) and has already been described as mechanisms of action employed by plant defensins and several other AMPs (Mello et al., 2011; Soares et al., 2017), as with *Candida tropicalis* yeasts treated with *Ca*CPin-II and *Ca*CDef-like (Cherene et al., 2023a). Our data suggest that *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides do not induce a pathological response in human cells, since they do not lead to an increase in ROS in MDA-MB-231, HeLa and PC-3 cell lines.

The amphipathic and cationic character of AMPs and their secondary structure are the main factors that determine the interactions between AMPs and their targets. (Buck et al., 2019; Choi et al., 2016). AMPs toxic effects usually involve electrostatic interactions with the cell surface. Most AMPs have positive net charges at neutral pH,

and their cellular selectivity is associated to their high affinity for the anionic lipid components of the membranes of microorganisms (Huang, 2006) and tumor cells (Harris et al., 2011; Schweizer, 2009). The outer layer of the cell membrane of mammalian cells, on the other hand, is closer to neutral and consists mainly of zwitterionic phospholipids and cholesterol, the latter further stabilizes the membranes to the action of AMPs (Huang, 2006; Sok et al., 1999). Furthermore, intracellular targets for AMPs have also been described, adding even more complexity to the mechanisms of action of these peptides (Freire et al., 2015; Li et al., 2021; Wei and Zhang, 2022; Yeaman et al., 2018). Therefore, to assess whether *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 peptides can interact with the outer surface of mammalian cell membranes, zeta potential of SKBR3, HeLa and RAW 264.7 cell lines was determined. Our data show that the peptides, except *Ca*CLTP2, had no electrostatic interaction with the surface of the membranes of these mammalian cells. Nonspecific LTPs (nsLTPs) are a class of AMPs found in all land plants. LTPs have a tunnel-like hydrophobic cavity that enables them to bind and transport various types of lipids (Edqvist et al., 2018; Melnikova et al., 2023). Thus, the cell membrane is considered a potential target for LTP antimicrobial action via hydrophobic and electrostatic interactions (Finkina et al., 2016).

No single general mechanism can be applied to explain the membrane effects of all cationic AMPs. Membrane permeabilization caused by cationic AMPs can occur by direct interaction with the membrane, as showed by four main models established to describe membrane-pore formation, *i.e.*, barrel-stave, toroidal-pore, carpet and aggregate models or by indirect intracellular action mode for acting in the regulation of important enzymes (Wei and Zhang, 2022). Plant defensins (Gebara et al., 2020; Mello et al., 2011; Soares et al., 2017) and lipid transfer proteins (LTPs) (Finkina et al., 2016; Salminen et al., 2016) can permeabilize membranes of microorganisms. Plant protease inhibitors can

affect cell membranes too, since they inhibit enzymes involved in important cellular events (Rudzińska et al., 2021). As many plant AMPs can permeabilize microorganisms cell membranes and *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 are able to permeabilize cell membrane of some *Candida* species (Cherene et al., 2023a), we evaluated the ability of these peptides to permeabilize mammalian cell membranes having an unwanted toxic effect. Furthermore, *Ca*CPin-II and *Ca*CLTP2 also have weak hemolytic activity (Cherene et al., 2023a). Our data suggests that peptides do not permeabilize mammalian cell membranes, while in the membrane permeabilization assay with the SYTOX Green probe there was no increase in fluorescence in SKBR3, HeLa or RAW 264.7 cells lines incubated with these peptides. Although *Ca*CLTP2 interacted with the surface of these cells lines according to the zeta potential assay data, this peptide did not cause membrane permeabilization. In this way, we suggest that the hemolytic effect already described for *Ca*CPin-II and *Ca*CLTP2 may have occurred due to the fact that erythrocytes are more vulnerable than other mammalian cell lines grown *in vitro*. Together, these data suggest that the peptides have some selective toxicity.

In order to obtain a more accurate toxicological analysis, it is important to correlate the *in vitro* toxicity results with an *in vivo* situation. *In vivo* tests allow the assessment of systemic toxicity, which is more complex and not achievable through cellular assays. Factors associated with metabolism of compound such as absorption, biotransformation, distribution and excretion may not be simulated in cell culture tests (Allegra et al., 2018; Van Vliet, 2011). For natural and synthetic membrane active AMPs, the hemolytic and cytotoxic experiments are often insufficiently backed up to provide an accurate prediction of an *in vivo* situation (Greco et al., 2020). Cationic and amphiphilic compounds, such as AMPs, are prone to rapidly associate with both major exogenic transport plasma proteins serum albumin and α-1 glycoprotein. Recent studies show the

