CARACTERIZAÇÃO E ATIVIDADE ANTIMICROBIANA DE PEPTÍDEOS COM AFINIDADE À QUITINA DE SEMENTES DE PLANTAS DO GÊNERO *Capsicum*

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CAMPOS DOS GOYTACAZES – RJ
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Tese apresentada ao Centro de Biociências e Biotecnologia da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Doutora em Biotecnologia Vegetal.

Orientadora: Profª. Drª. Valdirene Moreira Gomes.

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SUMÁRIO

RESUMO

GONÇALVES, Gabriella Rodrigues. D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro. Março de 2024; **Caracterização e atividade antimicrobiana de peptídeos com afinidade à quitina de sementes de plantas do gênero *Capsicum***. Orientadora: Profª Valdirene Moreira Gomes.

Nos últimos anos tem havido vários relatos da presença de proteínas antimicrobianas em espécies vegetais cultivadas ou silvestres, que estão implicadas nos mecanismos de defesa das plantas. A existência destas proteínas levanta a possibilidade de aplicações biotecnológicas dando origem ao desenvolvimento de novas técnicas para combater doenças causadas por fungos. Neste contexto, temos as proteínas de ligação à quitina. A quitina é um polímero de N-acetil-D-glucosamina, componente essencial da parede celular de fungos, sendo, portanto, as proteínas de ligação à quitina importantes no controle do crescimento desses organismos. Dessa forma, o objetivo desta tese foi isolar, caracterizar e avaliar o efeito antifúngico *in vitro* de peptídeos com propriedades de ligação à quitina, isolados de sementes de quatro espécies de *Capsicum*, no crescimento de fungos dos gêneros *Candida* e *Fusarium* e *in vivo* no modelo invertebrado de *Galleria mellonella*. Essa tese é dividida em três capítulos, sendo que o primeiro capítulo aborda a identificação e atividades biológicas *in vitro* e *in vivo* de uma fração de *C. annuum* ligante à quitina, denominada *Ca*-F2. O segundo capítulo refere-se a identificação e atividades biológicas *in vitro* e *in vivo* de frações com ligação à quitina isoladas de *Capsicum*, sendo *Cb*-F2 de *C. baccatum* e *Cf*-F2 de *C. frutescens.* E o terceiro e último capítulo da tese descreve o isolamento, caracterização e atividade de uma fração peptídica ligante à quitina isolada de *C. chinense*, denominada *Cc*-F2. Resumidamente, os resultados indicam que as frações identificadas nestes estudos possuem atividade antifúngica, causam a diminuição da viabilidade celular e podem causar permeabilização nas membranas de leveduras do gênero *Candida*. Peptídeos isolados de cada fração foram submetidos à espectrometria de massas. Os resultados mostraram que *Ca*-F2 apresentou similaridade com proteínas da família das albuminas 2S, as frações *Cb*-F2 e *Cf*-F2 apresentaram similaridade com proteínas transportadoras de lipídios (LTPs) e a fração *Cc*-F2 apresentou similaridade com heveína e endoquitinase. *Ca*-F2 não causou hemólise em células de mamíferos, e quando testadas *in vivo*, as frações *Ca*-F2, *Cb*-F2 e *Cf*-F2 não foram tóxicas em larvas de *G. mellonella*. Conclui-se que as frações contendo as proteínas identificadas possuem potencial para o desenvolvimento de novas moléculas antifúngicas, em especial contra espécies de importância médica do gênero *Candida.*

ABSTRACT

GONÇALVES, Gabriella Rodrigues. D.Sc., North Fluminense State University – Darcy Ribeiro. March 2024; **Characterization and antimicrobial activity of peptides with affinity for chitin from seeds of *Capsicum plants***. Advisor: Prof. Valdirene Moreira Gomes.

In recent years, there have been several reports of the presence of antimicrobial proteins, which are implicated in plant defense mechanisms, in cultivated or wild plant species. The presence of these proteins raises the possibility of biotechnological applications leading to the development of new techniques to combat diseases caused by fungi. In this context, we have chitin-binding proteins. Chitin is a polymer of N-acetyl-D-glucosamine, an essential component of the fungal cell wall, and chitin-binding proteins are therefore important for controlling the growth of these organisms. Thus, the objective of this thesis was to isolate, characterize and evaluate the *in vitro* antifungal effects of peptides with chitin-binding properties isolated from seeds of four *Capsicum* species on the growth of fungi of the genera *Candida* and *Fusarium* and *in vivo* on the model invertebrate *Galleria mellonella*. This thesis is divided into three sections, with the first section addressing the identification and *in vitro* and *in vivo* biological activities of a *C. annuum* chitin-binding fraction called *Ca*-F2. The second chapter refers to the identification and *in vitro* and *in vivo* biological activities of chitin-binding fractions from *C. baccatum* called *Cb*-F2 and from *C. frutescens* called *Cf*-F2, and the third and final chapters of the thesis discuss the isolation, characterization and activity of a chitin-binding peptide fraction isolated from *C. chinense*, here called *Cc*-F2. Briefly, the results indicate that the fractions identified in this study have antifungal activity, cause a decrease in cell viability and can cause permeabilization of the membranes of yeasts of the genus *Candida*. Peptides isolated from each fraction were subjected to mass spectrometry. The results showed that *Ca*-F2 was similar to proteins from the 2S albumin family, that the *Cb*-F2 and *Cf*-F2 fractions were similar to lipid transfer proteins (LTPs), and that the *Cc*-F2 fraction was similar to hevein and endochitinase. *Ca*-F2 did not cause hemolysis in mammalian cells, and when tested *in vivo*, the *Ca*-F2, *Cb*-F2 and *Cf*-F2 fractions were not toxic to *G. mellonella* larvae. It is concluded that the fractions containing the identified proteins have potential for the development of new antifungal agents, especially against medically important species of the genus *Candida*.

1 – INTRODUÇÃO

1.1 – Introdução geral

Infecções fúngicas sempre foram um grande problema mundial e nos últimos anos estas têm se agravado de forma sistemática. Elas variam de infecções superficiais a doenças sistêmicas que podem ser fatais, comumente são desafiadoras de serem diagnosticadas e tratadas nos estágios iniciais da doença, devido à complexidade dos fungos. Isso resulta no agravamento das infecções, tornando os procedimentos de tratamento mais complicados (Ramage *et al*., 2009; Reddy *et al*., 2022). Essas infecções, geralmente oportunistas, tendem a se manifestar principalmente em pacientes com sistema imunológico comprometido, como aqueles com HIV e câncer (Cui *et al*., 2022; Reddy *et al*., 2022).

O cenário ficou ainda pior devido à resistência crescente dos fungos aos medicamentos antifúngicos disponíveis, tanto para infecções humanas como animais, bem como para fitopatógenos, que interferem na produção de alimentos (Cui *et al*., 2022; Guillot; Bond 2020; Wang *et al*., 2022). Um dos fatores que contribuem para esse cenário de resistência, é o uso indevido e descontrolado de medicamentos, tornando ineficazes os tratamentos disponíveis atualmente (Akram *et al*., 2023; Sharma *et al*., 2018). A resistência microbiana emerge como uma grave preocupação à saúde pública (Berman; Krysan 2020), já que os fungos podem reduzir os efeitos dos medicamentos através de vários mecanismos de resistência, como o redirecionamento da droga, alteração da via metabólica, redução da absorção da concentração da droga, adaptação e alteração da parede celular e formação de biofilmes (Cui *et al*., 2022).

As espécies do gênero *Candida*, em especial, representam uma ameaça global à saúde pública, com frequentes surtos em hospitais e altas taxas de mortalidade (Pinto-Magalhães *et al*., 2019). Existem aproximadamente 150 espécies de *Candida* spp., mas um número pequeno foi considerado como patógeno humano. Essas leveduras fazem parte da microbiota humana normal e podem ser encontradas em membranas mucosas (30-60%), em tratos gastrointestinal e geniturinário de pessoas saudáveis, sendo que a interação microrganismo-hospedeiro ocorre ao longo da vida. Dessa forma, é possível que uma pessoa interaja com essa levedura diversas vezes sem grandes complicações (Perlroth *et al*., 2006; Blanco; Garcia 2008; da Fonseca *et al*., 2022). Entretanto, alterações na defesa do hospedeiro constituem fatores que podem favorecer a invasão sistêmica desse gênero de levedura em humanos, representando os principais agentes

envolvidos em infecções nosocomiais com alta taxa de mortalidade, principalmente em países subdesenvolvidos e em desenvolvimento (Salama; Gerstein, 2022).

Atualmente, existe um número limitado de medicamentos antifúngicos eficazes disponíveis, sendo que sua grande maioria causa efeitos adversos nos pacientes, limitando as opções terapêuticas (de Ullivarri *et al*., 2020). A partir dessas informações, existe uma grande necessidade de desenvolvimento de novas moléculas ativas e eficazes para tratamentos bem-sucedidos (Campoy; Adrio 2017; Amaral *et al*., 2021). Neste cenário, os peptídeos antimicrobianos (AMPs) estão entre os candidatos promissores para o desenvolvimento de novos agentes antifúngicos (Konakbayeva; Karlsson, 2023). Sua rápida ação, seletividade e por serem menos propícios a induzir seleção de resistência, tornam esse grupo um bom candidato ao desenvolvimento de novos agentes antifúngicos (Bradshaw, 2003). Esses peptídeos apresentam característica comuns, como pequenas sequências de aminoácidos (entre 10 e 100 resíduos), possuem carga líquida positiva e são moléculas anfipáticas, podendo interagir com diversas membranas e diversos alvos biológicos (Torres *et al*., 2019).

Nesse contexto, entre as várias famílias em que os AMPs estão inseridos, temos a família dos peptídeos e proteínas de ligação à quitina, também chamadas de "chitin binding proteins" (*CBP family*). Essas moléculas possuem capacidade de ligação reversível à quitina, um polissacarídeo estrutural presente em diversos organismos, incluindo a parede celular fúngica. Estudos relatam a atividade antifúngica dos CBPs de plantas contra diversos patógenos fúngicos (Loo *et al*., 2021; Saeed *et al*., 2021) e *Candida* spp. (Gonçalves *et al*., 2024; Lopes *et al*., 2020).

Os peptídeos e/ou proteínas de ligação à quitina podem ser utilizados no controle de combate a microrganismos patogênicos, devido a sua atividade antimicrobiana. Também podem ser isolados de diversas fontes e de diversos órgãos de plantas (Al Akeel *et al*., 2017). Um exemplo é a planta *Moringa oleifera*, que apresenta alto valor nutricional e medicinal. Suas sementes são coagulantes naturais, utilizados para purificar água turva, seus extratos aquosos e etanólicos de folhas são conhecidos por exibirem propriedades anti-inflamatórias, antimicrobianas, antidiabéticas e possuem efeito na redução do colesterol e na estabilização da pressão arterial. Existem diversos relatos na literatura de peptídeos de ligação à quitina isolados e caracterizados a partir de órgãos dessa planta. Em 2017, Kini *et al*., caracterizaram dois novos peptídeos heveína-símile da casca da árvore de *M. oleifera*, denominados mO1 e mO2, que inibiram o crescimento de fungos

fitopatogênicos, corroborando com os relatos mencionados acima. Dessa forma, reforça-se o grande potencial biotecnológico dessas moléculas.

1.2 – Proteínas e peptídeos de ligação à quitina com potencial antimicrobiano

A quitina, um homopolímero insolúvel e formado por monômeros do tipo N-acetil-D-glucosamina unidos por ligações glicídicas β-1-4, é encontrado em exoesqueletos de insetos, carapaça de crustáceos, cascas de ovos de nematóides e representa um dos principais constituites das paredes celulares dos fungos. A elevada taxa de produção de quitina por esses organismos, faz com que ela seja o segundo polissacarídeo mais abundante da natureza, perdendo apenas para a celulose (Hashimoto *et al*., 2000; Kadokura *et al*., 2007; Kurita, 2001). Em fungos, a quitina constitui entre 3 a 60% da massa total da parede celular e é responsável pela rigidez, força física e formato específico da parede celular (Lipke; Ovalle, 1998).

Essas proteínas se ligam à quitina por apresentarem domínios de ligação à quitina (*Chitin binding domain* - CBD) (Asensio *et al*., 2000). Este domínio de ligação à quitina cuja sequência de aminoácidos é conhecida contêm um motivo estrutural comum de 30 a 43 aminoácidos com diversas cisteínas e glicinas em posições conservadas, também conhecidas como domínio heveínico (Raikhel; Lee, 1993; Trindade *et al*., 2006). As proteínas que fazem parte dessa família não se ligam única e exclusivamente à quitina, podendo ligar-se a vários complexos glicoconjugados contendo N-acetil-D-glicosamina ou ácido N-acetil-D-neuramínico. As plantas, especialmente nas sementes, desenvolveram um sistema de imunidade inata que atua como a primeira linha de defesa contra uma ampla variedade de patógenos microbianos, desempenhando um papel crucial na proteção contra várias agressões (Li *et al*., 2021). Essa defesa conta com a presença de metabólitos secundários e uma vasta gama de proteínas e peptídeos, entre estes as proteínas de ligação à quitina (CBP). Como exemplo de uma das principais famílias de proteínas de ligação à quitina, podemos citar as enzimas quitinolíticas, as quais hidrolisam as ligações glicosídicas β-1,4 e degradam a quitina. As quitinases mais conhecidas contêm duas partes distintas: um domínio de ligação à quitina (CBD) e um domínio catalítico. O CBD auxilia na ligação da enzima à quitina presente na parede celular fúngica, aumentando a atividade lítica do domínio catalítico (Huang *et al*., 1997; Iseli *et al*., 1993; Taira *et al*., 2002; Yan *et al*., 2008). Além das quitinases, os CBDs estão presentes em

outras famílias de proteínas, como na heveína, em algumas lectinas e em algumas famílias de peptídeos antimicrobianos. Essas proteínas possuem suas características singulares, mas além disso, possuem atividade contra uma ampla gama de patógenos microbianos, incluindo patógenos fúngicos. Entre os peptídeos antimicrobianos, os *Ac*-AMP1 e o *Ac*-AMP2 foram os primeiros descritos. Estes apresentam 29 e 30 aminoácidos, respectivamente, e possuem sequências idênticas, exceto por um resíduo adicional de arginina na região C-terminal do *Ac*-AMP2. Os *Ac*-AMPs são principalmente ricos em cisteína (seis resíduos), glicina (sete resíduos) e aminoácidos básicos. A sequência de aminoácidos dos *Ac*-AMPs possui grande similaridade com os domínios ricos em cisteína/glicina das proteínas de ligação à quitina e apresentam três pontes dissulfeto (Broekaert *et al*., 1992; De Bolle *et al*., 1993). Os *Ac*-AMPs são as proteínas antifúngicas mais potentes da família de ligação à quitina. Estudos realizados por Broekaert *et al*., (1992) abrangendo diferentes fungos fitopatogênicos, revelaram que as concentrações requeridas para obter 50% de inibição do crescimento fúngico variaram de 2 a 10 $\mu g.mL^{-1}$, dependendo do fungo testado.

Em relação a estes peptídeos, podemos citar e descrever algumas de suas importantes propriedades, tais como: *Ac*-AMP1 e *Ac*-AMP2, que foram isolados de sementes de *Amaranthus caudatus* e inibiram o crescimento de diferentes fungos fitopatogênicos, além de mostrarem atividade contra bactérias (Broekaert *et al*., 1992); e *Pp*-AMP1 e *Pp*-AMP2, que foram purificados a partir de brotos de *Phyllostachys pubescens* e tiveram sua atividade antimicrobiana avaliada contra *Erwinia carotovora*, *Agrobacterium radiobacter*, *A. rhizogenes*, *Clavibacter michiganensis*, *Curtobacterium flaccumfacien*, *Fusarium oxysporum* e *Geotrichum candidume* na concentração necessária para inibir 50% do crescimento (IC50) dos microrganismos, que variaram entre 2 a 25 (μg/mL) (Fujimura *et al*., 2005). Podemos citar ainda o *Lm*CBP1 extraído de *Brassica napus* (Liu *et al*., 2020), o CBL (lectina ligante à quitina) extraído de *Solanum integrifolium*, que apresentou inibição do crescimento contra *Colletotrichum gloeosporioides* e *Rhizoctonia solani* com um diâmetro de inibição de zona de 8 mm e 12 mm, respectivamente (Chen *et al*., 2018), o *Sm*AMP3 extraído de folhas de *Stellaria media*, que apresentou atividade antifúngica contra *F. solani*, *Alternaria alternata*, *Botrytis cinerea*, entre outros, com IC50 de 3,7, 5,0 e 1,6 mM, respectivamente (Rogozhin *et al*., 2015), e aSG1-3 e aSR1-3 extraídos de *Alternanthera sessilis*, que não tiveram atividade antibacteriana significativa em concentrações ≤100 μM. (Kini *et*

al., 2015). Tratando-se de plantas de pimenta, foi reportado o gene *Ca*ChiIV1, isolado de plantas de *Capsicum annuum* L., regula a resposta ao fungo *Phytophthora capsici* e estresses abióticos, como metil-jasmonato (MeJA), peróxido de hidrogênio (H2O2), melatonina e manitol (Ali *et al*., 2019).

Outro exemplo é a proteína *Iu*-CBP, identificada e caracterizada a partir de sementes de *Iberis umbellata*. Ela apresenta baixa massa molecular e possui similaridade com albumina 2S e atividade antibacteriana contra *Bacillus subtilis*, *Xanthomonas oryzae*, *Staphylococcus aureus*, *Escherichia coli* e *Pseudomonas aeruginosa*, em concentrações de 5,0 e 10,0 µg/disco. Esta proteína também inibiu o crescimento micelial de *Aspergillus flavus* na concentração de 20 µg/disco (Saeed *et al*., 2021). Na Tabela 1 estão apresentados alguns dos principais peptídeos de ligação à quitina e proteínas de baixa massa molecular isolados de plantas nos últimos anos, bem como algumas das principais atividades biológicas.

Tabela 1. Identificação de alguns peptídeos e proteínas de ligação à quitina isolados de plantas e algumas de suas atividades biológicas.

CBP	Planta	Atividade	Referências
Pn_AMP1 e 2	*Pharbitis nil*	Atividade antifúngica	Slavokhotova *et al*., 2017
Mo-CBP2	*M. oleífera*	Atividade contra *Candida*	Silva Neto *et al*., 2020
Mo-CBP3	*M. oleífera*	Atividade contra fitopatógenos	Freire *et al*., 2015
Mo-CBP4	*M. oleífera*	*Trichophyton* e *Candida*	Lopes *et al*., 2020
WSMoL	*M. oleífera*	Atividade inseticida	de Oliveira *et al*., 2017
mO1 e mO2	*M. oleífera*	Atividade contra fitopatógenos	Kini *et al*., 2017
Ee_CBP	*Euonymus europaeus*	Atividade contra fitopatógenos	Slavokhotova *et al*., 2017
CJP-4	*Cryptomeria japônica*	-	Takashima *et al*., 2018
6-Cys-CB-HLPs	*Chenopodium quinoa*	Atividade antifúngica	Loo *et al*., 2021
Iu-CBP	*I. umbellata*	Atividade antibacteriana	Saeed *et al*., 2021
DrChit	*Drosera rotundifolia*	*Trichoderma viride*	Durechova *et al*., 2019
EuCHIT2	*E. ulmoides*	*Erysiphe cichoracearum*	Dong *et al*., 2017
CaChiIV1	*C. annuum*	*Phytophthora capsici*	Ali *et al*., 2019
PsV	*Pisum sativum*	Atividade antifúngica	Saeed *et al*., 2022
CBL	*S. integrifolium*	Atividade antifúngica	Chen *et al*., 2018
MdPR4	*Malus domestica*	Atividade antifúngica	Zhou *et al*., 2021
Ca-F2	*C. annuum*	Atividade contra *Candida*	Gonçalves *et al*., 2024

Em seguida, serão abordadas algumas famílias de peptídeos e proteínas que foram identificadas nesse trabalho e que apresentaram propriedades de ligação à matriz de quitina.

1.3 – Albumina 2S

O termo albumina foi dado para um grupo restrito de proteínas solúveis em água (Osborne, 1924). Com o avanço dos estudos foi indicado a existência de um tipo de proteínas de armazenamento de sementes (SSPs) chamado albuminas 2S (Youle; Huang 1979). Posteriormente, comparações revelaram que albuminas 2S fazem parte de um grupo de proteínas classificadas na superfamília das prolaminas, na qual também estão incluídas as proteínas transportadoras de lipídeos (LTPs), inibidores bifuncionais de α-amilase/serino proteases e prolaminas de cereais (Radauer; Breiteneder, 2007; Shewry *et al*., 1995).

As albuminas 2S são proteínas de baixa massa molecular e alto teor de glutamina, arginina, asparagina e cisteína. Além disso, compartilham propriedades físico-químicas semelhantes, como a presença de resíduos catiônicos e alta estabilidade devido às ligações dissulfeto (Souza, 2020). Sua função principal é a de armazenamento, para ser utilizada na germinação e no crescimento das plantas, como fonte de nitrogênio, carbono e enxofre (Shewry *et al*., 2006). Após a germinação, as albuminas 2S são degradadas a fim de fornecer aminoácidos para o desenvolvimento das sementes (Youle; Huang, 1979). Elas são divididas em duas subunidades: uma subunidade pequena, de 3–4 kDa, que contém os resíduos de cisteína 1 e 2, e uma subunidade grande, de 8–10 kDa, que contém os resíduos de cisteína 3 a 8 (Souza, 2020).

Várias albuminas 2S apresentam atividade inibitórias contra α-amilases, serino proteases, crescimento bacteriano e fúngico, bem como atividade inseticida, o que indica o papel das albuminas 2S na defesa de sementes (Souza, 2020). Foi observado que Rc-2S-Alb, uma albumina 2S purificada de sementes de *Ricinus communis*, apresentou alta atividade contra *P. aeruginosa, Klebsiella pneumonae* e *B. subtilis*, sendo que 0,26 $\mu g.mL^{-1}$ foi a concentração que apresentou a maior atividade inibitória sobre o crescimento de todas as bactérias testadas (Souza *et al*., 2016). Além disso, sabe-se que Rc-2S-Alb é rica em resíduos de arginina, o que pode favorecer o potencial eletrostático positivo da proteína, aumentando a interação eletrostática com a porção negativa da

membrana bacteriana, levando a permeabilização da membrana, perda do conteúdo citoplasmático e morte celular.

As albuminas 2S apresentam atividade sobre fungos patogênicos humanos, como leveduras do gênero *Candida* e dermatófitos do gênero *Trichophyton*, além de bactérias (Lopes *et al*., 2020). Uma albumina 2S foi purificada de sementes de pimenta (*C. annuum* L.) que apresentou atividade contra as leveduras *C. albicans*, *C. parapsilosis*, *C. tropicalis* e *C. guilliermondii*, responsáveis por infecções principalmente em pacientes imunossuprimidos (Ribeiro *et al*., 2007). Também foi purificada uma proteína de ligação à quitina de *M. oleífera*, denominada Mo-CBP2, que apresentou similaridade com albumina 2S e demonstrou atividade anti-*Candida*. Foi observado que Mo-CBP2 induziu a formação de poros nas membranas celulares de *Candida* e induziu a superprodução de espécies reativas de oxigênio (ROS) (Neto *et al*., 2017). Outro estudo revelou que proteínas de ligação à quitina isoladas de sementes de *C. annuum* apresentaram similaridade com albumina 2S e mostraram atividade sobre as leveduras *C. albicans* e *C. tropicalis*. Foi verificado nesse estudo que o possível mecanismo de ação pelo qual a proteína age sobre as leveduras é a permeabilização de membranas (Gonçalves *et al*., 2024).

As albuminas 2S destacam-se como inibidoras de fungos fitopatogênicos, responsáveis por significativas perdas na agricultura. A atividade antifúngica dessas albuminas tem sido intensamente estudada e os resultados têm demonstrado notável inibição fúngica (Souza, 2020). A albumina 2S citada anteriormente como Rc-2S-Alb, apresentou atividade inibidora de tripsina e além disso, apresentou atividade sobre os fitopatógenos *F. oxysporum* e *R. solani*, sendo capaz de aglutinar os esporos dos patógenos citados (Souza *et al*., 2016). Foi também relatado que a proteína de ligação à quitina Mo-CBP3 apresentou potencial inibitório sobre *F. solani*, *F. oxysporum*, *C. musae* e *C. gloesporioides*, todos eles de elevada importância para a agricultura. Mo-CBP3 apresentou ação fungicida e fungistática, de acordo com a concentração utilizada e além disso, o mecanismo de ação apresentado pela proteína é apoiado pela interação com a superfície celular do fungo por meio de interações eletrostáticas, uma vez que o sal reduz seu efeito inibitório. A proteína induziu o aumento da produção de ROS e causou a formação de poros na membrana plasmática. Além do exposto, Mo-CBP3 não apresentou efeitos tóxicos para células humanas, indicando grande potencial para o desenvolvimento de novos medicamentos antifúngicos (Mota *et al*., 2019).

1.4 – Proteínas transportadoras de lipídeos (LTPs)

As proteínas transportadoras de lipídios (LTP) foram inicialmente descritas em batata (*S. tuberosum*) por Kader, (1975). Elas possuem esse nome devido a sua capacidade em transportar fosfolipídeos e galactolipídeos entre um doador e uma membrana aceitadora durante ensaios *in vitro* (Kader, 1996). Elas possuem uma cavidade hidrofóbica similar a um túnel que é capaz de acomodar lipídeos e outros ligantes (Edqvist *et al*., 2018). Acredita-se que muitas das funções desempenhadas pelas LTPs estão associadas à sua capacidade de se ligar e transportar lipídios e outras moléculas hidrofóbicas (Amador *et al*., 2021). Existem vários tipos de nomenclaturas para difenciar as LTPs, porém, uma muito utilizada é de acordo com sua massa molecular. As LTP1 possuem massa molecular de aproximadamente 9 kDa, enquanto as LTP2 apresentam massa de 7 kDa. Além de serem diferentes na identidade da sequência primária (menos de 30% de similaridade), são também distintas na eficiência de transferência lipídica (Kader, 1996).

Sua região mais conservada é o motif 8CM, sendo que a diferença entre as LTPs é devida à natureza das ligações dissulfeto, com maior variação nas cisteínas 5 e 6, que auxiliam nos efeitos funcionais na estrutura terciária. Outra região conservada importante corresponde a uma glicina entre as hélices 1 e 2, que são conectadas por ligações dissulfeto entre C2 e C3 (Figura 1A). Resíduos que também são bastante conservados nas LTPs são a lisina e tirosina (Fleury *et al*., 2019). Diferenças nessas regiões e motif podem tanto interferir nos seus mecanismos, como podem contribuir para suas estruturas e propriedades biológicas (Amador *et al*., 2021).

A estrutura tridimensional das LTPs é identificada por possuir um domínio compacto que usualmente é composto por quatro hélices (H1-H4) conectadas por laços curtos (L1-L3), além de uma cauda C-terminal desestruturada (Amador *et al*., 2021) (Figura 1B). Elas apresentam cavidade interna em formato de túnel para acomodar os lipídeos e que também auxiliam na estabilidade contra processamento térmico e digestivo (Liu *et al*., 2010). Nas LTPs tipo 1, geralmente a H2 e H3 e o loop C-terminal são mais longos. O domínio é unido por diversas ligações de hidrogênio intramoleculares e por quatro pontes de enxofre conservadas nos padrões de C1-C6, C2-C3, C4-C7 e C5-C8 para LTPs1 (Gincel *et al*., 1994) e C1-C5, C2-C3, C4-C7 e C6-C8 para as LTPs tipo 2 (Pons *et al*., 2003).

As LTPs apresentam diferentes papéis nas plantas, destacando-se a defesa contra patógenos como um dos mais importantes. Diversos trabalhos relataram o isolamento e atividade dessas LTPs contra diversos microrganismos, como leveduras (Zottich *et al*., 2011) e fitopatógenos. McLaughlin e colaboradores (2021) superexpressaram uma LTP em trigo transgênico que foi capaz de diminuir o crescimento de *F. graminearum* em duas linhagens, reduzindo também o tamanho de uma lesão fùngica em ensaios realizados em folhas. As LTPs também apresentam atividade antimicrobiana contra bactérias, como as CmLTPs isoladas de *Chelidonium majus* que demostraram atividade contra *Campylobacter jejuni*, *Listeria greyi* e *Clostridium perfringens* (Nawrot *et al*., 2014). Seu mecanismo de ação também pode envolver a secreção destas proteínas no apoplasto permitindo que se liguem a moléculas lipídicas secretadas por plantas, como ácido jasmônico ou secretadas pelos patógenos (Finkina *et al*., 2016). Dessa forma, esses peptídeos se tornaram candidatos promissores para o desenvolvimento de novas drogas para combater patógenos humanos e de importância agrícola (Souza *et al*., 2018).

As LTPs também estão relacionadas com o desenvolvimento das sementes, germinação (Finkina *et al*., 2016; Safi *et al*., 2015), amadurecimento de frutos (Tomassen *et al*., 2007 e estão envolvidas no estresse abiótico das plantas. Hairat et al. (2018), por exemplo, demonstraram que duas LTPs de trigo que foram superexpressas em *A. thaliana*, aumentaram a tolerância desses indivíduos transformados ao estresse salino. Além desses, outros papéis são designados para as LTPs (Amador *et al*., 2021).

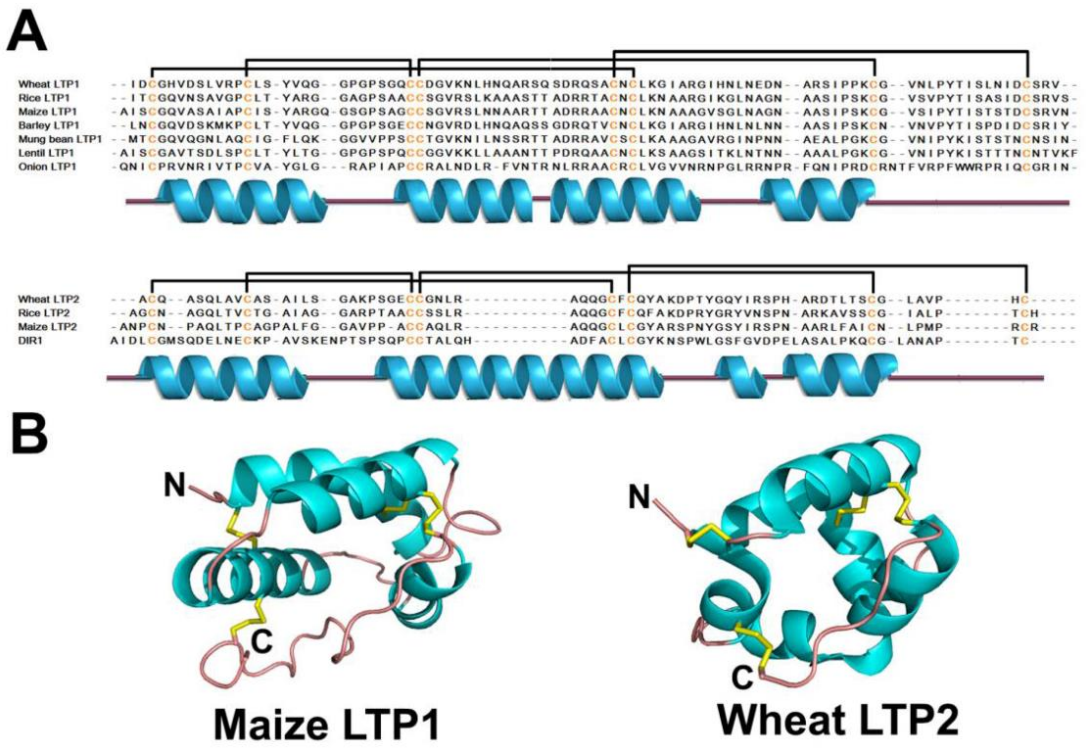

Figura 1. Sequências (A) e estruturas (B) de exemplos de proteínas transportadoras de lipídios. A estrutura secundária é representada por cores diferentes: azul - α-hélice; rosa – espiral aleatória; amarelo - ligações dissulfeto (Tam *et al*., 2015).

Devido a sua multifuncionalidade, essas moléculas são alvos de diversos estudos biotecnológicos para as plantas, já que estão envolvidas em importantes vias fisiológicas, defesa de plantas contra estresses bióticos e abióticos e sinalização celular (Van loon; Van strien, 1999). Também são utilizadas em estudos que envolvem tecnologias de transgenia e engenharia molecular, possibilitando melhorias em cultivares de interesse agronômico, tornando-os resistentes a estresses bióticos e abióticos (Amador *et al*., 2021). Podem desempenhar efeito protetor durante infecções bacterianas, diminuindo citocinas pró-inflamatórias (Souza *et al*., 2018); inibem α-amilase salivar humana, podendo ser utilizados no controle da obesidade, já que previnem a digestão pós-prandial de carboidratos em humanos (da Silva *et al*., 2018); possuem atividade antinociceptiva (Campos *et al*., 2016); estabilidade termodinâmica (Maghsoudi *et al*., 2013) e podem ser utilizados para fornecer estabilidade química e física a compostos farmacológicos, já que esses devem manter suas propriedades ao serem armazenados, até o momento da utilização pelo paciente (dos Santos *et al*., 2017). Com isso, novos estudos sobre essas moléculas são de grande importância, uma vez que elas possuem diversas aplicações biotecnológicas, incluindo a grande problemática da resistência microbiana.

1.5 – Heveína

Os peptídeos semelhantes à heveína fazem parte de uma família de peptídeos com aproximadamente 29 a 45 aminoácidos, ricos em cisteína (com 3 a 5 pontes dissulfeto), que se ligam à quitina. A heveína recebeu esse nome devido ao seu isolamento do látex da seringueira do Pará (*Hevea brasiliensis*; Família: Euphorbiaceae) em 1960 (Slavokhotova *et al*., 2017). Sua sequência de aminoácidos foi determinada anos depois (Walujono *et al*., 1975), sendo extremamente parecida com a aglutinina de ligação à quitina (UDA) da urtiga (*Urtica dioica*; Família: Urticaceae) (Chapot *et al*., 1986).

Esses peptídeos apresentam estruturas ricas em cisteína e glicina, juntamente com o domínio de ligação à quitina conservado, o que auxilia sua ligação com a quitina na parede celular dos fungos e no exoesqueleto dos artrópodes. As estruturas primárias e a quantidade de ligações dissulfeto (S-S) variam significativamente dentro dessa classe de peptídeos (Tam *et al*., 2015). Eles são divididos em três subfamilias de acordo com a quantidade de resíduos de cisteína: 6C, 8C e 10C, embora os mais clássicos contenham oito resíduos de cisteína (Figura 2A). Em relação à sua estrutura secundária, compreende-se um motif espiral-β1-β2-espiral-β3 com variações baseadas na presença de voltas curtas nas duas espirais longas e na fita β3 (Slavokhotova *et al*., 2017; Tam *et al*., 2015). A folha β central do motif heveína é formada por duas cadeias β antiparalelas, enquanto a região central é mantida pelas ligações S–S (Figura 2B) (Tam *et al*., 2015).

Nas plantas, essas moléculas são responsáveis pela defesa contra uma ampla gama de fungos patogênicos (Franco *et al*., 2006). Diversos estudos relatam a atividade antifúngica de peptídeos semelhantes à heveína, muitos relacionados à atividade antimicrobiana contra patógenos animais e fitopatógenos. O efeito antifúngico deve-se principalmente à presença de um domínio conservado de ligação à quitina. (Srivastava *et al*., 2021). Ar-AMP (Q5I2B2) isolada de *Amaranthus retroflexus* faz parte da subfamília 6C e possui atividade contra *B. cinerea, F. culmorum, Helminthosporium sativum, A. consortiale,* e *Rhizoctonia solani.* Em relação a subfamília 8C, podemos destacar Hevein (P02877) isolado de *H. brasiliensis*, que teve atividade sobre *B. cinerea, F. culmorum, F. oxysporum, Phycomyces blakesleeanus*, *Pyrenophora triticirepentis*, *Pyricularia oryzae*, *Septoria nodorum* e *Trichoderma hamatum.* Em relação a subfamília 10C, podemos citar EAFP2 isolado de *Eucommia ulmoides,* ativo contra *Aculops lycopersici, F. moniliforme, F. oxysporum* e *C. gossypii* (Slavokhotova *et al*., 2017). Além disso, esses peptídeos podem atuar em conjunto com outro grupo de proteínas protetoras, as quitinases.

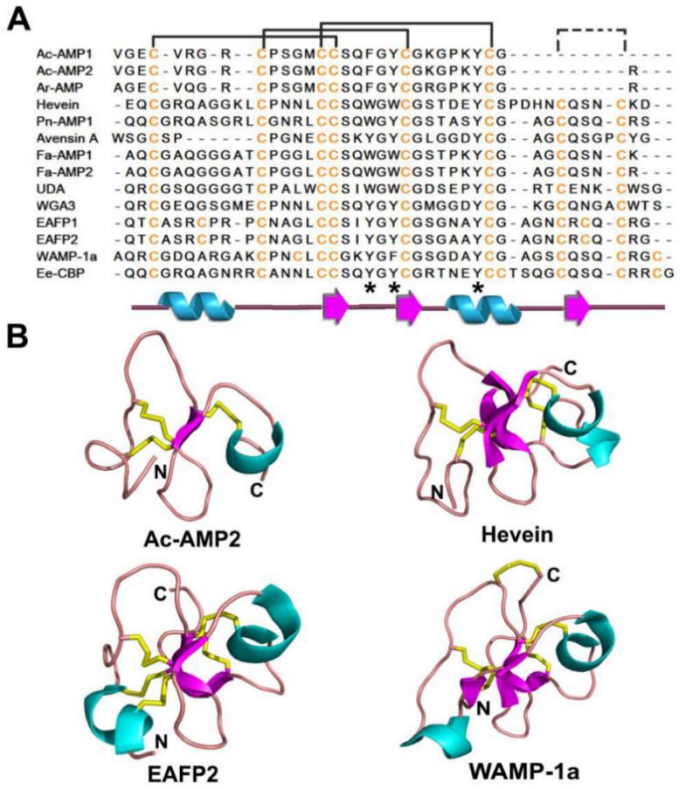

Figura 2. Sequências (A) e estruturas (B) de peptídeos semelhantes a heveína. A estrutura secundária é representada por cores diferentes: azul - α-hélice; rosa – espiral aleatória; amarelo - ligações dissulfeto (Tam *et al*., 2015).

Os peptídeos WAMP, por exemplo, são inibidores específicos da fungilisina, uma metaloprotease secretada pelos fungos do gênero *Fusarium*, protegendo uma quitinase vegetal da degradação pela enzima do patógeno fúngico (Odintsova *et al*., 2020). É importante mencionar que o mecanismo de ação dos peptídeos semelhantes à heveína é baseado na sua interação com a quitina fúngica presente na célula. Essa interação é apoiada pelo domínio heveína presente nesses peptídeos, responsável pela interação da quitina e pelos danos causados à parede celular do fungo (Tam *et al*., 2015), contudo, não se sabe se o mecanismo utilizado por esses peptídeos é ubíquo ou se é característico apenas para alguns deles.

Devido à grande atividade dos peptídeos semelhantes à heveina contra fungos fitopatogênicos, esses peptídeos são utilizados em biotecnologia para obtenção de plantas transgênicas com maior resistência a fitopatógenos, como, por exemplo, SmAMP1 e SmAMP2, que foram utilizados para expressão em plantas de tabaco e conferiram maior resistência contra os fungos *Bipolaris sorokiniana* e *Thielaviopsis basicola* (Shukurov *et al.*, 2012). São utilizadas regiões promotoras dos genes desses peptídeos para sua

produção de grandes quantidades, sendo uma classe de peptídeos promissora para o desenvolvimento de novos antimicrobianos naturais (Azmi *et al*., 2021).

1.6 – Quitinases

As quitinases (EC 3.2.1.14) são enzimas que hidrolisam as ligações β-1,4 na quitina. Com base na similaridade de sequência e diferentes mecanismos catalíticos, as quitinases são agrupadas nas famílias de glicosil hidrolases (GH) 18, 19 e 20 (Langner; Göhre, 2016). Os genes de quitinases são classificados em sete classes (I-VII) (Neuhaus, 1999; Sarma *et al*., 2012). As quitinases das classes I, II, IV, VI e VII pertencem à família GH19, enquanto as quitinases das classes III e V são membros da família GH18 (Ohnuma *et al*., 2012). Além disso, também são classificadas como proteínas relacionadas à patogênese (PR), sendo elas: PR-3, PR-4, PR-8 e PR-11 (Neuhaus, 1999).

Elas possuem massas moleculares bastante variadas e são bastante abundantes em plantas. Quitinases da classe I do feijão-caupi produzidas em *Pichia pastoris* apresentaram massa molecular de 34 e 37 kDa (Landim *et al*., 2017). Uma quitinase isolada de sementes de feijão-caupi, que se mostrou tóxica para fungos fitopatogênicos, apresentou massa molecular de 22 kDa (Gomes *et al*., 1996). Foi verificado também, que o genoma de *Arabidopsis* parece conter 25 quitinases ou proteínas semelhantes à quitinases. Seu tamanho varia de 211 a 430 aminoácidos, com massa molecular entre 20 e 50 kDa. Além disso, foi observado que o genoma do arroz apresenta 49 quitinases com 178 a 479 aminoácidos e massa molecular entre 18 e 50 kDa (Grover, 2012).

Algumas quitinases (especialmente as de classe I e que representam as PR-3) possuem um domínio de ligação à quitina (CBD), sendo responsável pela forte ligação da enzima ao substrato, aumentando assim sua atividade hidrolítica (Hoell *et al*., 2010). O domínio de ligação à quitina ou domínio heveínico, como também é conhecido, como já relatado, contêm um motif estrutural comum de 30 a 43 aminoácidos com diversas cisteínas e glicinas em posições conservadas (Figura 3) (Raikhel; Lee, 1993; Trindade *et al*., 2006). As quitinases não necessitam especialmente de um domínio de ligação à quitina para exercer atividade catalítica, como as de classe II, que não possuem CBD, mas possuem em contrapartida um domínio quitinolítico que se liga à quitina (Neuhaus, 1999).