various forms of binding of cationic AMPs with these carriers, which reduces the bioavailability of AMP so that it exerts its *in vivo* bioactivity and this is a parameter not accounted for in the cytotoxic experiments (Sivertsen et al., 2014; Svenson et al., 2007). Thus, some AMPs that showed cytotoxic activity may not have any pronounced toxic effects *in vivo* (Greco et al., 2020) while having physiological toxicity not revealed by cellular assays. We performed *in vivo* systemic toxicity tests using *G. mellonella* larvae as a study model. *G. mellonella* (greater wax moth) larvae is an established and widely used model for drug discovery, *in vivo* toxicity tests and host-pathogen interactions studies, as an intermediate invertebrate model between *in vitro* and mammalian *in vivo* trials (Serrano et al., 2023). They reflect aspects of the complexity present in mammals, such as an innate immune system that is structurally and functionally similar to that of mammals, and are accepted as an ethical alternative for research (Allegra et al., 2018; Cutuli et al., 2019; Piatek et al., 2021). The data obtained regarding the peptides *in vivo* toxicity are in agreement with the data already described for each one of them. The protease inhibitor *Ca*CPin-II despite not showing *in vitro* toxicity for the mammalian cells tested, showed toxicity in the *in vivo* test. The *Ca*CPin-II toxicity for *G. mellonella* larvae may be related to at least one of its properties already described in the literature: its ability to inhibit important enzymes and its insecticidal activity (Cherene et al., 2023a; Cherene et al., 2023b). Several Pin-II protease inhibitors have already been isolated and have shown insecticidal activity (Mishra et al., 2010; Yadav et al., 2021). Although some plant defensins (Kovaleva et al., 2020) and LTPs (Maximiano and Franco, 2021) have already been described with insecticidal activity, the defensin-like *Ca*CDef-like and the nsLTP *Ca*CLTP2 did not show *in vivo* toxicity to *G. mellonella* larvae. *Ca*CDef-like peptide has no cytotoxicity for any of the mammalian cell lines tested and are not hemolytic (Cherene et al, 2023a). In fact, currently some of the few AMPs that are in clinical testing are

defensins (Hein et al., 2022). *Pv*D_1 is a plant defensin that shows antifungal activity against four different *Candida* species and does not show *in vivo* toxicity to *G. mellonella* larvae (Skalska et al., 2020). Pezadeftide, a derivative of a plant defensin, is in phase IIa clinical trials for the treatment of fungal nail disease (Hein et al., 2022). Plant LTPs present a broad range of versatile bioactivities and most studies showed low or no toxicity to mammalian cells, but the biggest challenges pointed out for the use of LTPs in human health are the difference between the results found in *in vitro* assays and in pre-clinical or clinical tests, since these molecules can trigger side effects, such as strong allergic reactions (Edqvist et al., 2018; Maximiano and Franco, 2021).

5. Conclusion

Our data suggest that *Ca*CPin-II, *Ca*CDef-like and *Ca*CLTP2 may have biotechnological applications for the development of new drugs and therapies. Due to its *in vivo* toxicity and hemolytic activity, *Ca*CPin-II protease inhibitor can be used as a framework for the development of bioinspired AMPs with a higher selective toxicity and for the development of antimicrobials and insecticides that do not involve direct application in mammals. *Ca*CDef-like and *Ca*CLTP2 should thus be safe for animal use, as they showed little or no toxicity. Our data points to the *Ca*CDef-like peptide as the safest antifungal candidate, as it had good results in all tests. However, for this purpose, other toxicological tests using mammals as a study model are necessary. As *Ca*CLTP2 was able to bind to the cell surface without causing damage, we suggest that this peptide can still be used for the development of drug-delivering systems.

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6 - CONCLUSÕES GERAIS

Conclusões Gerais

A maioria dos AMPs de plantas do gênero *Capsicum* foram isolados de sementes e frutos. *Ca*CPin-II, *Ca*CDef-like e *Ca*CLTP2 são AMPs isolados de folhas de *C. annuum* cv. Carioquinha através de um método simples de extração e cromatografia em de fase reversa em HPLC. Uma fração enriquecida de *Ca*CPin-II pode ainda ser obtida através da centrifugação do extrato, sem necessidade de métodos cromatográficos, facilitando sua obtenção em maiores quantidades, o que torna possível a execução de ensaios que requerem grandes quantidades do peptídeo.

Os resultados deste trabalho demonstram que os peptídeos isolados possuem potencial como agentes antimicrobianos contra espécies do gênero *Candida*, o que abre caminho para testes com outros microrganismos patogênicos. Apesar de *Ca*CPin-II, *Ca*CDef-like e *Ca*CLTP2 não possuírem forte efeito fungicida com as leveduras testadas, a elucidação da suas estruturas e possíveis mecanismos de ação torna possível ainda a criação de novos peptídeos sintéticos bioinspirados com maior toxicidade seletiva.

*Ca*CPin-II possui atividade hemolítica e é tóxico para o modelo de estudo *in vivo* testado, no entanto, este peptídeo tem potencial para ser usado no desenvolvimento de antimicrobianos e pesticidas que não envolvam a aplicação direta em mamíferos ou para antimicrobianos de uso tópico. Este peptídeo possui ainda potencial para criação de métodos para o controle de pragas agrícolas menos tóxicos. *Ca*CDef-like e *Ca*CLTP2 parecem ser seguros para o desenvolvimento de fármacos para uso em animais, porém mais testes usando mamíferos como modelo de estudo precisam ser feitos.

Conclui-se que os peptídeos isolados possuem potencial biotecnológico para a criação de novas terapias frente a emergência de patógenos multi-resistentes e para a criação de insumos mais sustentáveis para a indústria e para cadeia produtiva de alimentos.