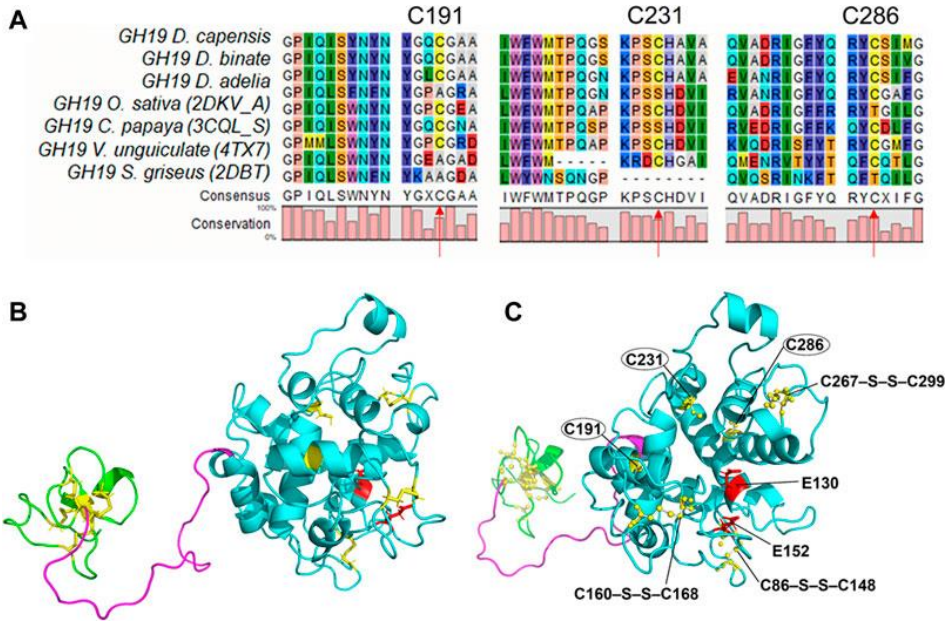

Figura 3. Alinhamento proteico de quitinases homólogas (A). Estrutura do modelo de homologia Chit19, destacando ligações dissulfeto em amarelo (B). (B) e (C) representam a mesma estrutura, mas em (C) o ângulo instantâneo é girado para destacar os supostos locais catalíticos e as ligações dissulfeto do domínio central, bem como as cisteínas livres (Sinelnikov *et al*., 2021).

As quitinases de plantas foram relatadas a partir de diversas fontes e espécies vegetais, como *Ficus carica* (Spano *et al*., 2015), *Euphorbia characias* (Martos *et al*., 2017), *C. annuum* (Liu *et al*., 2017), *Avena chinensis* (Li *et al*., 2019), *Carica papaya* L. (Lucas-Bautista *et al*., 2020) e *Trichosanthes dioica* (Kabir *et al*., 2016). Elas desempenham um papel durante o crescimento da planta e processos de desenvolvimento, como durante a germinação, senescência, polinização e embriogênese somática (Fráterová *et al*., 2013; van Hengel *et al*., 2001). Outra função das quitinases envolve respostas ao estresse abiótico. Alguns genes de quitinases são induzidos por uma variedade de estresses abióticos, incluindo estresse osmótico por frio, metais pesados e ferimentos (Dana *et al*., 2006; Mészáros *et al*., 2013; Guleria *et al*., 2017; van Loon *et al*., 2006). Muita atenção tem sido dada ao papel das quitinases na defesa contra a invasão de patógenos fúngicos (Graham; Sticklen, 1994).

Como exemplo de quitinase para controle de pragas e doenças, há um trabalho realizado por Durechova *et a*l., (2019), onde foi extraída uma quitinase de *Drosera rotundifolia* com posterior inserção em uma planta transgênica de tabaco para exploração do seu potencial antifúngico. A proteína encontrada foi denominada como DrChit, com

aproximadamente 32 kDa. A proteína purificada inibiu efetivamente o crescimento do fungo *T. viride*, indicando assim atividade quitinolítica e óbvio potencial antifúngico. Foi verificado ainda, que no ensaio antifúngico com as plantas transgênicas de tabaco, houve um atraso no crescimento fúngico em comparação com as plantas não transgênicas. Além dessa, outras quitinases isoladas que podem ser citadas são VcLysM1 e VcLysM2, extraídas de *Volvox carteri* (Kitaoku *et al*., 2019), e Mo-chi1, extraída de *M. oleífera* (Bezerra *et al*., 2018).

Outro trabalho relata uma quitinase purificada de sementes de aveia nua (*Avena chinensis*) usando técnicas cromatográficas simples. Sua massa molecular e seu ponto isoelétrico foram determinados em 35 kDa e 8,9, respectivamente. A análise parcial da sequência de aminoácidos e a pesquisa de homologia indicaram que provavelmente é uma quitinase de plantas de classe I, da família glicosil hidrolase 19. Com a quitina como substrato, o pH e a temperatura ótimos da quitinase foram pH 7,0 e 40 °C, respectivamente. Testes de atividade antifúngica *in vitro* demonstraram que esta quitinase purificada tinha atividade inibitória potente e dependente da dose sobre os fungos *Panus conchatus* e *T. reesei* (Li *et al*., 2019).

Além da sua utilização no controle de patógenos fúngicos, as quitinases também podem ser empregadas no controle de insetos pragas, como é o caso de uma quitinase extraída do látex de *Euphorbia characias*, denominada como *Euphorbia* latex chitinase (ELC). Essa quitinase foi purificada e sua capacidade de degradar o exoesqueleto quitinoso de *Drosophila suzukii* foi testada. Foi relatado pelos autores que o tratamento com ELC causou redução do crescimento larval, maior mortalidade e degradação notável das estruturas externas do inseto. Dessa forma, a quitinase pode induzir um duplo efeito nas larvas de *D. suzukii*, uma lesão direta nos corpos larvais e uma ação como antinutritiva (Martos *et al*., 2017).

1.7 – Plantas do gênero *Capsicum* como fonte de peptídeos antifúngicos

As pimentas e pimentões nativos da América Central, da América do Sul e do México são pertencentes à família Solanaceae e ao gênero *Capsicum.* O gênero abrange 35 espécies, sendo elas domesticadas, semidomesticadas e silvestres. Cinco espécies foram domesticadas e exploradas devido a seus frutos pungentes utilizados como especiarias, são eles: *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L.

e *C. pubescens* (Carrizo Garcia *et al*., 2016; Sun *et al*., 2014) (Figura 4). Atualmente estão dispersas por todo mundo, e no Brasil são cultivadas principalmente nos estados de Minas Gerais, Bahia, Goiás e Rio de Janeiro, sendo este último um importante centro de diversidade. As espécies do gênero possuem grande variabilidade quanto ao formato, tamanho, cor e posição de flores e frutos (Carvalho; Bianchetti, 2007; Bianchi *et al*., 2016).

A cultura da pimenta apresenta grande importância econômica. Em 2018, a produção mundial foi de 40,9 milhões de toneladas, comercializadas como pimenta fresca ou seca, apresentando o sétimo lugar mundial em vegetais colhidos (Fao, 2019). Ela é muito utilizada na culinária tradicional, proporcionando cor, sabor e aroma em diversos pratos, além de ser utilizada na indústria cosmética e de apresentar potencial na medicina, por conter compostos como flavonoides, vitaminas C e E, carotenoides, minerais e capsaicinoides com efeitos antimicrobianos, analgésicos, anticarcinogênicos e antiobesidade (Batiha *et al*., 2020; Hernández-Pérez *et al*., 2020).

Nos últimos anos o gênero *Capsicum* vem sendo utilizado como modelo de estudo em muitos trabalhos, como nos descritos por Santos *et al*., (2017), Baenas *et al*., (2019), Moulin *et al*., (2020), Bianchi *et al*., (2020), entre outros. Dentro do nosso grupo, já foram isoladas e caracterizadas diversas famílias de peptídeos antimicrobianos pertencentes a este gênero, como: tionina-símile (Taveira *et al*., 2014), defensina (Gebara *et al*., 2020), proteínas transportadoras de lipídeos (LTPs) (Diz *et al*., 2006); inibidores de proteinases (Silva *et al*., 2021), peptídeos de ligação à quitina (Gonçalves *et al*., 2024), entre outros. Na Tabela 2 estão listados peptídeos antimicrobianos e genes de peptídeos antimicrobianos identificados em diversos órgãos de plantas do gênero *Capsicum*. Já foi demonstrado que esses peptídeos apresentam atividade contra diversos tipos de microrganismos, incluindo bactérias, fungos fitopatogênicos e leveduras (Oliveira *et al*., 2022).

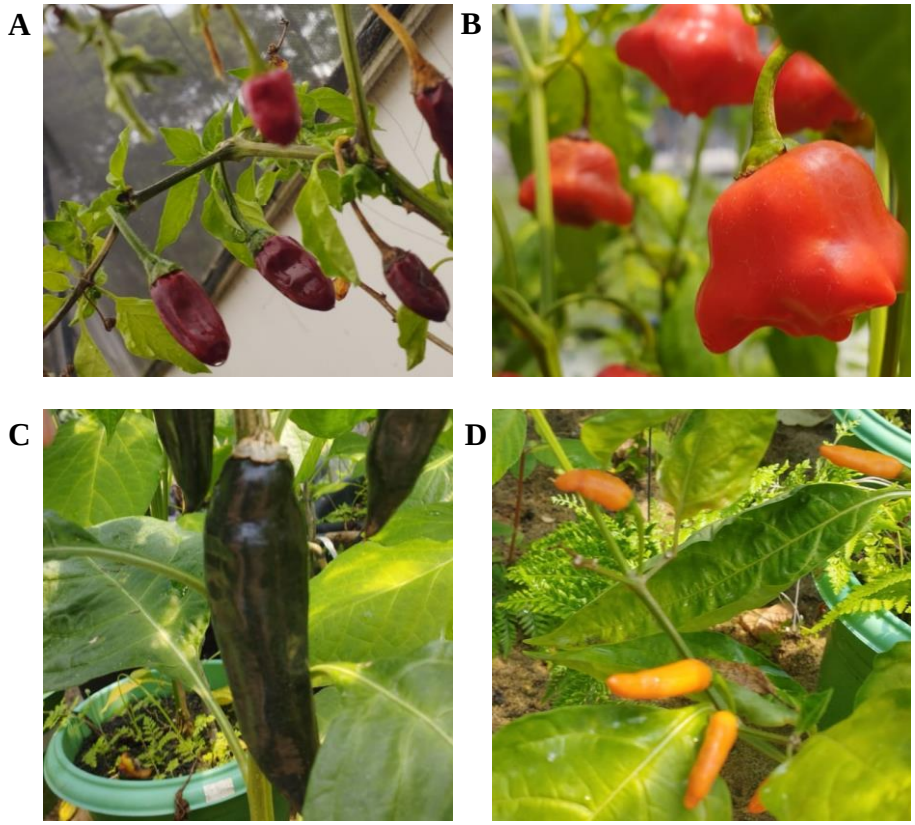

Figura 4. Espécies de pimenta utilizadas nessa pesquisa. *C. annuum* (Acesso UENF 1381) (A), *C. baccatum* (Acesso UENF 1732) (B), *C. chinense* (Acesso UENF 1755) (C) e *C. frutescens* (Acesso UENF 1775) (D) (Fonte: Autor, 2023).

Para a criação de novos produtos biotecnológicos e ajudar a conter doenças microbianas, é de suma importância conhecer e preservar a flora, além de conhecer a etnofarmacologia do nosso país, como é o caso da utilização de plantas do gênero *Capsicum*. Sendo assim, o objetivo dessa tese foi isolar e caracterizar peptídeos de ligação à quitina de sementes de quatro espécies de *Capsicum*: *C. annuum*, *C. baccatum*, *C. chinense* e *C. frutescens* e posteriormente avaliar suas atividades antifúngicas e sua toxicidade *in vitro* e *in vivo* utilizando larvas de *Galleria mellonella* como modelo de estudo. A tese está organizada em três capítulos referentes a artigos científicos produzidos durante o doutorado. Cada capítulo conta com uma introdução, com os métodos utilizados, resultados e discussão. No final da tese estão apresentadas as principais conclusões do trabalho.

Tabela 2. Peptídeos antimicrobianos e genes de peptídeos antimicrobianos identificados no gênero *Capsicum* (Adaptado de 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. anunum*	not determined	Oh et al. (1999)
CATHIO N1	-	-	-	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. oxsporum* *B. cinérea*	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.
CanThio2	CanThio2	72	8,36 kDa					(2019)
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 CaDeF2.2	40 40	5 kDa ~ 6 kDa	Defensin	Fruit	*C. annuum*	*C. buinensis* *C. tropicalis* *C. parapsilosis* *M. tuberculosis*	Gebara et al. (2020)
-	*Cc*Def3	-	~ 6,5 kDa	Defensin	Fruit	*C. chinense*	*C. albicans* *C. buinensis* *C. tropicalis*	Aguieiras et al. (2021)
CALTPI *CALTPII* *CALTPIII*	- - -	- - -	- - -	LTP	Leaves, Stalks, Fruit	*C. annuum*	not determined	Jung et al. (2003)

2 – OBJETIVOS

2.1 Objetivo geral

Isolar, caracterizar e avaliar a atividade antimicrobiana de peptídeos com propriedade de ligação à quitina sobre fungos fitopatogênicos e leveduras de interesse médico a partir de sementes do gênero *Capsicum*.

2.2 Objetivos específicos

A: Purificar peptídeos de ligação à quitina de sementes de *C. annuum*, *C. baccatum*, *C. chinense* e *C. frutescens*;

B: Avaliar a atividade antimicrobiana dos peptídeos ligantes à quitina sobre leveduras do gênero *Candida* e fungos do gênero *Fusarium* e avaliar alguns dos possíveis mecanismos de ação;

C: Determinar a massa molecular e a estrutura primária dos peptídeos identificados;

D: Determinar a estrutura tridimensional dos peptídeos de ligação à quitina identificados e selecionados;

E: Analisar a hemotoxicidade *in vitro* dos peptídeos de ligação à quitina identificados e selecionados contra eritrócitos de carneiro;

F: Analisar a toxicidade *in vivo* dos peptídeos de ligação à quitina identificados e selecionados sobre larvas de *Galleria mellonella*.

3 – CAPÍTULO 1

Chitin-binding peptides from *Capsicum annuum* with antifungal activity and low toxicity to mammalian cells and *Galleria mellonella* larvae

Chapter 1: Manuscript published

Chitin-binding peptides from *Capsicum annuum* with antifungal activity and low toxicity to mammalian cells and *Galleria mellonella* larvae

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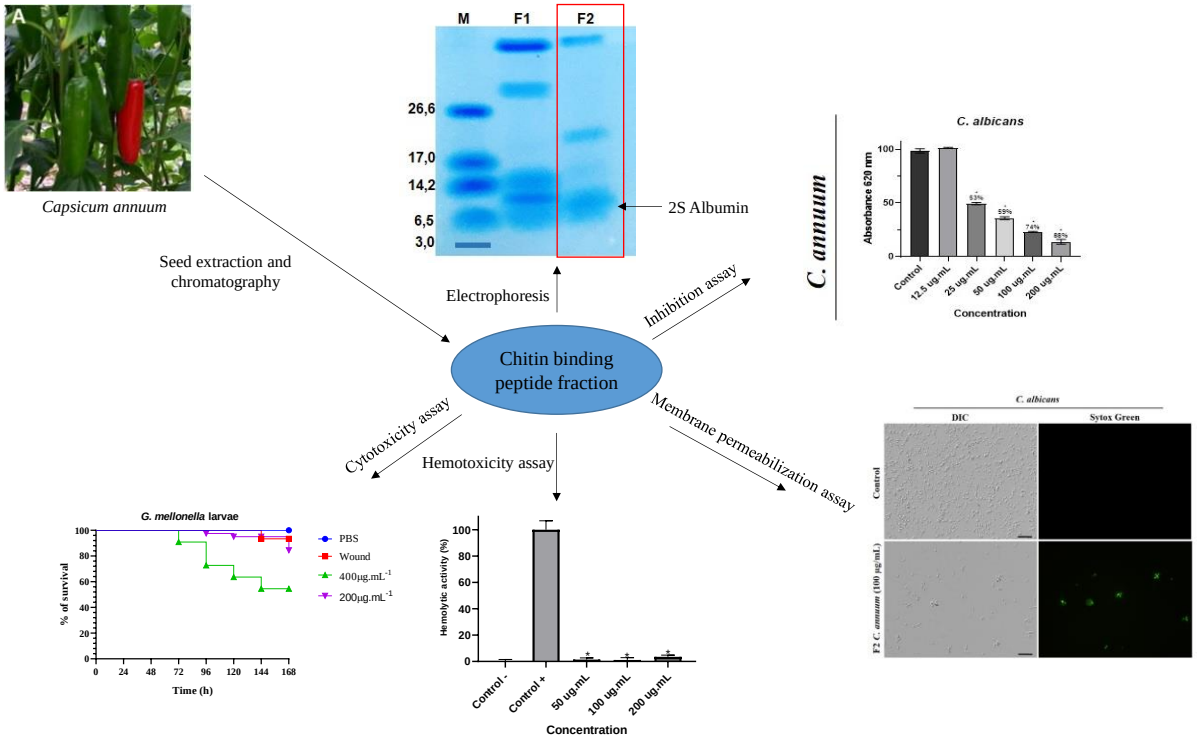
Graphical Abstract



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ARTICLE

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Chitin-binding peptides from *Capsicum annuum* with antifungal activity and low toxicity to mammalian cells and *Galleria mellonella* larvae

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Abstract

In recent years, there have been several reports of the presence of toxic proteins in cultivated or wild plant species, which are implicated in plant defense mechanisms. The existence of these proteins raises the possibility of biotechnological applications originating from the development of new techniques to combat diseases caused by fungi. In this context, there are chitin-binding proteins. Chitin is an essential component of the fungal cell wall, so chitin-binding proteins are important in controlling fungal growth. Thus, the objective of this study was to characterize and evaluate the *in vitro* antimicrobial effect of peptides with chitin binding properties isolated from *Capsicum annuum* seeds on the growth of the genus *Candida*. Initially, proteins were extracted in phosphate pH 5.4, and a chitin column was equilibrated with sodium acetate (0.08 M, pH 4.5), where 50 mg of the peptide-rich heated fraction from each species was applied. Subsequently, the retained material was eluted with 0.1 M HCl. Tricine SDS–PAGE was used to visualize the peptides. After chromatography, two fractions, F1 (not retained in the chitin column) and F2 (retained in the chitin column, named *Ca*-F2), were obtained. Electrophoresis showed major protein bands between 3 and 14 kDa. Electrophoresis from chitin affinity chromatography also showed major bands between 3 and 14 kDa, especially for *Ca*-F2 retained in the column. One peptide obtained from the F2 fraction was identified by mass spectrometry and showed

similarity to seed 2S albumin, named *Ca*-Alb2S. *Ca*-F2 inhibited the growth of *C. albicans* and *C. tropicalis*, was not toxic to mammalian cells and still had a high survival rate when tested in vivo on *Galleria mellonella* larvae. This is the first report of chitin-binding peptides isolated from *Capsicum* seeds through an affinity column and their biological activities. These studies are at an early stage; therefore, other tests are needed to study the mechanism of action of the fraction, since the findings indicate great potential for the development of new antifungal molecules.

KEYWORDS
2S albumin, antimicrobial activity, *Capsicum*, fungi

1 | INTRODUCTION

For many centuries, diseases caused by bacteria, viruses and protozoa have been considered major public health problems. In the last century, fungal diseases did not have as much impact on human health, but this has changed as the number of immunocompromised patients has increased, as these patients are highly susceptible to fungal infections.[1] In this context, the incidence of candidiasis has been observed in systemic infections (candidemia), which is a serious infectious disease with high mortality caused by yeasts of the genus *Candida* that are more susceptible in immunosuppressed patients.[2] It is predicted that if antimicrobial resistance continues to grow worldwide, by the year 2050, approximately 10 million people will die annually from drug-resistant infections.[3] In addition to the limited number of effective drugs available, most cause adverse effects in patients, preventing their prolonged use.[4] Yeasts of the *Candida* genus are highly adaptable microorganisms that are able to develop resistance following prolonged treatment with antifungals.[5] In this sense, it is necessary to study alternative compounds for efficient treatments of infectious diseases.

Currently, research focused on biotechnology must be thought within the idea of One Health, since human health is interdependent of other species and the environment,[6] which requires the creation of new substances of biotechnological interest that can provide sustainable development and positive impacts on the Brazilian and world economy.

Plants synthesize a variety of proteins capable of reversibly binding to matrices that have chitin in their composition. Chitin, an insoluble, β-1,4-linked homopolymer of N-acetyl-D-glucosamine, is found in insect exoskeletons, crustacean shells, and eggshells of nematodes and is one of the main constituents of fungal cell walls. The high rate of chitin production from these organisms makes it the second most abundant polysaccharide in nature, second only to cellulose.[7–9]

Classical members of this chitin-binding protein (CBP) family contain one or more chitin-binding domains. These proteins are found in different families, such as lectins, chitinases, heveins, vicilins and peptide families as the Ac-AMP type (*Amaranthus caudatus* antimicrobial peptides).[10] Previous studies have reported the antifungal activity of plant CBPs against phytopathogens[11,12] and *Candida* spp.[13,14] In Table 1, some chitin-binding peptides have been identified.

More recently, Mo-CBP3 and Mo-CBP4, two small chitin-binding albumin 2S-like proteins, were purified from *M. oleifera* seeds. Mo-CBP4 was able to cause an increase in permeability of the microconidia membrane and was shown to be a potent antidermatophytic protein causing membrane permeability, ROS overproduction and damage to the cell wall in fungi.[18,19] These works opened the door for the study of peptides or small proteins similar to reserve proteins and their antifungal properties, such as vicilins, vicilin-like and 2S albumins.

In the case of pepper plants, only a chitin-binding protein (CBP) family member *CaChiIV1* from *C. annuum* L. was reported. Evidence suggests that the *CaChiIV1* gene plays a prominent role in the defense mechanism of pepper plants against *P. capsici* infection.[23] In recent years, within our group, several *Capsicum* peptides have already been isolated and characterized, such as thionine-like,[24] defensin,[25] lipid transport proteins (LTPs)[26] and proteinase inhibitors.[27] In this study, for the first time, we used a chitin-binding column to purify peptide-rich fractions from seeds of *C.annuum* with chitin-binding activity and antimicrobial activity against yeasts.

2 | MATERIALS AND METHODS

2.1 | Plant material

The seeds of *C. annuum* (Access UENF 1381) were provided by the Laboratório de Melhoramento Genético Vegetal (LMGV), Universidade Estadual do Norte Fluminense Darcy Ribeiro, Brazil. The seeds of *C. annuum* were sown in 72-cell polystyrene trays with substrate fertilized with formulation (4 N/14P/8 K) (Vivatto®). The trays were kept in a growth chamber at 28°C and irrigated once a day. After the emergence of two pairs of definitive leaves, the seedlings were transplanted individually into plastic pots (5 L) containing a mixture of soil and substrate (in a 2:1 ratio). Then, the pots were transferred to an acclimatized greenhouse. The plants were irrigated once a day until the harvest of the ripe fruits.

TABLE 1 Identification of chitin-binding peptides, source from which they were extracted and microorganisms to which they have activity.

Chitin bindind peptides	Plant	Activity	Reference
Pn_AMP1 and Pn_AMP2	*Pharbitis nil*	Activity against *Botrytis cinerea, Colletotrichum langenarium, Fusarium oxysporum, Phytophthora capsici, Saccharomyces cerevisiae*	[15]
EAFP1 and EAFP2	*Eucommia ulmoides*	*Aculops lycopersici, F. moniliforme, F. oxysporum, C. gossypii*	[15]
Fa_AMP1 and Fa_AMP2	*Fagopyrum esculentum*	*Clavibacter michiganensis, Agrobacterium rhizogenes, F. oxysporum, Geotrichum candidum*	[15]
WSMoL	*Moringa oleifera*	Negative effect on the development of *Anagasta kuehniella*	[16]
mO1 and mO2	*Moringa oleifera*	Inhibits the growth of phytopathogenic fungi	[17]
Mo-CBP2	*M. oleifera*	Activity against *Candida*	[14]
Mo-CBP3	*M. oleifera*	Inhibits germination and mycelial growth of phytopathogenic fungus	[18]
Mo-CBP4	*M. oleifera*	*In vitro* activity against *Trichophyton mentagrophytes*	[19]
EuCHIT2	*E. ulmoides* Oliver	Increased resistance to *Erysiphe cichoracearum* in Tobacco plants	[20]
DrChit	*Drosera rotundifolia*	*Trichoderma viride* growth inhibition	[21]
CJP-4	*Cryptomeria japônica*	—	[22]
CaChiIV1	*C. annuum*	*P. capsici*	[23]

2.2 | Microorganisms

The yeast cells were provided by were obtained of the biological collection of fungi from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The yeast species *Candida albicans* (CE022) and *C. tropicalis* (CE017) were cultivated in Sabouraud medium and kept in a refrigerator at 4°C in the Laboratório de Fisiologia e Bioquímica de Microrganismos (LFBM), Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes–RJ.

2.3 | Protein extraction and precipitation with ammonium sulfate

Initially, 5 g of seeds was ground with the aid of a mortar, pestle, and liquid nitrogen to form very fine-grained flour. After obtaining the flour, the extraction of the proteins immediately followed. The proteins were extracted from the flour according to the methodology described by Diz et al.[26] First, the proteins were extracted in phosphate buffer (0.01 M Na_2HPO_4, 0.015 M NaH_2PO_4, 0.1 M KCl, 1.5% EDTA), pH 5.4, at a ratio of 1:10 (5 g flour: 50 mL buffer), for 3 h under constant stirring at 4°C. The homogenate was subjected to centrifugation (15,000*g* for 30 min), the residue was discarded, and ammonium sulfate was added to the supernatant for precipitation at 90% saturation. The solution was kept overnight at 4°C. After this process, the suspension was centrifuged again at 15,000 x g for 30 min. Proteins precipitated in the 0–90% ammonium sulfate saturation range were recovered by resuspension in 10 mL of distilled water and heated at 80°C for 15 min. Then, they were submitted to the last centrifugation (10.000*g* for 10 min). The supernatant was dialyzed against distilled water (avg. flat width 32 mm, 1.27 in.) at 4°C for 3 days and finally lyophilized.

2.4 | Protein quantification

Quantitative protein determinations were performed using the bicinchoninic acid method, according to Smith et al.,[28] with ovalbumin (Sigma) used as the standard protein.

2.5 | Isolation of proteins and peptides with affinity for chitin

The chitin (poly-(1 → 4)-β-N-acetyl-D-glucosamine) used for this study was obtained from shrimp shells—practical grade, powder and commercially available from Sigma–Aldrich. Chitin was chemically treated according to Miranda et al.,[29] where 25 g of industrial chitin was acidified with 500 mL of 100 mM HCl. This mixture was incubated for 24 h at 4°C with periodic shaking. After 24 h, all HCl was removed, and 250 mL of 100 mM NaOH was added to the resulting precipitate. This mixture was heated at 100°C for 16 h. After heating, the NaOH was removed, and another 250 mL of NaOH was added to the precipitate for another 16 h of heating. This step was repeated once again, resulting in three 16 h warm-ups. After the last heating, all NaOH was removed, and 200 mM HCl was added to the precipitate. After the last acidification, the HCl was removed from the mixture, and distilled water was added to the precipitate for storage. A chitin column was equilibrated with sodium acetate buffer (0.1 M, pH 5.5), and a 50 mg peptide-rich heated fraction (PRHF), solubilized in the same sodium acetate buffer, was applied to the column. Chromatography was initially performed with equilibrium buffer. The retained fraction was eluted with HCl (0.05 M) solution, and the absorbance of the desorbed proteins was monitored at 280 nm. The flow rate used was 1 $mL.min^{-1}$, and the volume used was 3 mL per tube. Protein peaks were collected and recovered after dialysis and lyophilization.[30]

2.6 | Tricine gel electrophoresis in the presence of SDS

Tricine-SDS–PAGE was performed according to the methodology described by Schägger and von Jagow.[31] For gel assembly, 8 × 10 cm and 7 × 10 cm glass plates and 0.75 mm spacers were used. The separating gel was prepared at a 16.4% acrylamide/bis-acrylamide concentration, and the concentration gel was prepared at 3.9%. The samples were heated for 5 min at 100°C and centrifuged at 15,000*g* for 5 min. After these procedures, 20 μL of sample was applied to the gel. The run was performed at a constant voltage of 24 V for approximately 16 h. M3546 (Sigma–Aldrich) was used as a protein molecular weight marker. Molecular mass of proteins in Da (26.600; 17.000; 14.200; 6.500; 3.496; 1.060).

2.7 | Mass spectrometry

The identification of peptides present in *Ca*-F2 was carried out in collaboration with the Marine Biochemistry Laboratory (BioMar-Lab), Department of Fisheries Engineering, Federal University of Ceará (UFC), Ceará, Brazil. After tricine-SDS–PAGE, one band present in *Ca*-F2 was extracted, and then, tryptic peptides were obtained according to Shevchenko et al.[32] and subjected to LC–MS/MS analysis. The instrument used was a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp, MA, USA). Data collection and processing parameters were adjusted as described by Carneiro et al.[33] The interpretation of the spectra was performed using the Mascot program, and the sequenced peptides were submitted to alignment using the BLASTp tool.[34] Sequences with a high percentage of identity were chosen and subjected to multiple alignments using the CLUSTAL Multiple Sequence Alignment by MUSCLE program (3.8).[35] The calculation of the percentages of positive and identical residues was carried out considering only the residues obtained by mass spectrometry.

2.8 | Docking of 2S albumin with the tetramer of N-acetylglucosamine $(NAG)_4$

The peptide sequences identified were aligned using BLASTp with proteins deposited in the Protein Data Bank (PDB). The models of the protein with the greatest identity were used for the docking experiments. The docking of 2S albumin with $(NAG)_4$ was performed using the DockThor v.2 program.[36] The blind docking strategy was performed using a search space defined by a 40 Å × 40 Å × 40 Å cube, covering 100% of the protein surface. The NAG_4 model was created from PyMol, with the Azahar plug-in to design oligosaccharides. All rotations of the ligand were free to rotate while the atoms of the protein were kept rigid. The standard search algorithm of the site was used with 1,000,000 evaluations and 24 executions per experiment. The best 2S Albumin-ligand complex was evaluated using affinity energy value and submitted to the PLIP Web Tool program,[37] and to the LigPlot+ v program. 2.2.4.[38] Noncovalent interactions between 2S albumin and $(NAG)_4$ were identified by the programs.

2.9 | Trypsin enzyme inhibition assay

The residual trypsin activity in the presence of *Ca*-F2 was measured based on the hydrolytic activity of commercial bovine trypsin on the Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA) substrate after incubation with the fractions. For the assay, Tris–HCl buffer (50 mM, pH 8.0), 25 μL of BApNA substrate (5 mM), 10 μL of trypsin (1 $\mu g.mL^{-1}$) and aliquots of fraction (50 $\mu g.mL^{-1}$) in a final volume of 200 μL were used. The sample was incubated in a water bath (Quimis) at 37°C for 30 min. To stop the reaction, 30% acetic acid (100 μL) was added. Subsequently, photometric readings of the reactions were performed based on the p-nitroanilide released at a wavelength of 405 nm (Spectroquant Pharo 100, Merck).[39]

2.10 | Effect of peptides on fungal growth

With the aid of a seeding loop, an aliquot was removed from the petri dish containing *C. albicans* colonies, and another aliquot was removed from the petri dish containing *C. tropicalis* colonies. Subsequently, these aliquots were placed in new petri dishes containing Sabouraud agar (10 g. L peptone, 40 g. L D(+)glucose, 15 g. L agar) (Merck) to generate striations in the middle. These new plates were kept in an oven at 30°C for 24 h. After growth, the cells were removed from the petri dish with the aid of a seeding loop and homogenized in 10 mL of Sabouraud broth (10 g. L peptone, 20 g. L D(+)glucose) (Merck) for quantification in a Neubauer chamber (LaborOptik) with the aid of an optical microscope (Axiovison A2, Zeiss). Subsequently, the yeast (1×10^4 $cel.mL^{-1}$) were incubated in Sabouraud broth containing different concentrations of the fractions obtained from the seeds. The assay was performed in cell culture microplates (96 wells) at 30°C for a period of 24 h. Cell growth was determined by optical density, monitored every 6 h, in a microplate reader at a wavelength of 620 nm. Each test was performed in triplicate. The entire procedure was performed under aseptic conditions in a laminar flow hood.[40] Cell growth without the addition of proteins was also determined.

2.11 | Plasma membrane permeabilization

The membrane permeabilization of *C. albicans* cells treated with 100 $\mu g.mL^{-1}$ *Ca*-F2 was measured by SYTOX Green uptake. After 24 h of yeast cell incubation with protein fractions, control and treated cells were incubated with the fluorescent probe SYTOX Green (0.2 μM) (Invitrogen, Carlsbad, CA, USA) for 10 min at 30°C and analyzed by DIC using an optical microscope (Axioplan. A2, Zeis, Germany) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths 450–490 nm; emission 500 nm).[41] The percentage of permeabilized cells was calculated based on the

total number of cells in the differential interference contrast (DIC) and fluorescence images of five random microscopic fields for each sample, assuming the total cell number in bright field of each sample as 100%. The percentage of permeabilized cells was statistically determined using a one-way ANOVA with means differences at $p < 0.05$ considered to be significant.

2.12 | Cell viability analysis

To analyze the effect of *Ca*-F2 on the cell viability of *C. albicans* and *C. tropicalis* yeasts, a growth inhibition assay was initially performed to obtain colonies. For plating, after 24 h of incubation, the control cells (without *Ca*-F2) and the test cells (with *Ca*-F2) were diluted 1000×. A 60 µL aliquot of the dilution was spread with a Drigalski loop over the surface from a Petri dish containing Sabouraud agar and cultivated at 30°C for 36 h. At the end of this period, the colony forming units were determined, and the Petri dishes were photographed.[42] The experiments were performed in triplicate, and the results are shown assuming that the control represents 100% cell viability.

2.13 | Hemolytic activity

The hemolytic activity of *Ca*-F2 was determined against defibrinated sheep red blood cells (sRBC) using the methodology described by Oren and Shai with modifications.[43] Fresh defibrinated sRBCs were rinsed with saline (0.15 M NaCl), followed by centrifugation for 10 min at 2400g and immediate resuspension in saline solution. *Ca*-F2 was prepared in microtubes containing saline solution. Subsequently, Ca-F2 at concentrations of 200, 100, and 50 µg.mL^{-1} was incubated at 37°C for 1 h with the sRBC suspension (50 µL) (final erythrocyte concentration, 1% vol/vol). After incubation, 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 at 405 nm (ABS_{405}). A solution containing Triton X-100 (1%) was used as a positive hemolysis control (C+), and erythrocytes in saline were used as a negative control (C−). The percentage of hemolytic activity was calculated assuming that the positive control represents 100% hemolysis according to the formula: % of hemolytic activity = 100 × (*Ca*-F2 ABS_{405} − C− ABS_{405})/(C+ ABS_{405} − C− ABS_{405}). The results presented are mean values obtained in the experiment, carried out in triplicate. Dose–response curves were constructed, and $^{*}p < 0.05$ compared to the group treated with Triton X-100 (100% hemolytic activity) by Tukey's test.

2.14 | Effect of *Ca*-F2 toxicity on *G. Mellonella* larvae

Assays were performed as described by Mylonakis et al.[44] with modifications. Twenty last-instar *G. mellonella* larvae ranging from 250 to 300 mg in weight and 2 cm were used in each of the treatment and control groups. A Hamilton syringe was used to inject 10 µL of each concentration of *Ca*-F2 (200 and 400 µg/mL^{-1}) into the hemocoel of each caterpillar through the last proleg. Two groups were included as controls for the general viability of the larvae: one group was inoculated with PBS, and the other suffered only the injury of the injection needle. After the injection, the larvae were incubated in Petri dishes at 37°C, and the number of dead larvae was counted every 24 hours for a period of 7 days. During this incubation period, the larvae were kept without any type of nutrition. The 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 Software, Inc., California, CA, USA). The assay was performed in duplicate.

2.15 | Statistical analysis

Data from yeast and filamentous fungi growth inhibition assays were evaluated by one-way ANOVA. Differences in means of $p < 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism software (version 5.0 for Windows).

3 | RESULTS AND DISCUSSION

3.1 | Chitin affinity chromatography and electrophoretic profile

Chitin binding proteins were obtained from aqueous extracts of *C. annuum* by chitin affinity chromatography. The chromatogram showed that the extract was fractionated into two major peaks called F1 and F2 (named *Ca*-F2), with F1 being the nonretained fraction in the column and F2 being the fraction retained in the column for *C. annuum* (Figure 1a). These results indicate the presence of proteins and peptides with chitin binding capability. The electrophoretic profile of the fractions obtained by chromatography can be seen in Figure 1b. We can observe that the bands in both fractions (F1 and F2) have molecular masses in the marker range between 3.0 and 26.6 kDa (Figure 1b); however, there are bands in the two fractions with molecular masses above the 26.4 kDa band.

Other works have already demonstrated the presence of different peptides with antimicrobial activity in *Capsicum* seeds, but these peptides have been isolated through methodologies other than chitin affinity columns.[27,39,45,46] Therefore, this is the first work that uses a chitin column to obtain peptide-rich fractions with antimicrobial activity from the seeds of the *Capsicum* genus. In other plant species, low molecular weight proteins have been purified using a chitin affinity column. One of the main examples is the antifungal protein Mo-CBP3. This is a thermostable chitin-binding protein (14.3 kDa) with antifungal activity isolated from *M. oleifera* seeds. Mo-CBP3 is a potent fungicidal agent inhibiting both the spore germination and

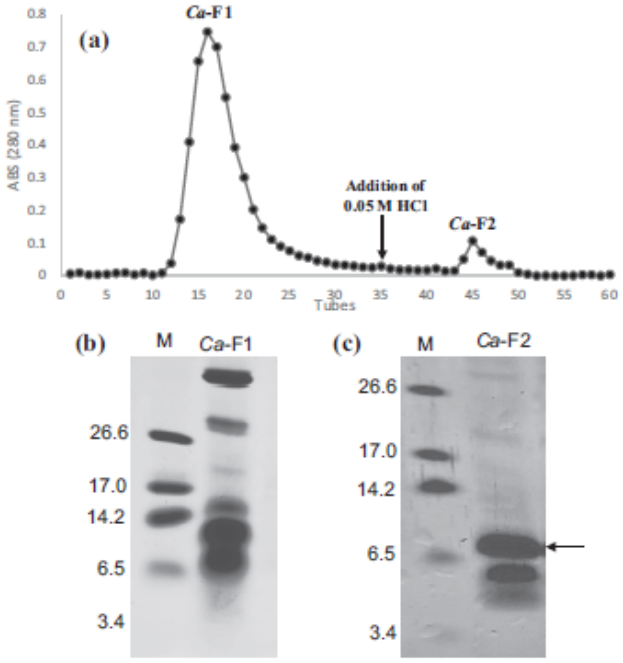


FIGURE 1 Chromatogram obtained after chitin affinity chromatography of fractions of *C. annumm*. (a) Protein elution was monitored by absorbance at 280 nm. The flow was 0.5 mL.min^{-1}, and the volume used was 3 mL per tube. Electropheheretic visualization by SDS-tricine-PAGE of the peptide-enriched F1 fraction (b) and peptide-enriched F2 fraction (c). M: Low mass molecular marker (kDa). Arrows indicate the bands that were subjected to mass spectrometry.

mycelial growth of *F. solani*.[18,47] The *Ca*-F2 described in this work showed similar molecular weights to Mo-CBP3.

3.2 | Mass spectrometry sequencing

For the identification of some peptides obtained in *Ca*-F2, some main bands were submitted to mass spectrometry. However, only one sample obtained a result with similarity to proteins in the databases: the major band of *Ca*-F2 (above 6.5 kDa) named Ca.1 (20 µg). The spectra were interpreted by Mascot software, and fragments of peptide residue sequences were obtained. All residues were subjected to identification of similar proteins in the NCBI BLASTP database. The obtained peptides MMMMRMQQEEMQPR (Ca.1) were similar to 2S albumin, a seed storage protein (Figure 2). After this process, we named the peptide *Ca*-Alb2S.

Mass spectrometry sequencing of the Ca.1 band of *Ca*-F2 showed similarity with proteins of the 2S albumin family (Figure 2). Seed 2S albumins constitute an important class of low-molecular-weight, multifunctional reserve proteins that generally constitute 20–50% of the total protein content of seeds.[48] They have been reported as proteins that have toxic properties against several organisms, including parasites.[49] Recent studies report that chitin-binding proteins may show similarities with 2S albumins, as shown for Mo-CBP3 and Mo-CBP4 proteins.[12,19]

3.3 | Docking of 2S albumin with the tetramer of N-acetylglucosamine $(NAG)_4$

The peptide sequences obtained from *C. annuum*, MMMMRMQQEEMQPR (Figure 2), showed 85,71% identity and 92% coverage with the 2S albumin of *Bertholletia excelsa* (2LVF_A). The 2S albumin structure was submitted to a blind molecular docking with $(NAG)_4$, and the best model showed negative values of affinity energy (−7.075 kcal/mol), indicating spontaneous binding. The results from docking experiments showed that the 2S albumin amino acids GLU92, ASN93, SER96, and PHE114 form hydrogen bonds with $(NAG)_4$ (Figure 3b; Table 2). These amino acids are present in the 5ª alpha helix exposed in the model surface (Figure 3a). Hydrophobic interactions of 2S Albumin residues MET42 and ARG97 with $(NAG)_4$ were identified by LigPlot+ v. 2.2.4 program (Table 2). These amino acids are present in loop regions (Figure 3c).

In silico molecular modeling of 2S albumin isolated from *C. annuum* was performed using 2S albumin from *B. excelsa* as a template. The docking of 2S albumin with $(NAG)_4$ showed negative affinity energy values (−7.075 kcal/mol), indicating spontaneous binding and interactions by hydrogen bonds and hydrophobic interactions. The affinity energy value obtained here can be compared, for example, to the bond between the chitinase enzyme that hydrolyzes its substrate formed by N-acetylglucosamine monomers, between −4.7 and −9.2 kcal/mol, depending on the amount of monomers in the oligomer.[50] Previous work, such as that by Miranda et al.,[29] and Ventury et al.,[51] also demonstrated molecular docking, however, with the vicilin protein. In the work carried out by Miranda et al.,[29] the chitin binding site with $(NAG)_4$ also indicated spontaneous binding, with negative energy values (−6.793 kcal/mol). Interactions by hydrogen bonds, salt bridges and hydrophobicity involving amino acids from this chitin binding site and $(NAG)_4$ have also been demonstrated. The docking performed by Ventury et al.[51] also demonstrated spontaneous binding, with energy values of (−7.527 kcal/mol), and interactions by hydrogen bonds, salt bridges and hydrophobicity. In the work carried out, an alpha-helix region was bound to chitin. Similar results were observed in the work carried out by Miranda et al.[29]

3.4 | Effect of *Ca*-F2 on the inhibition of trypsin enzyme activity

A residual trypsin activity assay for *Ca*-F2 was performed (data not shown). *Ca*-F2 showed 15% inhibition of trypsin enzymatic activity at a concentration of 50 µg.mL^{-1}.

This work also evaluated the ability of *Ca*-F2 obtained after affinity chromatography to inhibit the activity of trypsin, an enzyme of the serine protease class that can be found in pathogenic organisms that colonize plant tissues.[52] According to the results obtained, it was

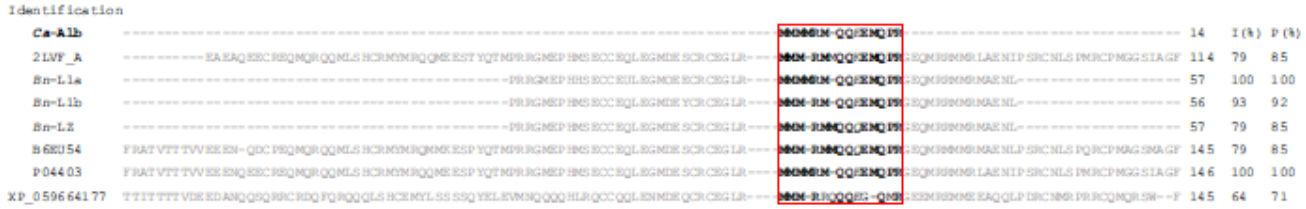


FIGURE 2 Alignment of amino acid residues of the tryptic peptide of a *Ca*-F2 protein band obtained from *C. annuum* seeds by chitin affinity chromatography (the band subjected to mass spectrometry is indicated by an arrow in Figure 1c). The band showed similarity with 2S albumin proteins from *Bertholletia excelsa* (UniProt accession code: 2LVF_A), *Bertholletia excelsa* H.B. K (Bn-L1a, Bn-L1b, Bn-LZ), *Bertholletia excelsa* – Brazil Nut (UniProt accession code: B6EU54 and P04403) and *Cornus florida* (UniProt accession code: XP_059664177.1). The red box indicates the region of similarity between the peptide identified by masses and some 2S albumin. I indicates the percentage of identical residues highlighted in the red box region, and P indicates the percentage of positive residues, that is, those that have the same biochemical characteristics.

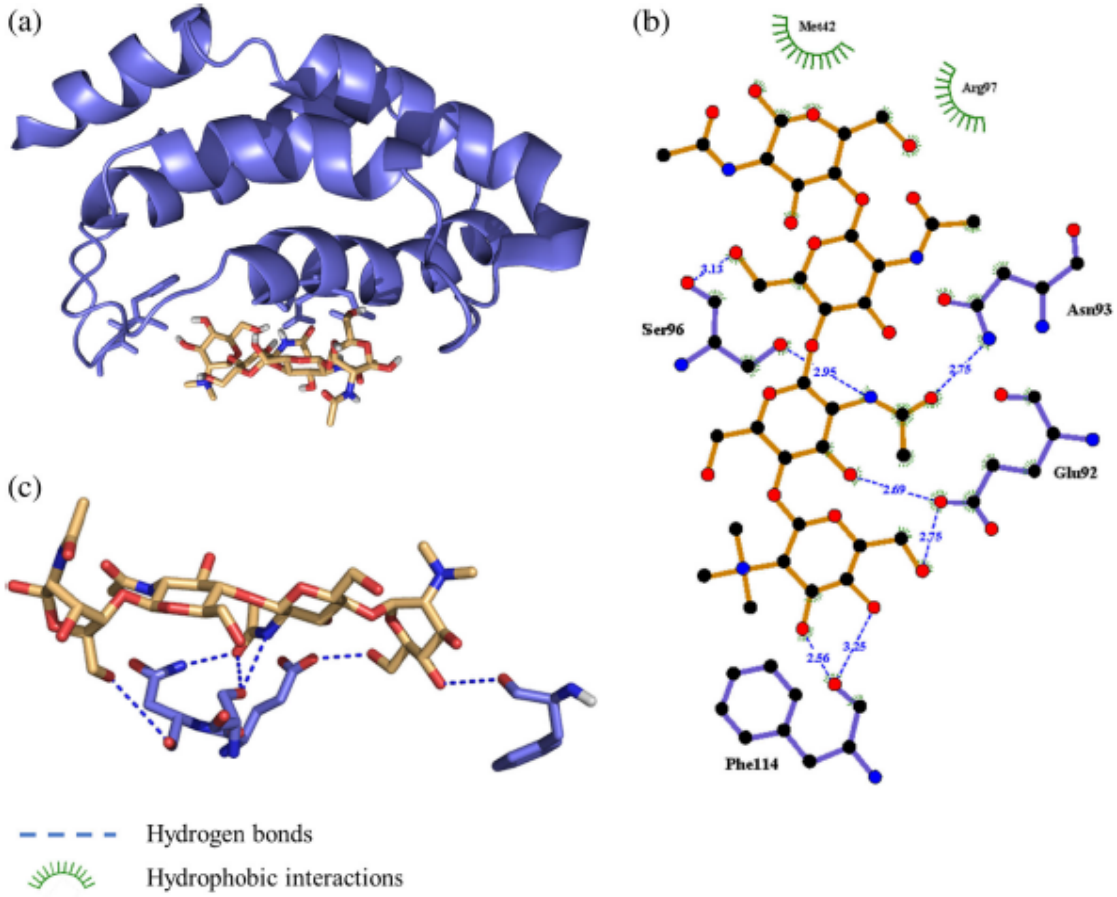


FIGURE 3 Docking of 2S albumin with the tetramer of N-acetylglucosamine $(NAG)_4$. (a) Three-dimensional structure of the 2S albumin protein interacting with $(NAG)_4$. (b) Zoom of the region of the 2S albumin-$(NAG)_4$ interaction. Dashed blue lines = hydrogen bonds, dark blue rods = nitrogen atoms, red rods = oxygen atoms. (c) 2D diagram representing the interactions between amino acid residues of 2S albumin with $(NAG)_4$ (LigPlot + program v 2.2.4). Green arcs = hydrophobic interactions, blue dotted lines = hydrogen bonds, the numbers express the distance (Å) of the hydrogen bonds, black spheres = carbon atom and blue spheres = nitrogen atoms, red spheres = oxygen atoms.

clear that of the fractions tested, *Ca*-F2 was able to inhibit the activity of the trypsin enzyme (data not shown). It has already been described that peptides isolated from *Capsicum* seeds can present inhibitory activity against serine proteases, such as the peptide isolated by Ribeiro et al.,[53] a peptide from *C. annuum* seeds. Some works describe that plant peptides can act as bifunctional inhibitors with

inhibitory activity for trypsin and α-amylase. Lipid transfer proteins, for example, can act as inhibitors, especially inhibiting α-amylase, as already reported for LTPs from *Capsicum* seeds.[54] Studies also point to the antimicrobial effects of low molecular weight trypsin inhibitors on different species of fungi. Ribeiro et al.[53] and Silva et al.[55] isolated trypsin inhibitors from seeds of the genus *Capsicum* capable of inhibiting the growth of yeasts and phytopathogenic fungi, with the exception of the growth of fungi of the genus *Fusarium*.

3.5 | Effect of *Ca*-F2 on the growth of yeast

Initially, an antimicrobial assay was carried out to assess the effect of *Ca*-F2 on the growth of *C. albicans* and *C. tropicalis* yeasts in the presence of 12.5, 25, 50, 100, and 200 μg.mL^{-1} for 24 h (Figure 4). *Ca*-F2 inhibited the growth of *C. albicans* and *C. tropicalis*, especially at concentrations of 50, 100, and 200 μg.mL^{-1}, where we can see a percentage close to 100% inhibition for *C. tropicalis*. At low concentrations, the peptide may induce the formation of pseudohyphae, which will result in a higher absorbance reading since there is no presence of pseudohyphae in control cells (Figure 4).

Concerning antimicrobial activity, chitin-binding proteins have antimicrobial activity, especially against fungi.[19,56,57] Reserve proteins such as the 2S albumin family are also characterized by antimicrobial activity against a wide spectrum of pathogens. It has *in vitro* inhibitory activity against several microorganisms.[58] In addition, it is also important to emphasize that these proteins, in addition to presenting different functions, ranging from storage, enzyme inhibition and lipid transfer to cell wall structure, can be grouped in relation to a defined number of cysteine residues (eight-cysteine motif) located in distinct positions of their sequences, suggesting a common ancestor for these families.[59]

TABLE 2 Interactions between 2S albumin amino acids residues and $(NAG)_4$.

Residue	AA	Distance
Hydrophobic interactions[a]		
42	MET	3.56
97	ARG	3.55
Hydrogen bonds[b]		
92	GLU	2.38
93	ASN	3.06
93	ASN	2.10
96	SER	2.19
96	SER	2.06
114	PHE	2.58

[a]Distance between interactions carbon atoms.
[b]Distance between hydrogen and acceptor atoms.

3.6 | Plasma membrane permeabilization

Since *Ca*-F2 showed greater inhibition potential for all tested yeasts, we investigated whether it alters the membrane integrity of the yeast *C. albicans* by fluorescence microscopy. We observed that the *C. albicans* cells treated with 100 μg.mL^{-1} *Ca*-F2 presented prominent labeling for Sytox green fluorescent dye, indicating that this peptide was able to affect the structural integrity of the plasma membrane, allowing the entry and labeling of the dye. In the control (in the absence of *Ca*-F2), no fluorescence was observed. We observed that, in addition to the reduction in the number of cells, the yeast *C. albicans* had 25.0% permeabilization (Figure 5). We also observed cells forming clumps when treated with *Ca*-F2.

In a recent work carried out by Lopes et al,[19] chitin binding proteins with fungicidal activity, called Mo-CBP4, which has similarity with 2S albumin, have an inhibition mechanism for the fungus

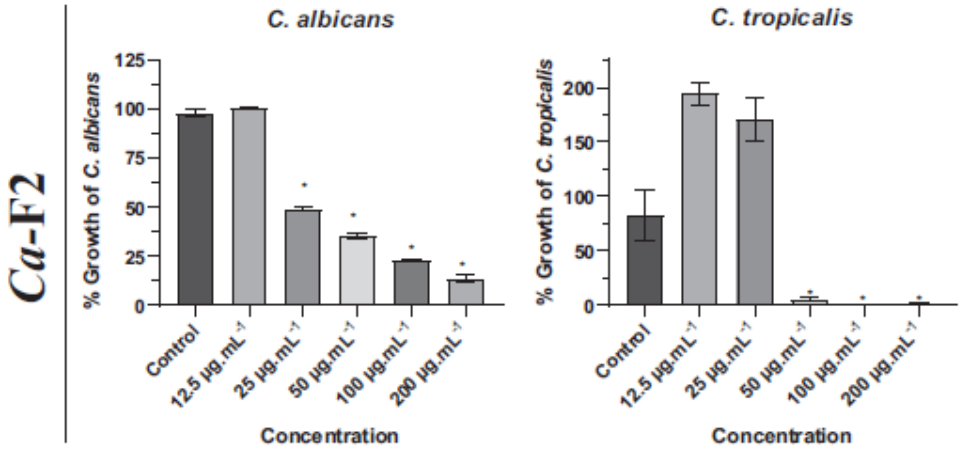
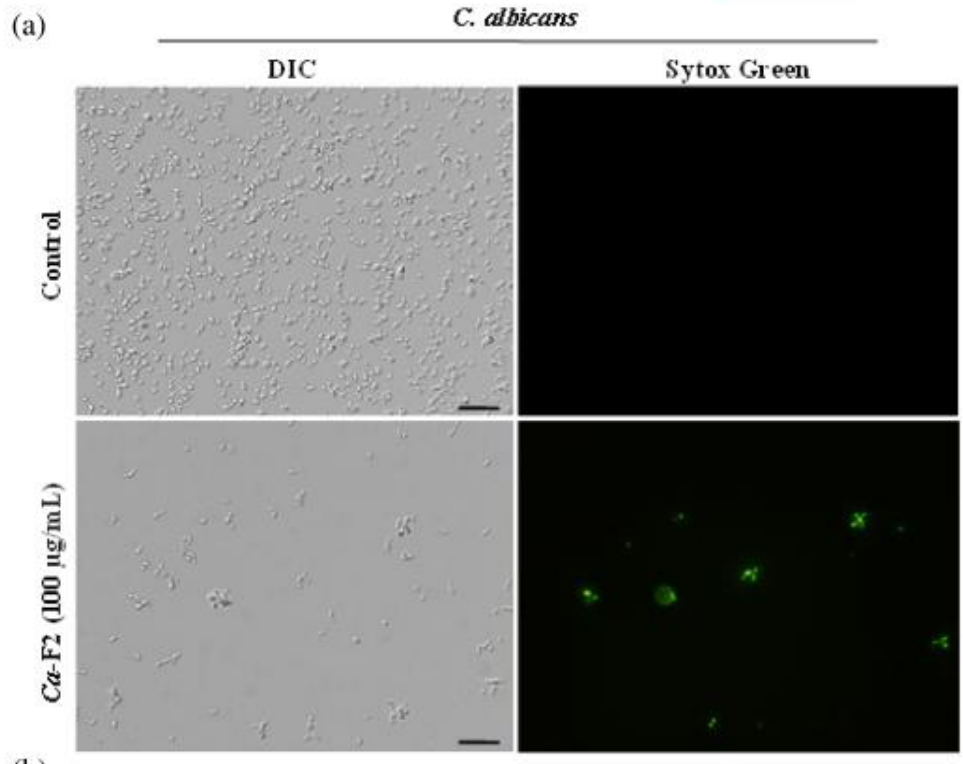


FIGURE 4 Effect of *Ca*-F2 on the growth of *C. albicans* and *C. tropicalis* at concentrations of 12.5, 25, 50, 100 and 200 μg.mL^{-1} for 24 h. Values are the means (± SD) of triplicates. Asterisks indicate significant differences ($p < 0.05$) between treatments and controls. Values above the bars indicate the percentage of growth inhibition.



(b)

Species of yeast	Sample	Cell number in DIC	Cell number in fluorescence	% of permeabilized cells (Sytox Green+)
C. albicans	Control	1409.0±412.1	0±0	0
	***Ca*-F2**	122.2±21.04	30.6±3.5	25.0*

FIGURE 5 Membrane permeabilization assay. (a) Images of *C. albicans* cells after membrane permeabilization using the fluorescent probe SYTOX green. Cells were treated with *Ca*-F2 for 24 h. Control cells were treated only with SYTOX green. Bars = 20 µm. (b) The percentage of permeabilized cells was quantified by the cell number in five random fields of the DIC and fluorescence views of the samples, with the cell number in the DIC of each sample considered 100%. * significant differences by one-way ANOVA, $p < 0.05$.

T. mentagrophytes associated with an increase in membrane permeability, overproduction of ROS and damage to the cell wall leading to the death of microconidia. Chitin-binding proteins are rich in positively charged residues (such as arginine) and can interfere with negatively charged cell membrane components that cause membrane disarrangement and lead to cell lysis.[60,61] We observed the ability of *Ca*-F2 to induce plasma membrane permeabilization of *C. albicans* (Figure 5). SYTOX Green is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes and does not cross intact cell membranes. For this reason, it can be used to investigate whether certain agents act as fungicides causing cell death by promoting leakage of intracellular content, leading to an imbalance in cell homeostasis. Our findings corroborate other works, such as Lopes et al.,[19] showing that this class of proteins can maintain the integrity of the membrane, leading to the death of different fungal species. It was also observed that another chitin-binding protein called Iu-CBP was isolated from *Iberis umbellata* seeds, and in addition to demonstrating activity against several bacteria, it also showed activity against the fungus *Aspergillus flavus*.[62] In addition to the families reported above showing activity against phytopathogenic yeasts and fungi,[19] other chitin-binding proteins, such as lectins, vicilins and chitinases, have been reported to be toxic to insects.[16,29,63]

3.7 | Cell viability

Based on the antimicrobial activity of *Ca*-F2, the cells were plated in a new culture medium, and after 36 h, the colony forming units (CFU) were counted, and the viability of *C. albicans* and *C. tropicalis* yeasts was analyzed (Figure 6). *Ca*-F2 at 200 µg.mL^{-1} produced a severe reduction in the number of colony forming units (CFU) for *C. albicans* after 36 h of incubation, indicating a lethal dose of 82%. For *C. tropicalis*, *Ca*-F2 also produced a reduction in the number of CFUs of 100%, indicating that the inhibitory effect of *Ca*-F2 on this yeast is fungicidal.

After obtaining the binding fraction on chitin (*Ca*-F2), antifungal tests were performed to evaluate the antimicrobial activity against yeasts. According to the results obtained (Figure 4), *Ca*-F2 showed

antimicrobial activity against the yeasts *C. albicans* and *C. tropicalis* at the different concentrations tested. The inhibitory effect of *Ca*-F2 on *C. tropicalis* yeast was fungicidal. The fact that *Ca*-F2 exerts a fungicidal effect on the tested yeasts is an important feature when thinking about the development of new pathogen control measures, since fungistatic compounds can contribute to the development of resistant microorganisms.[64]

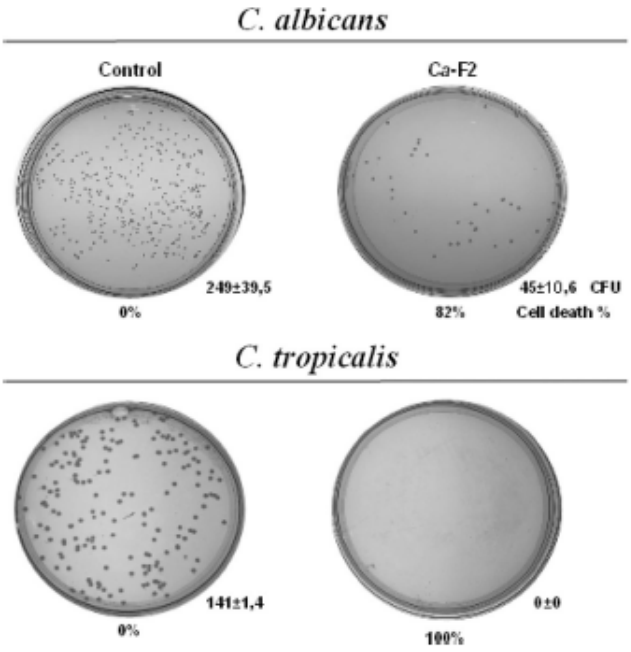


FIGURE 6 Cell viability of the yeasts (a) *C. albicans* and (b) *C. tropicalis*. Petri dish images showing the growth of colonies in the control (without the addition of *Ca*-F2) and after treatment with 200 μg.mL^{-1} *Ca*-F2 for 36 h. The cell death percentage was calculated in relation to the control, untreated cell (cell viability–100%). The experiments were performed in triplicate. CFU = colony forming unit. Numbers under the images indicate the percentage of cell death.

3.8 | Hemolytic potential on sheep red blood cells and toxicity effect on *G. mellonella* larvae of *Ca*-F2

The ability of *Ca*-F2 to cause lysis in mammalian cells was evaluated. It was verified that *Ca*-F2 has very low hemotoxicity against mammalian cells, even when dealing with high concentrations. A percentage of hemolysis of 1.37%, 1.37%, and 3.5% was verified for the concentrations of 50, 100 and 200 μg.mL^{-1}, respectively, as shown in Figure 7a. The ability of *Ca*-F2 to cause toxicity in *G. mellonella* larvae was also evaluated. Larvae inoculated with *Ca*-F2 had a low rate of toxicity. At a concentration of 200 μg.mL^{-1}, there was a 15.6% rate of toxicity; however, when the concentration was increased, there was a greater increase in the rate of *Ca*-F2 toxicity on *G. mellonella* larvae. When the concentration of 400 μg.mL^{-1} was tested, the toxicity rate became 45.5% (Figure 7b), showing that at very high concentrations, *Ca*-F2 can be toxic.

A major concern in the development of therapeutic drugs is their high toxicity to mammalian cells, even when dealing with natural peptides extracted from plants.[65] With the results obtained, we observed that even at high concentrations (200 μg.mL^{-1}), *Ca*-F2 did not cause hemolysis in the cells tested (Figure 7a). Taveira et al.[66] found similar results in their work. They tested a bioinspired peptide on defense from *C. annuum* fruits called CaDef2.1G27-K44. Four concentrations were evaluated (200, 100, 50, and 25 μM), and the results indicated that the peptide has very low cytotoxicity and hemotoxicity against mammalian cells, even at the highest concentration tested, with the percentage of hemolysis being 19% for the concentration of 200 μM. In another work carried out by Toledo et al.,[67] two peptides

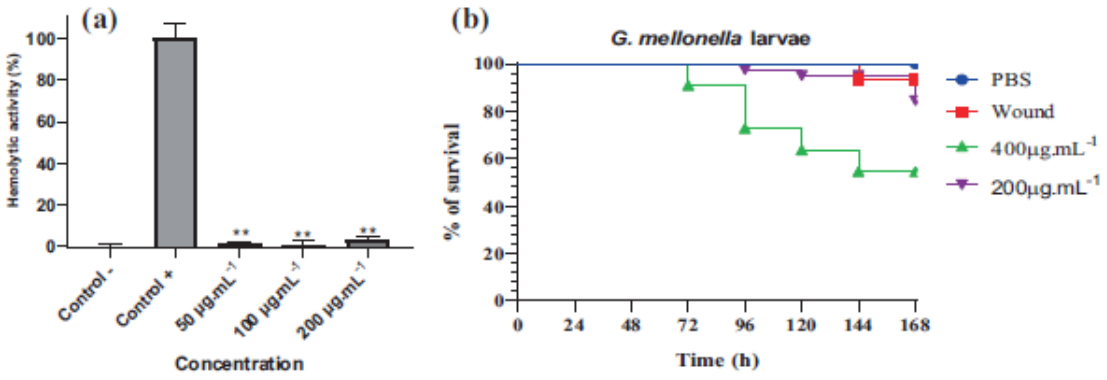


FIGURE 7 (a) Effect of *Ca*-F2 on sheep red blood cells. Data are presented as a percentage of hemolytic activity of cells treated with *Ca*-F2 (50, 100 and 200 μg.mL^{-1}), untreated as a negative control (Control– [0.15 M NaCl]) and positive control (Control +) treated with 1% (vol/vol) Triton X-100 detergent at 37°C for 1 h. Hemolytic activity percentage was calculated in relation to the positive control (hemolytic activity–100%) and to the negative control (hemolytic activity–0%). The results presented are mean values obtained over one experiment, performed in triplicate. Asterisks indicate significant differences ($p < 0.05$) between treatments and Control +. (b) Effect of *Ca*-F2 in vivo on *G. mellonella* larvae. Survival curve of *G. mellonella* larvae inoculated with *Ca*-F2 at 200 and 400 μg.mL^{-1}. Phosphate saline buffer (PBS) and needle wounds were used as controls. Statistical significance was calculated using the Gehan-Breslow-Wilcoxon test $p < 0.05$.

called RR and RW were designed, where the charge and hydrophobicity were increased, resulting in better antifungal activity and less toxicity for mammalian cells, and the antifungal activity was even more highlighted in the more hydrophobic and cationic peptide (WR).

Figure 7b shows that the concentration of 200 $\mu g.mL^{-1}$ *Ca*-F2 showed a low rate of toxicity for *G. mellonella* larvae; however, when we increased the concentration, the mortality rate of the larvae increased significantly. These results are probably because the peritrophic membrane (PM) present in the midgut of the larvae is rich in chitin; thus, *Ca*-F2 may bind to this matrix, breaking it. PM has often become the target of studies that discuss plant defense against insects because by interfering with chitin metabolism, nutrients are neither absorbed nor transported on a regular basis, impairing insect development.[68] In work carried out by Qin et al.,[69] it was observed that a chitin-binding protein CBPA present in *Bacillus thuringiensis* bacteria could help direct the bacteria to the PM of *G. mellonella* larvae, confirming that chitin-binding proteins interfere with the PM of insects. However, it is important to emphasize that our objective is the low toxicity of *Ca*-F2; therefore, further studies will be carried out with the concentration that had low toxicity on the larvae. With the information presented and with further studies, *Ca*-F2 presents important characteristics for the development of new drugs.

4 | CONCLUSION

For the first time, chitin affinity chromatography was carried out in pepper plants. We evaluated the *in vitro* and *in vivo* activity of the chitin-binding fraction *Ca*-F2 from *C. annuum*. *Ca*-F2 had antimicrobial activity against *C. albicans* and *C. tropicalis* and did not present toxicity to mammalian cells and *G. mellonella* larvae, indicating a promising candidate in the development of new treatments for fungal control.

AUTHOR CONTRIBUTIONS

The study was conceived by Valdirene Moreira Gomes and Gabriella Rodrigues Gonçalves. Experimental procedures were carried out by Gabriella Rodrigues Gonçalves, Marciele Souza Silva, Layrana Azevedo dos Santos, Thomas Zacarone Afonso Guimarães, Sarah Rodrigues Ferreira, Celso Shiniti Nagano, and Renata Pinheiro Chaves. Data analyses were performed by Valdirene Moreira Gomes, André de Oliveira Carvalho, Gabriel Bonan Taveira, Rosana Rodrigues, and Olney Vieira da Motta. The article was written by Gabriella Rodrigues Gonçalves, Valdirene Moreira Gomes, and Gabriel Bonan Taveira. All authors reviewed the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors state that there is no conflict of interest in this work.

DATA AVAILABILITY STATEMENT

Data are available upon request to the authors.

ETHICS STATEMENT

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

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4 – CAPÍTULO 2

Structural and functional characterization of of lipid transfer proteins (LTPs) with chitin-binding properties: insights from protein structure prediction, molecular docking and antifungal activity

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Structural and functional characterization of of lipid transfer proteins (LTPs) with chitin-binding properties: insights from protein structure prediction, molecular docking and antifungal activity

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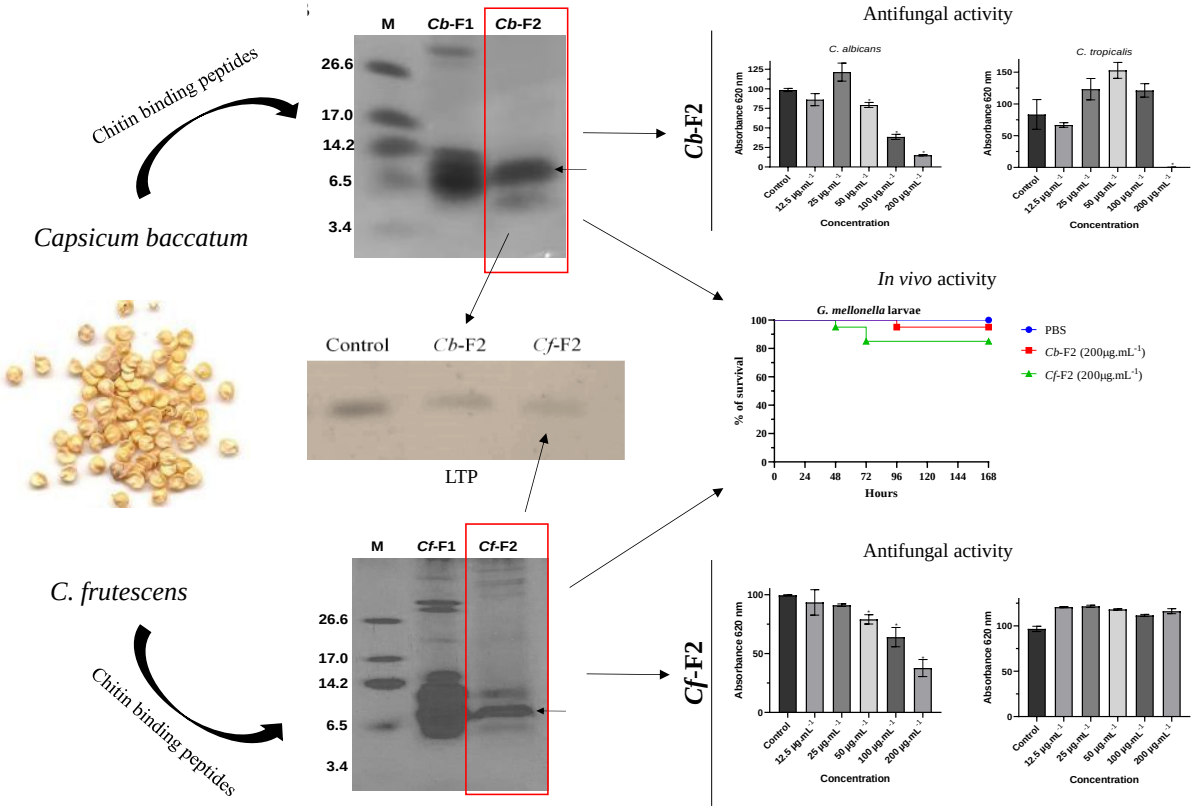
Graphical Abstract



Abstract

Faced with the emergence of multiresistant microorganisms that affect human health, microbial agents have become a serious global threat, affecting human health and various plant crops. Antimicrobial peptides (AMPs) have attracted significant attention in research for the development of new microbial control agents. The objective of this work was to characterize and evaluate the antimicrobial activity of peptides with affinity for chitin isolated from seeds of *Capsicum baccatum* and *C. frutescens* on the growth of *Candida* and *Fusarium* species. Proteins were extracted in phosphate buffer pH 5.4 and subjected to chitin column chromatography. After chitin affinity chromatography, two fractions were obtained for each species, *Cb*-F1 and *Cf*-F1 and *Cb*-F2 and *Cf*-F2, respectively. The *Cb*-F1 (*C. baccatum*) and *Cf*-F1 (*C. frutescens*) fractions did not bind to the chitin column. The electrophoresis obtained after chromatography showed two major protein bands between 3.4 and 14.2 kDa for *Cb*-F2. For *Cf*-F2, three major bands were identified between 6.5 and 14.2 kDa. One band from each species was subjected to mass spectrometry and both bands showed similarity to non-specific lipid transfer protein (LTP). *Cb*-F2 inhibited the growth of *C. albicans* and *C. tropicalis*. *Cf*-F2 inhibited the growth of *C. albicans* but did not inhibit the growth of *C. tropicalis*. Both fractions were unable to inhibit the growth of *Fusarium* species. The toxicity of the fractions was tested *in vivo* on *Galleria mellonella* larvae, and both showed a low toxicity rate at high concentrations. Therefore, the fractions have great potential for the development of new antifungal molecules.

KEYWORDS chitin binding peptides, antimicrobial peptides, *Capsicum*, *Galleria mellonella*, *Candida*

1 | INTRODUCTION

Antimicrobial peptides (AMPs) are a group of molecules that occur spontaneously in all living organisms and act in defense against pathogens, including fungi and bacteria.[1] They have a low molecular weight and net positive charge and are rich in hydrophobic amino acids.[2,3] In plants, they are part of the innate defense, acting as the first line of defense against infections caused by pathogenic microorganisms and are present in all plant organs.[4] This group includes thionins, defensins, cyclotides and lipid transport proteins (LTPs). Another class of plant-isolated proteins with antifungal activity is the chitin-binding proteins (CBPs). These molecules have the ability to reversibly bind chitin, a structural polysaccharide present in various organisms, such as the cell wall of fungi. Studies have reported the antifungal activity of plant CBPs against phytopathogens[5,6] and *Candida* spp.[7,8] This group includes hevein-like, 2S albumin and chitinases. To date, the presence of chitin-binding LTPs has not been reported so far.

LTP, also known as nonspecific lipid transfer protein (nsLTP), is produced by several types of plants. LTPs are quite abundant, rich in cysteine residues with conserved positions, are secretory and soluble and have a molecular mass of less than 10 kDa.[9-12] Structurally, LTPs have four to five α-helices, which are stabilized through four relatively conserved disulfide bonds with the pattern of C – Xn – C – Xn – CC – Xn – CXC – Xn – C – Xn – C. Helix folding results in a central hydrophobic cleft suitable for binding to various lipids, including fatty acids and phospholipids.[13] Based on their molecular weight, LTPs are divided into two groups: LTP type 1, which has approximately 7 kDa, and LTP type 2, which has approximately 10 kDa.[14] The disulfide bonding pattern in type 1 is C1–C6 and C5–C8, whereas in type 2, C1–C5 and C6–C8. They differ in tertiary structure, such as the long tunnel hydrophobic cavity in type 1, but in type 2, LTPs are composed of two head-to-head hydrophobic cavities.[15,16]

LTPs perform several functions simultaneously. One of the most reported functions is the defense function in plants against pathogens. It is possible that the response mechanism involves the secretion of nsLTP in the apoplast so that they bind to lipid molecules secreted by the plants or to molecules secreted by the microorganisms.[17] nsLTP are also involved in biological processes such as fruit ripening,[18] seed growth and germination,[17,19] lipid barrier assembly,[13,17] and lipid transport[20] and are also associated with abiotic stress in plants, what was demonstrated by the increase of overexpression tolerance in a saline stress environment.[21] Furthermore, there are no reports of nsLTP exhibiting toxicity against plant or animal cells.

Several works have reported the isolation of these biomolecules from different species of plants and from different organs. Schmitt et al.[22] isolated BrLTP2.1, which was extracted from the nectar of *Brassica rapa*, and its antifungal activity was reported. McLTP1 was extracted from *Morinda citrifolia* seeds showing antibacterial and anti-inflammatory activity and improved survival in lethal sepsis.[23,24] The nsLTP1 protein isolated from *Trachyspermum ammi* seeds has also been described, but there is no description of antimicrobial activities.[25] Regarding plants of the genus *Capsicum*, we can highlight an LTP isolated from *C. annuum* seeds with inhibitory activity against α-amylase, serine proteinases and fungi.[26]

Microbial resistance to drugs continues to rise around the world increasing the demand for new bioactive molecules that have a wide spectrum of activities. Therefore, the discovery of new antifungal agents, particularly those produced by plants, is of great importance.[27] In this scenario, research on plant chitin binding proteins with applications in the medical field was intensified. Thus, the objective of this work was to isolate and characterize chitin-binding peptides from seeds of *C. baccatum* (Accession UENF 1732)

and *C. frutescens* (Accession UENF 1775) and to analyze their possible biological activities.

2 | MATERIALS AND METHODS

2.1 | Plant material

The seeds of *C. baccatum* (Access UENF 1732) and *C. frutescens* (Access UENF 1775) were provided by the Laboratory for Plant Genetic Improvement (LMGV), Center for Agricultural Sciences and Technologies, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Brazil. The seeds were sown in 72-cell polystyrene trays with substrate fertilized with formulation (4 N/14P/8K) (Vivatto®). The trays were kept in a growth chamber at 28°C and irrigated once a day. After the emergence of two pairs of definitive leaves, the seedlings were transplanted individually into plastic pots (5 L) containing a mixture of soil and substrate (in a 2:1 ratio). Then, the pots were transferred to an acclimatized greenhouse. The plants were irrigated once a day until the ripe fruits were harvested.

2.2 | Microorganisms

The yeast species *C. albicans* (CE022), *C. tropicalis* (CE017) and the filamentous fungi *F. oxysporum* and *F. solani* were cultivated in Sabouraud medium and kept in a refrigerator at 4°C in the Laboratory of Physiology and Biochemistry of Microorganisms (LFBM) of the Center for Biosciences and Biotechnology (CBB), from the State University of North Fluminense Darcy Ribeiro, Campos dos Goytacazes - RJ.

2.3 | Protein extraction and precipitation with ammonium sulfate

First, the seeds (5 g) were ground until a very fine-grained flour was obtained. The proteins were extracted in phosphate buffer (0.01 M Na_2HPO_4, 0.015 M NaH_2PO_4, 0.1 KCl, 1.5% EDTA), pH 5.4, at a ratio of 1:10 (5 g flour: 50 mL buffer) for 3 h under constant stirring at 4°C. The homogenate was subjected to centrifugation (15,000 x *g* for 30 min), the precipitate was discarded, and ammonium sulfate was added to the supernatant for precipitation at 90% saturation. The solution was kept overnight at 4°C. After this process, the suspension was centrifuged again at 15,000 x *g* for 30 min. Proteins precipitated in the 0-90% ammonium sulfate saturation range were recovered by resuspension in 10 mL of distilled water and heated at 80°C for 15 min. Subsequently, a final centrifugation (10,000 x *g* for 10 min) was performed. The supernatant was dialyzed against distilled water (avg. flat width 32 mm, 1.27 in. – Sigma–Aldrich) at 4°C for 3 days and finally lyophilized[28].

2.4 | Protein quantification

Quantitative protein determinations were performed using the bicinchoninic acid method,[29] with ovalbumin (Sigma–Aldrich) used as the standard protein.

2.5 | Isolation of proteins and peptides with affinity for chitin

Poly-(1→4)-β-N-acetyl-D-glucosamine (chitin) was obtained from shrimp shells – practical grade, powder and commercially available from Sigma–Aldrich. Initially, a chemical treatment of chitin was performed,[30] in which 25 g of industrial chitin was acidified with 500 mL of 100 mM HCl. This mixture was incubated for 24 h at 4°C with constant shaking. After 24 h, all HCl was removed, and 250 mL of 100 mM NaOH was

added to the resulting precipitate. This mixture was heated at 100°C for 16 h. After heating, the NaOH was removed, and another 250 mL of NaOH was added to the precipitate for another 16 h of heating. This step was repeated once again, totaling three 16 h warm-ups. After the last heating, all NaOH was removed, and 200 mM HCl was added to the precipitate. The HCl was then removed from the mixture, and distilled water was added to the precipitate for storage. Subsequently, a chitin column was equilibrated with sodium acetate buffer (0.1 M, pH 5.5), and a 50 mg peptide-rich heated fraction, solubilized in the same sodium acetate buffer, was applied to the column. The chromatography was performed with equilibration buffer and the retained fraction was eluted with 0.05 M HCl solution. The absorbance of the desorbed proteins was monitored at 280 nm. The flow rate used was 1 $mL.min^{-1}$. Protein peaks were collected and recovered after dialysis and lyophilization.

2.6 | Tricine gel electrophoresis in the presence of SDS

To perform the tricine-SDS–PAGE gel, a gel was mounted using glass plates of 8 x 10 cm and 7 x 10 cm, and 0.75 mm spacers were used. The separating gel was prepared at a 16.4% acrylamide/bis-acrylamide concentration, and the gel concentration was 3.9%. The samples were mixed with the sample buffer and then heated for 5 min at 100°C and centrifuged at 15,000 x *g* for 5 min. Subsequently, the equivalent to 20 $\mu g/mL^{-1}$ of sample was applied to the gel. A constant voltage of 24 V was used for running for approximately 16 h.[31] M3546 (Sigma–Aldrich) was used as a protein molecular weight marker. Molecular mass of proteins in kDa (26.600; 17.000; 14.200; 6.500; 3.496; 1.060).

2.7 | Western blotting

Western blotting was performed according to the methodology described by Towbin et al.[32] After electrotransfer, the membranes were incubated in blocking buffer containing 2% skimmed-milk poder in phosphate-buffered saline for 16 h at 4°C. The membranes were washed ten times for 5 min in phosphate-buffered saline (PBS) ($NaH_2PO_4$10 μM, NaCl 0.15 M, pH 7.4) at room temperature and then incubated with a primary anti-LTP antibody (1:1000) in blocking buffer. After that, the membranes were washed as before and incubated with the secondary antibody (1:500) in blocking buffer for 1 h at room temperature under gentle agitation and washed as described above. The membranes were developed with 3,30'-diaminobenzidine (DAB) in a developing solution (40 μM Tris-HCl, pH 7.5, 1 mg/mL^{-1} DAB, 100 μM imidazole, and 0.03% hydrogen peroxide) until the stained bands were visualized.

2.8 | In-gel digestion

Using a scalpel, the gel bands were cut into slices and then into tiny cubes of ~1 mm^3. Each cubed slice was placed in a separate 1.5 mL tube, and 1000 μL of destaining solution (50 mM AmBic/50% ACN - 1:1) was added to each sample. The tubes were gently agitated on a Thermomixer at room temperature overnight. After removing the solution, 200 μL of fresh destaining solution was added for 1 h before removal. Gel bands were then dehydrated by adding 500 μL of 100% ACN to each tube for 1 min, followed by a repeated step. For protein reduction, 200 μL of a solution containing 10 mM DTT/100 mM AmBic was added to each tube and incubated at 55°C for 30 min with gentle agitation on a Thermomixer. Subsequently, 500 μL of 100% ACN was added to each tube for dehydration, followed by the addition of 200 μL of alkylation solution (55 mM

iodoacetamide-IAA/100 mM AmBic). The tubes were kept in the dark on a Thermomixer at room temperature for 30 min.

For protein digestion, 200 µL of cold trypsin solution (digestion solution containing trypsin in 10 mM AmBic/10% ACN) was used. The tubes were kept at 4°C for 30 min and then transferred to a Thermomixer at 37°C overnight for complete digestion. Then, 200 µL of extraction buffer (containing 1:2 of 5% formic acid to 100% ACN) was added and incubated at 37°C for 30 min in a Thermomixer. The samples were then evaporated in a SpeedVac until completely dried. Prior to mass spectrometry analysis, the samples were resuspended in 50 µL of 0.1% formic acid in 50 mM AmBic.[33]

2.9 | Mass spectrometry analysis

The identification of peptides present in *Cb*-F2 was carried out in collaboration with the Marine Biochemistry Laboratory (BioMar-Lab), Department of Fisheries Engineering, Federal University of Ceará (UFC), Ceará, Brazil. After tricine-SDS–PAGE, one band present in *Cb*-F2 was extracted, and then, tryptic peptides were obtained[33] and subjected to LC–MS/MS analysis. The instrument used was a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp, MA, USA). Data collection and processing parameters were adjusted as described by Carneiro et al.[34] Trypsin-digested peptides were analyzed by matrix-assisted laser desorption (ESI-Quad-ToF) mass spectrometry. The interpretation of the spectra was performed using the Mascot program, and the sequenced peptides were submitted to alignment using the BLASTp tool.[35] Sequences with a high percentage of identity were chosen and subjected to multiple alignments using the CLUSTAL Multiple Sequence Alignment by MUSCLE program (3.8).[36] The

calculation of the percentages of positive and identical residues was carried out considering only the residues obtained by mass spectrometry.

For identification of peptides present in *Cf*-F2, ESI-LC–MS/MS analyzes were performed at Unit of Integrative Biology, Genomic and Proteomics Sector (UENF), using a nanoAcquity UPLC coupled to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, United Kingdom) . During separation, samples were loaded onto the nanoAcquity UPLC 5 µm C18 trap column (180 µm x 20 mm) at 5 µL/min^{-1} for 3 min and then onto the nanoAcquity HSS T31 1.8 µm analytical reversed-phase column (75 µm x 150 mm) at 400 nL/min^{-1}, with a column temperature of 45°C. For peptide elution, a binary gradient was used, with mobile phase A (water and 0.1% formic acid) and mobile phase B (acetonitrile and 0.1% formic acid). The gradient elution started at 7% B, then ramped from 7% B to 40% B until 92.72 min, then remained at 99.9% until 106.00 min, then decreased to 7% B until 106.1 min, and finally remained at 7% B until the end of the experiment at 120 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility and independent-data acquisition mode (HDMS^{E}). The ion mobility wave was adjusted to a velocity of 600 m s^{-1}; the transfer collision energy ramped from 19 V to 55 V in high-energy mode; the cone and capillary voltages were 30 V and 2750 V, respectively; and the source temperature was 70°C. For the TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range from 50 to 2000 Da. Human [Glu1]-fibrinopeptide B (Sigma–Aldrich) at 100 fmol/µL^{-1} was used as an external calibrant, and lock mass acquisition was performed every 30 s. Mass spectra were acquired by MassLynx v4.0 software.[37]

2.10 | Proteomic data analysis

For spectral processing and database searching, the ProteinLynx Global Server (PLGS; version 3.0.2) (Waters, USA) and ISSO Quant workflow software were used.[38,39] The PLGS was processed using a low-energy threshold of 150 (counts), an elevated-energy threshold of 50 and an intensity threshold of 750. In addition, the analysis was performed using the following parameters: two missed cleavages, a minimum fragment ion per peptide equal to 3, a minimum fragment ion per protein equal to 7, a minimum peptide per protein equal to 2, fixed modifications of carbamidomethyl and variable modifications of oxidation and phosphoryl. The false discovery rate was set to a maximum of 1%. The proteomics data were processed against the *C. annumm* protein database from UniProt (https://www.uniprot.org/proteomes/UP000222542) for *C. frutescens*.

2.11 | Protein structure analysis

The predicted structural model for the identified proteins was generated using the FASTA sequences in the AlphaFold Protein Structure Database. AlphaFold is an AI system that predicts a protein's 3D structure from its amino acid sequence. The generated models were further edited using the UCSF Chimera molecular graphics program (https://www.rbvi.ucsf.edu/chimera) to highlight the regions of interest in the protein structure.

2.12 | Docking of LTP with the tetramer of N-acetylglucosamine $(NAG)_4$

The docking of LTP with $(NAG)_4$ was performed using the DockThor v.2 program.[40] The blind docking strategy was performed using a search space defined by a 40 Å x 40 Å

x 40 Å cube, covering 100% of the protein surface. The $(NAG)_4$ model was created from PyMol with the Azahar plug-in to design oligosaccharides. All rotations of the ligand were free to rotate while the atoms of the protein were kept rigid. The standard search algorithm of the site was used with 1,000,000 evaluations and 24 executions per experiment. The best lipid transfer protein (LTP)-ligand complex was evaluated using the affinity energy value and submitted to the PLIP Web Tool program[41] and to the LigPlot+ v program. 2.2.4.[42] Noncovalent interactions between lipid transfer protein (LTP) and $(NAG)_4$ were identified by the programs.

2.13 | Effect of peptides on fungal growth

An aliquot was removed from the petri dish containing *C. albicans* colonies, and another aliquot was removed from the petri dish containing *C. tropicalis* colonies. Subsequently, these aliquots were placed in new petri dishes containing Sabouraud agar (10 g. L peptone, 40 g. L D(+)glucose, 15 g. L agar) (Merck) to generate striations in the middle. These new plates were kept in an oven at 30°C for 24 h. Subsequently, the cells were removed from the petri dish and homogenized in 10 mL of Sabouraud broth (10 g. L peptone, 20 g. L D(+)glucose) (Merck) for quantification in a Neubauer chamber (LaborOptik) with the aid of an optical microscope (Axiovison A2, Zeiss). Regarding filamentous fungi, the inocula were transferred from the stock and placed to grow in petri dishes containing Sabouraud agar (Merck) for 11 days at 30°C. After growth, the spores were also removed and homogenized in 10 mL of Sabouraud broth for further quantification in a Neubauer chamber. Yeast (1×10^4 cel/mL^{-1}) and fungal cells (1×10^3 cel/mL^{-1}) were incubated in Sabouraud broth containing different concentrations of the fractions obtained from the seeds. The assay was performed in cell culture microplates

(96 wells) at 30°C for a period of 24 h for yeast and 36 h for filamentous fungi. Cell growth was determined by optical density, monitored every 6 h, in a microplate reader at a wavelength of 620 nm. Each test was performed in triplicate. The entire procedure was performed under aseptic conditions in a laminar extractor according to the methodology adapted from Broekaert et al.[43] Cell growth without the addition of proteins was also determined.

2.14 | Cell viability analysis

To evaluate the effect of *Cb*-F2 and *Cf*-F2 on the cell viability of *C. albicans* and *C. tropicalis* yeasts, a growth inhibition assay was initially performed to obtain colonies. For plating, after 24 h of incubation, the control cells (without *Cb*-F2 and *Cf*-F2) and the test cells (with *Cb*-F2 and *Cf*-F2) were diluted 1000x. A 60 µL aliquot of the dilution was spread with a Drigalski loop over the surface from a Petri dish containing Sabouraud agar and cultivated at 30°C for 36 h. At the end of this period, the colony forming units were determined, and the Petri dishes were photographed.[44] The experiments were performed in triplicate, and the results were presented considering that the control represents 100% cell viability.

2.15 | Effect of *Cb*-F2 and *Cf*-F2 toxicity on *G. mellonella* larvae

For this assay, twenty last instar larvae of *G. mellonella* were used for the control and treatments, and larvae that weighed between 250 and 300 mg were selected. To inject 10 µL of *Cb*-F2 and *Cf*-F2 (200 µg/mL^{-1}) into the larval hemocoel through the last proled, a Hamilton syringe was used. Two control groups were used: one group was inoculated

with PBS, and another group suffered only injury caused by the injection needle. Subsequently, the larvae were incubated at 37°C in Petri dishes for 7 days, and the number of dead larvae was counted at every 24 hours. Larvae were considered dead when they did not show any 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 the GraphPad software (Inc., California, CA, USA). The assay was performed in duplicate.[45]

2.16 | Statistical analysis

Data from yeast and filamentous fungi growth inhibition assays were evaluated by one-way ANOVA. Differences in means of $p < 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism software (version 5.0 for Windows).

3 | RESULTS AND DISCUSSION

3.1 | Chitin affinity chromatography, electrophoretic profile and detection of LTP by western blotting

The chromatogram reveals that protein extracts from the *C. baccatum* and *C. frutescens* species were fractionated, yielding two major peaks for each species (Figure 1). The fractions called *Cb*-F1 (*C. baccatum*) and *Cf*-F1 (*C. frutescens*) did not bind to the chitin column. The fractions named *Cb*-F2 (*C. baccatum*) and *Cf*-F2 (*C. frutescens*) were the fractions that bound to the chitin column (Figure 1A, C). Thus, the presence of peptides that have the ability to bind to chitin was verified.

The electrophoretic profile of the fractions obtained by the chromatography can be visualized in Figure 1B, D. In Figure 1B, which indicates the electrophoretic profile of *C. baccatum*, we can observe the protein bands for both fractions. *Cb*-F1 has bands in the marker range between 3.4 and 26.6 kDa, but there is a band above the 26.6 kDa marker. For *Cb*-F2, two major protein bands are demonstrated, between 3.4 and 14.2 kDa (Figure 1B). For *C. frutescens*, we can observe the electrophoretic profile in Figure 1D. In *Cf*-F1, we can observe major bands in the molecular marker range and bands above the 26.6 marker. For *Cf*-F2, we observed major protein bands between 6.5 and 14.2 kDa (Figure 1D).

The presence of LTP in the *Cb*-F2 and *Cf*-F2 fractions was demonstrated using anti-nsLTP serum by western blotting. The control used was an LTP of *C. chinense* seeds (Figure 2).

Other studies reported the presence of chitin-binding peptides isolated from plant seeds, such as Fa_AMP1 and Fa_AMP2, extracted from *Fagopyrum esculentum*, were purified by gel filtration on Sephadex G75, ion-exchange HPLC on SP COSMOGEL, and reverse-phase HPLC. They had molecular masses of 3,879 Da and 3,906 Da respectively[46] and Mo-CBP4 (11.78 kDa) was purified after protein extraction and chromatography on chitin and CM Sepharose™ columns, from *Moringa oleifera*[6] In the case of species of the genus *Capsicum*, little is known about chitin-binding peptides and their possible activities.

3.2 | Peptide identification by mass spectrometry

For the identification of peptides obtained in the *Cb*-F2 and *Cf*-F2 fractions, major bands obtained by electrophoresis (20 μg) were submitted to mass spectrometry. The bands subjected to the technique are identified by an arrow in Figure 1B, D. The spectra were

interpreted by Mascot and PLGS software, and fragments of peptide residue sequences were obtained. The residues were subjected to identification of similar proteins in the NCBI and UniProt BLASTP databases. The obtained peptides CGVQLSVPISR (*C. baccatum*), TLSGLAQSTDERRYANLKDDAAQALPGKCGVALNVPISR and CEQQFQRTCDDYLRCEGLTQIIHQEQQAAVLQGRAEAFQTAQALPGLCRHCSIP SLS (*C. frutescens*) were similar to the type 1 lipid transfer protein (LTP-1) (Figure 3), and the results were reinforced by western blotting.

Mass spectrometry sequencing of the bands of the *Cb*-F2 and *Cf*-F2 fractions showed similarity with proteins of the LTP family (Figure 3). The presence of peptides related to the LTP family was also confirmed by western blotting (Figure 2). This result shows for the first time the property of LTPs to associate with the chitin matrix. The relevance of this finding will be investigated later.

Other works reported the isolation and characterization of nsLTPs, which involves extraction of proteins with saline buffer or acidic solution, fractionation in ammonium sulfate (~80%)[47-49, 25] and subsequent chromatography, which may include ion exchange (DEAE-Sephadex), size exclusion chromatography (Sephadex G-50) or reversed-phase high-performance liquid chromatography (RP- HPLC).[47,50,51,12] This is the first work that reports the isolation of LTPs through chitin affinity chromatography.

3.3 | Three-dimensional structure

Three LTPs were identified in this work, one from *C. baccatum* and two from *C. frutescens*. Based on the amino acid sequence, a search was performed on the NCBI for *C. baccatum* and the UniProt database for *C. annuum*, where we performed a prediction of the three-dimensional structure of the peptides (Figure 4). For all proteins, in green,

we have the region identified from mass spectrometry; in orange, we have an area highlighted that concerns the alpha helices found, while the regions in gray represent the coil regions.

The most conserved region is the 8CM motif, and the difference between the types of LTP is defined by the nature of the disulfide bonds, with variation in the C5 and C6 cysteines, leading to functional effects on the tertiary structure. Some amino acids are also important to define type I nsLTPs from the others, such as the conservation of a glycine between helix 1 and 2, connected by the disulfide bridge between C2 and C3. It is also known that lysine and tyrosine residues are conserved among type I nsLTPs. These differences contribute to the structures and can interfere with their mechanisms.[52]
Generally, the three-dimensional structure of nsLTPs presents a compact domain composed of four helices (H1-H4) connected by short loops (L1-L3) and an unstructured C-terminal tail.[52] Helices 1 and 2 and the C-terminus are generally longer in type I nsLTPs. The domain is joined by several intramolecular hydrogen bonds and by four disulfide bridges conserved in the patterns: (1): C1-C6, C2-C3, C4-C7 and C5-C8 for type I nsLTPs[53] and (2): C1-C5, C2-C3, C4-C7 and C6-C8 for type II nsLTPs.[54] Furthermore, the three-dimensional structures have an internal tunnel-shaped cavity that will accommodate different types of lipids, in addition to being stable against thermal and digestive processing.[55]

3.4 | Docking of the LTPs with $(NAG)_4$

The LTP three-dimensional structure most similar to *Cb*-F2 (Acession: PHT58787) was submitted to a blind molecular docking with $(NAG)_4$, and the best model showed negative values of affinity energy (-8.085 kcal/mol), indicating spontaneous binding (Figure 5A).

Amino acid residues participating in the interaction with $(NAG)_4$ VAL83, ALA84, LEU85, ASN86 and PRO88 are located in the 5° loop region. ARG52 is present in the 3ª alpha helix, while PRO78 and SER79 are exposed in the 4ª alpha helix of the model surface (Figure 5A). The results from docking experiments showed that the LTP amino acids VAL83, LEU85 and ASN86 form hydrogen bonds with $(NAG)_4$ (Figure 6A, Table 1). Hydrophobic interactions of LTP residues ARG52, PRO78, SER79, VAL83, ALA84 and PRO88 with $(NAG)_4$ are represented in Figure 6-B, Table 1.

The best docking of the LTP three-dimensional structure similar to *Cf*-F2 (Acession: A0A2G3AMC5) with $(NAG)_4$ also showed a negative value of affinity energy (-7.516 kcal/mol), indicating spontaneous binding (Figure 5B). Amino acid residues participating in the GLN6, THR7, GLY8 and ILE9 interactions are present in the first loop region of the protein model, while ASP96 is present in the last loop region. CYS11 is located in the 1ª alpha helix, and the amino acid residues THR54, ASN57, CYS58 and LYS60 are in the 3ª alpha helix exposed in the model surface (Figure 5B). The LTP-$(NAG)_4$ complex suggests that the amino acid residues GLN6, THR7, ILE9, CYS11, ASN57 and ASP96 form hydrogen bonds with $(NAG)_4$. (Figure 6C, Table 1). Amino acid residues THR7, GLY8, CYS11, THR54, ASN57, CSY58 and LYS60 interact hydrophobically with $(NAG)_4$ (Figure 6D, Table 1).

Amino acid residues identified from *Cf*-F2 were also similar to another LTP (Acession: A0A2G3AE21). The docking performed showed a negative interaction energy (-7.749 kcal/mol), indicating spontaneous binding (Figure 5C). Amino acid residues participating in the interaction with $(NAG)_4$ ILE2, ALA3, ASP4, ASN5 and GLU9 are present in the first loop region of the protein model. ARG32 is present in the 2ª alpha helix, while GLN81, GLU82, GLN84 and ALA85 are present in the 6ª alpha helix exposed in the model surface (Figure 5C). The results from docking experiments showed

that amino acid residues ASP4, ASN5, GLU9, ARG32, GLN81, GLU82 and GLN84 form hydrogen bonds with $(NAG)_4$ (Figure 6E, Table 1). Amino acid residues ILE2, ALA3, ASN5, ARG32, GLN81 and ALA85 interact with $(NAG)_4$ through hydrophic interactions (Figure 6E, Table 1).

The LTP three-dimensional structures most similar to *Cb*-F2 (Acession: PHT58787), *Cf*-F2 (Acession: A0A2G3AMC5) and *Cf*-F2 (Acession: A0A2G3AE21) isolated from *C. baccatum* and *C. frutescens*, respectively, were submitted to a blind molecular docking with $(NAG)_4$. The docking of LTP with $(NAG)_4$ showed negative affinity energy values (-8,085 kcal/mol), (-7,516 kcal/mol) and (-7,749 kcal/mol), respectively, for the previously described accessions, indicating spontaneous binding and interactions by hydrogen bonds and hydrophobic interactions. The amino acids participating in the interaction with $(NAG)_4$ were VAL83, ALA84, LEU85, ASN86, PRO88, ARG52, PRO78 and SER79 for the first access, GLN6, THR7, GLY8, ILE9 ASP96, CYS11, THR54, ASN57, CYS58 and LYS60 for the second access and ILE2, ALA3, ASP4, ASN5, GLU9, ARG32, GLN81, GLU82, GLN84 and ALA85 for the third access.

In a previous work, Ventury et al.[56] demonstrated that vicilin from *Vigna unguiculata* seeds (50 kDa), with chitin-binding, presented spontaneous binding, with energy values of (-7,527 kcal/mol), and interactions by hydrogen bonds, salt bridges and hydrophobicity. In another study carried out by Nazeer et al., [25] LTP was isolated from *T. ammi* seeds. The three-dimensional structure predicted for the isolated protein is composed of four α-helices stabilized by four disulfide bonds and a long C-terminal tail. Docking was also carried out with two different ligands (myristic acid and oleic acid). The amino acids Leu11, Leu12, Ala55, Ala56, Val15, Tyr59 and Leu62 are suggested to be essential for the binding of lipid molecules. This binding pattern involving specific

residues with ligands is variable, what has been proven by many 3D structures of LTPs solved.

3.5 | Effect of *Cb*-F2 and *Cf*-F2 on the growth of yeast and fungi

To verify whether the fractions have antimicrobial activity, a growth inhibition assay was performed on the yeasts *C. albicans* and *C. tropicalis* at concentrations of 12.5, 25, 50, 100 and 200 μg/mL^{-1} for 24 h and on the fungi phytopathogenic *F. oxysporum* and *F. solani* at a concentration of 200 μg/mL^{-1} for 36 h. *Cb*-F2 inhibited the growth of *C. albicans* at concentrations of 50, 100 and 200 μg/mL^{-1} and inhibited the growth of *C. tropicalis* only at a concentration of 200 μg/mL^{-1}; however, it inhibited 100% of the growth of this yeast. *Cf*-F2, on the other hand, only inhibited the growth of the yeast *C. albicans* at concentrations of 50, 100 and 200 μg/mL^{-1}. It was also found that the retained (*Cb*-F2 and *Cf*-F2) and nonretained (*Cb*-F1 and *Cf*-F1) fractions did not show activity against phytopathogenic fungi at the tested concentrations (Figure 7).

The nsLTPs have been the subject of several antimicrobial studies. Although its *in vivo* studies are little explored, it is known that they are capable of inhibiting the growth of important pathogenic microorganisms *in vitro*. For example, nsLTPs from *C. anuumm* seeds demonstrated activity against *Colletotrichum lindemunthianum* and *C. tropicalis* with efficacy demonstrated at a concentration of 400 μg/mL^{-1} for both species. This concentration corresponds to a lethal dose for 70% of *C. tropicalis* cells. In contrast, when using *Cb*-F2, we observed 100% cell mortality using only half the concentration (200 μg/mL^{-1}) compared to *C. annuum* nsLTP.[57] In another study with the same species, the authors demonstrated that the identified LTP had activity against *Saccharomyces cerevisiae, Pichia membranifaciens, C. tropicalis* and *C. albicans*,[58] while the LTP

identified in *Coffea canephora* demonstrated activity against *C. albicans* at a concentration of 400 μg/mL$^{-1,}$ inhibiting only 50% of cell growth,[59] while *Cb*-F2 and *Cf*-F2 inhibited 84 and 62% at a concentration of 200 μg/mL$^{-1,}$ respectively.

The defense response mechanism probably involves the secretion of nsLTP in the apoplast, allowing its connection with molecules secreted by microorganisms.[17] Therefore, they interact with receptors such as serine/threonine protein kinases, which have an extracellular leucine-rich repeat (LRR) domain, as well as a transmembrane region and a cytoplasmic protein kinase (PK). This interaction activates a cascade of protein kinases (MAPKs), inducing protective factors, such as PR proteins (related to pathogenesis), AMPs and SARs (acquired systemic resistance).[52]

3.6 | Cell viability

Due to the antimicrobial activity of the fractions, the cell viability of the yeasts *C. albicans* and *C. tropicalis* was determined at 200 μg/mL^{-1} with an incubation period of 36 h (Figure 8). *Cb*-F2 reduced the number of colony forming units (CFU) for *C. albicans*, showing 85.15% loss of viability. For *C. tropicalis*, the fraction showed an even greater reduction of 99.4%, indicating a fungicidal effect for this species. *Cf*-F2, on the other hand, showed a lethal dose of 84.3% for *C. albicans*. As expected, there was no CFU reduction for *C. tropicalis*.

To be considered fungicidal, an antifungal must reduce the number of colonies by 99% in CFU.mL, and to be considered fungistatic, it must reduce the number of colonies by <99% in CFU.mL in relation to the initial inoculum.[60]

3.7 | Toxicity effect on *G. mellonella* larvae of *Cb*-F2 and *Cf*-F2

The ability of *Cb*-F2 and *Cf*-F2 to cause toxicity in *G. mellonella* larvae at a concentration of 200 μg/mL^{-1} was evaluated. Both fractions demonstrated a low level of toxicity in inoculated larvae. For *Cb*-F2, it was verified that at the tested concentration, there was a 95% larval survival rate. For the *Cf*-F2 fraction, it was verified that at the same concentration, there was a lower survival rate, of 85% (Figure 9).

The toxicity of fractions to *G. mellonella* larvae was assessed in this study. These larvae are used as an alternative to traditional experimental models that use murines, as there are positive correlations between the results obtained in *G. mellonella* and murine models. This approach reduces experimental time and costs in addition to eliminating the need for authorization by ethics committees.[61] Although the peritrophic membrane (PM) present in the midgut of larvae is rich in chitin, we found that at this concentration, the fractions do not have the capacity to fully bind to this matrix and rupture it. Other studies report that chitin-affinity proteins can interfere with insect PM. The CBP fraction from *V. unguiculata* seeds was able to interfere with the development of *Callosobruchus maculatus*, reducing larval mass and length.[62] In another work, it is reported that CBPA, a chitin-binding protein, was expressed and purified from *Escherichia coli*. The interaction of this protein with the intestine and the PM *in vivo* was evaluated in *G. mellonella*, focusing on the initial objectives of the infection. From the results, the authors indicated that there is a possible interaction between CBPA and chitin-rich PM. Chitinases or other proteins can be produced and destabilize the chitin structure of the PM, along with the role of active pore-forming toxins known to damage midgut cells, resulting in reduced PM turnover.[63] As it has already been reported that chitin-binding proteins can interfere with PM, further analyses at different concentrations must be performed to confirm their low toxicity.

Infectious diseases and problems with antimicrobial resistance are increasingly serious, raising a demand for new antimicrobial molecules that have a broad spectrum of activity with few side effects.[64] As a result, AMPs have been sought as a promising alternative to traditional antibiotics, including nsLTPs, which have several actions, such as antibacterial, antiviral, enzymatic and antifungal activity.[65] Its positive charge favors its binding to negatively charged molecules, such as phospholipids and lipopolysaccharides, resulting in cell death.[15] As seen in this work, the *Cb*-F2 and *Cf*-F2 fractions are promising candidates for studying the development of effective methods for fungal control. However, further studies must be carried out to improve the results obtained.

4 | CONCLUSION

Two fractions of the species *C. baccatum* and *C. frutescens* were identified after chitin affinity chromatography, and their fungicidal activity and cytotoxicity *in vitro* and *in vivo*, respectively, were evaluated. Both fractions demonstrated *in vitro* antimicrobial activity against *C. albicans*, and only the *Cf*-F2 fraction did not show activity against *C. tropicalis*. After mass spectrometry, it was verified that both fractions were similar to LTPs, a group that has several functions in plants, including antifungal action. The *in vivo* toxicity of the fractions towards *G. mellonella* larvae was also evaluated. It was verified that at the concentration tested, neither fraction presented relevant a toxic effect on the larvae, indicating that they are candidates for the development of new therapeutic agents.

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DATA AVAILABILITY All data generated or analyzed during this study are included in this published article.

CONFLICT OF INTEREST The authors declare no conflicts of interest.

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Legends

FIGURE 1 Chromatograms obtained after chitin affinity chromatography of fractions *C. baccatum* (A) and *C. frutescens* (C). Protein elution was monitored by absorbance at 280 nm. The flow was 0.5 $mL.min^{-1}$. Electropheheretic visualization by SDS-tricine-PAGE of peptide-enriched fractions. *Cb*-F1 (1) and *Cb*-F2 (2): *C. baccatum* (B) and *Cf*-F1 (1) and *Cf*-F2 (2): *C. frutescens* (D). M: Low mass molecular marker (kDa). Arrows indicate the bands that were subjected to mass spectrometry

FIGURE 2 Western blotting of F2 fraction proteins extracted from different *Capsicum* species, *C. baccatum* (*Cb*-F2) and *C. frutescens* (*Cf*-F2), revealed by anti-LTP antibody. The positive control consisted of an LTP-rich fraction of *C. chinense* seeds

FIGURE 3 Alignment of amino acid residues of the tryptic peptides of the major protein bands from *Cb*-F2 and *Cf*-F2 seeds obtained by chitin affinity chromatography (protein band marked with an arrow in Figure 1B, D) showed similarity with LTP protein type 1. Gray highlights represent regions of similarity, while black highlights represent identical regions

FIGURE 4 Three-dimensional structure of the proteins identified in this work. PHT58787.1_NsLTP1, A0A2G3AE21, and A0A2G3AMC5 refer to nonspecific lipid transfer proteins. The fasta sequences of each identified protein were used as input for protein modeling in the SWISS-MODEL server. The PHT58787.1_NsLTP1 accession was obtained from NCBI for *C. baccatum*. Both A0A2G3AE21 and A0A2G3AMC5 were obtained from the UniProt database for *C. annuum*. The peptide sequences identified in this work are highlighted in green. The structures colored in orange refer to alpha-helix regions, while the gray color refers to the coil regions

FIGURE 5 Three-dimensional structure of the LTP protein interacting with $(NAG)_4$. A: LTP with similarity to *Cb*-F2, *C. baccatum*, accession: PHT58787. Alpha helices are represented in blue; loop regions are represented in gray. B: LTP with similarity to *Cf*-F2, *C. frutescens*, accession: A0A2G3AMC5. Alpha helices are represented in green; loop regions are represented in gray. C: LTP with similarity to *Cf*-F2, *C. frutescens*, accession: A0A2G3AE21. Alpha helices are represented in pink; loop regions are represented in gray. The N-acetylglucosamine tetramer is represented in orange. Dashed blue lines = hydrogen bonds

FIGURE 6 Docking of LTP with the tetramer of N-acetylglucosamine $(NAG)_4$. Region of LTP: PHT58787 – $(NAG)_4$ interaction through (A) hydrogen bonds and (B) hydrophobic interactions. Region of LTP: A0A2G3AMC5 – $(NAG)_4$ interaction through (C) hydrogen bonds and (D) hydrophobic interactions. Region of LTP: A0A2G3AE21 – $(NAG)_4$ interaction through (E) hydrogen bonds and (F) hydrophobic interactions. Amino acid residues in lines participate in the interaction through hydrogen bonds; dashed blue lines are hydrogen bonds; amino acid residues in spheres participate in the interaction hydrophobically

FIGURE 7 Effect of the *Cb*-F2 and *Cf*-F2 fractions from *C. baccatum* and *C. frutescens* on the growth of *C. albicans* and *C. tropicalis* at concentrations of 12.5, 25, 50, 100 and 200 $\mu g.mL^{-1}$ for 24 h and the effect of *Cb*-F1, *Cf*-F1, *Cb*-F2 and *Cf*-F2 on the growth of *F. oxysporum* and *F. solani* at concentrations of 200 $\mu g.mL^{-1}$ for 36 h. Values are the means (± SD) of triplicates. Asterisks indicate significant differences ($p < 0.05$) between treatments and controls. Values above the bars indicate the percentage of growth inhibition

FIGURE 8 Cell viability of the yeasts *C. albicans* and *C. tropicalis*. Petri dish images showing the growth of colonies in control (without addition of the F2 fraction) and after

treatment with 200 μg.mL^{-1} of *Cb*-F2 and *Cf*-F2 fraction for 36 h. Percentage of cell death was calculated in relation to the control, untreated cell (cell viability – 100%). The experiments were performed in triplicate. CFU = colony forming unit. Numbers under the images indicate the percentage of cell death

FIGURE 9 Effect of *Cb*-F2 and *Cf*-F2 *in vivo* on *G. mellonella* larvae. Survival curve of *G. mellonella* larvae inoculated with *Cb*-F2 and *Cf*-F2 200 μg.mL^{-1}. Phosphate-buffered saline (PBS) and needle wounds were used as controls. Statistical significance was calculated using the Gehan-Breslow-Wilcoxon test $p < 0.05$

TABLE 1 LTP amino acid residues involved in the interaction with the N-acetylglucosamine tetramer.

	PHT58787	A0A2G3AMC5	A0A2G3AE21
Hydrogen Bonds	VAL83 LEU85 ASN86	GLN6 THR7 ILE9 CYS11 ASN57 ASP96	ASP4 ASN5 GLU9 ARG32 GLN81 GLU82 GLN84
Hydrophobic Interactions	ARG52 PRO78 SER79 VAL83 ALA84 PRO88	THR7 GLY8 CYS11 THR54 ASN57 CYS58 LYS60	ILE2 ALA3 ASN5 ARG32 GLN81 ALA85

FIGURE 1

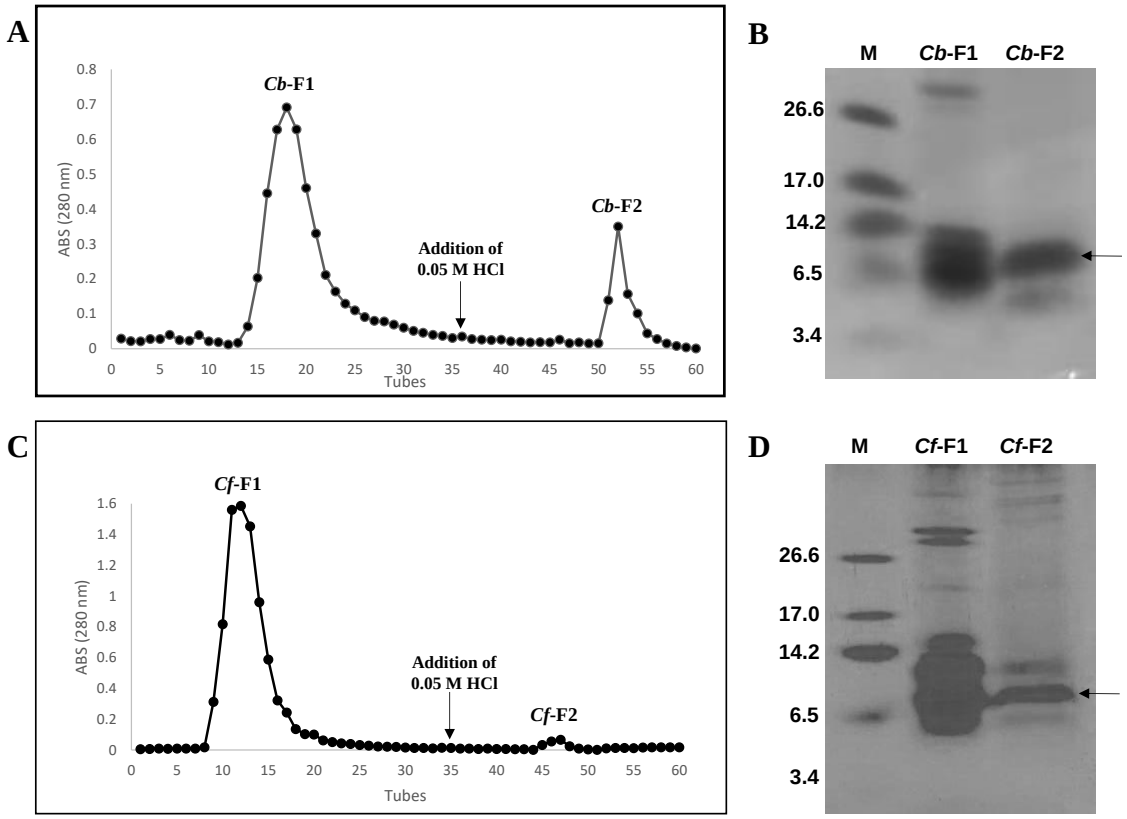

A
Cb-F1
Cb-F2
Addition of
0.05 M HCl
ABS (280 nm)
Tubes
B
M
Cb-F1
Cb-F2
26.6
17.0
14.2
6.5
3.4
C
Cf-F1
Cf-F2
Addition of
0.05 M HCl
ABS (280 nm)
Tubes
D
M
Cf-F1
Cf-F2
26.6
17.0
14.2
6.5
3.4

FIGURE 2

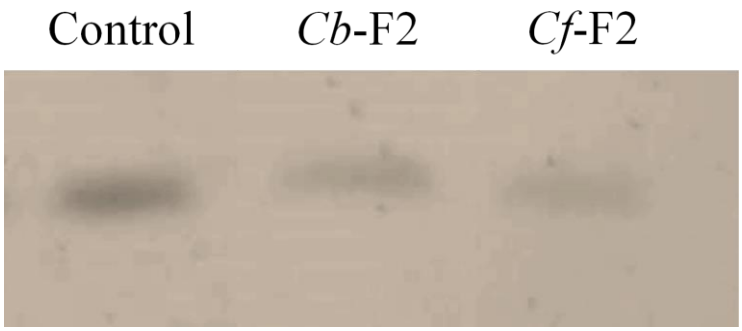


FIGURE 3

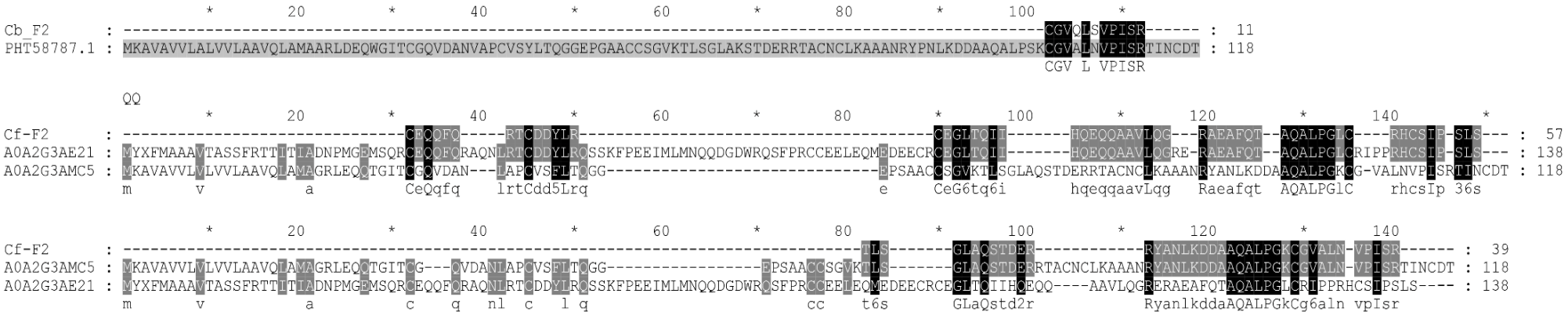

Cb_F2 : ---------- : 11
PHT58787.1 : MKAVAVVLALVVLAAVQLAMAARLDEQWGITCGQVDANVAPCVSYLTQGGEPGAACCSGVKTLSGLAKSTDERRTACNCLKAAANRYPNLKDDAAQALPSKCGVALNVPISRTINCDT : 118
Cf-F2 : ---------- : 57
A0A2G3AE21 : MYXFMAAAVTASSFRTTITLADNPMGEMSQRCEQQFQRAQNLRTCDDYLRQSSKFPEEIMLMNQQDGDWRQSFPRCCEELEQMEDEECRCEGLTQII-------HQEQQAAVLQGRE-RAEAFQT--AQALPGLCRIPPRHCSIP-SLS--- : 138
A0A2G3AMC5 : MKAVAVVLVLVVLAAVQLAMAGRLEQQTGITCGQVDAN---LAPCVSFLTQGG----------EPSAACCSGVKTLSGLAQSTDERRTACNCLKAAANRYANLKDDAAQALPGKCG-VALNVPISRTINCDT : 118
Cf-F2 : ---------- : 39
A0A2G3AMC5 : MKAVAVVLVLVVLAAVQLAMAGRLEQQTGITCG---QVDANLAPCVSFLTQGG----------EPSAACCSGVKTLS-------GLAQSTDERRTACNCLKAAANRYANLKDDAAQALPGKCGVALN-VPISRTINCDT : 118
A0A2G3AE21 : MYXFMAAAVTASSFRTTITLADNPMGEMSQRCEQQFQRAQNLRTCDDYLRQSSKFPEEIMLMNQQDGDWRQSFPRCCEELEQMEDEECRCEGLTQIIHQEQQ----AAVLQGRERAEAFQTAQALPGLCRIPPRHCSIPSLS---- : 138

FIGURE 4

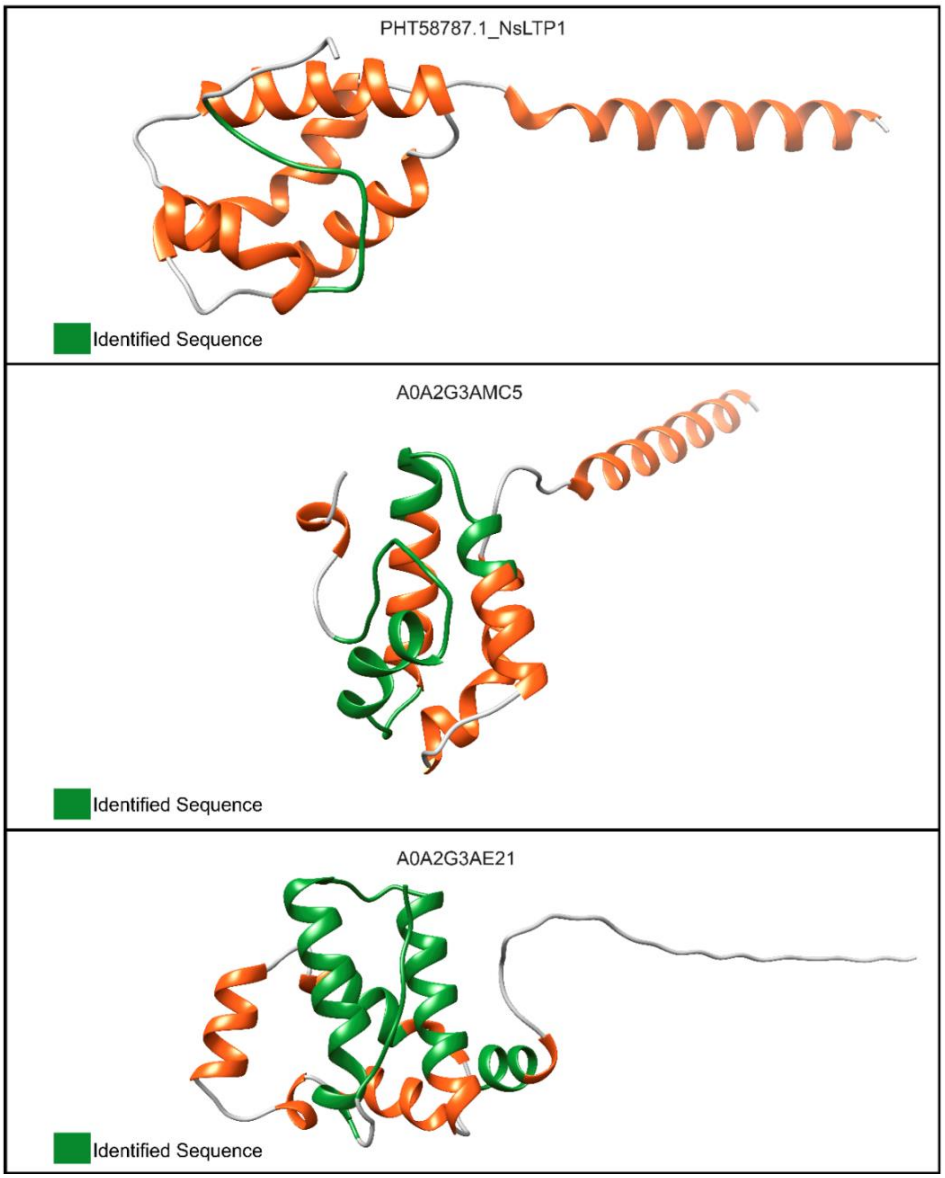

PHT58787.1_NsLTP1
Identified Sequence
A0A2G3AMC5
Identified Sequence
A0A2G3AE21
Identified Sequence

FIGURE 5

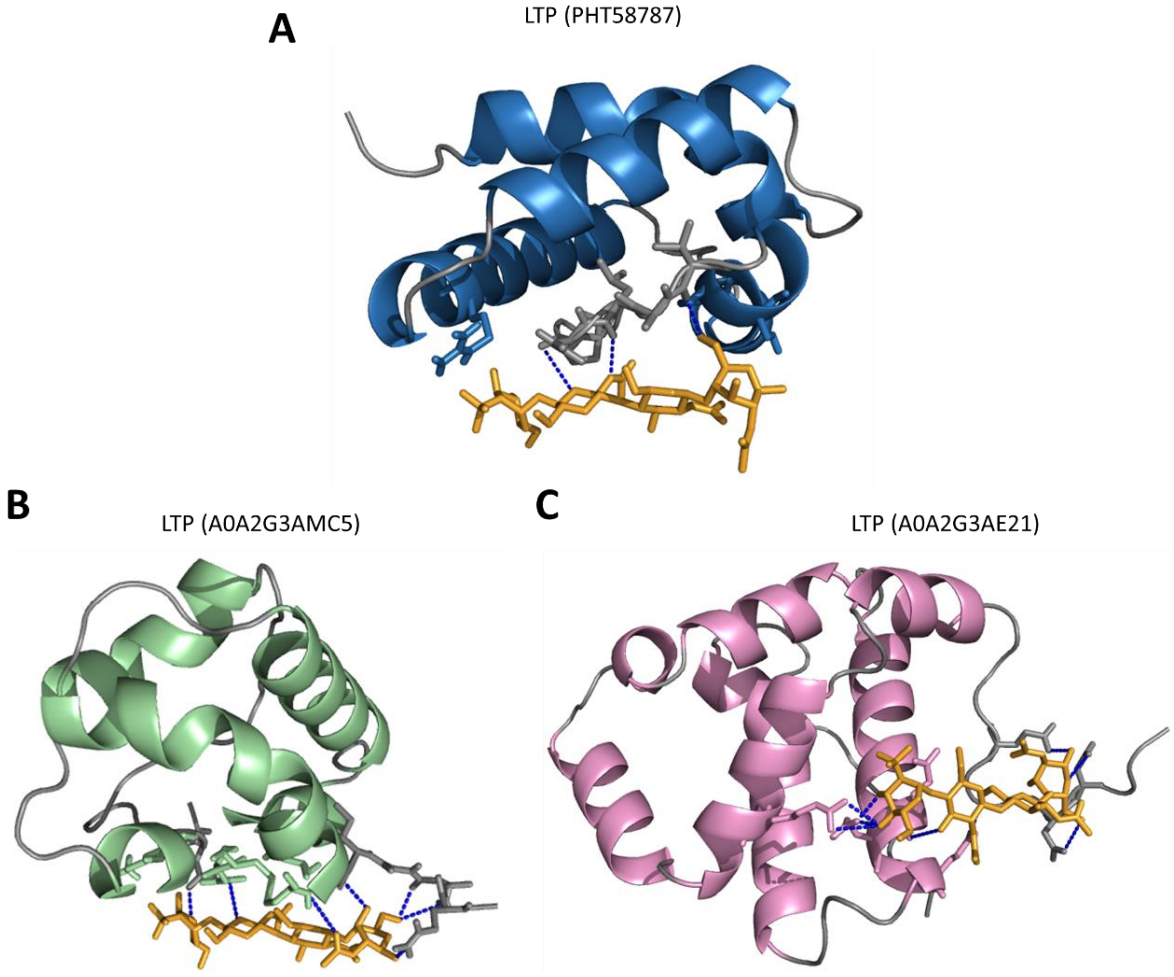


FIGURE 6

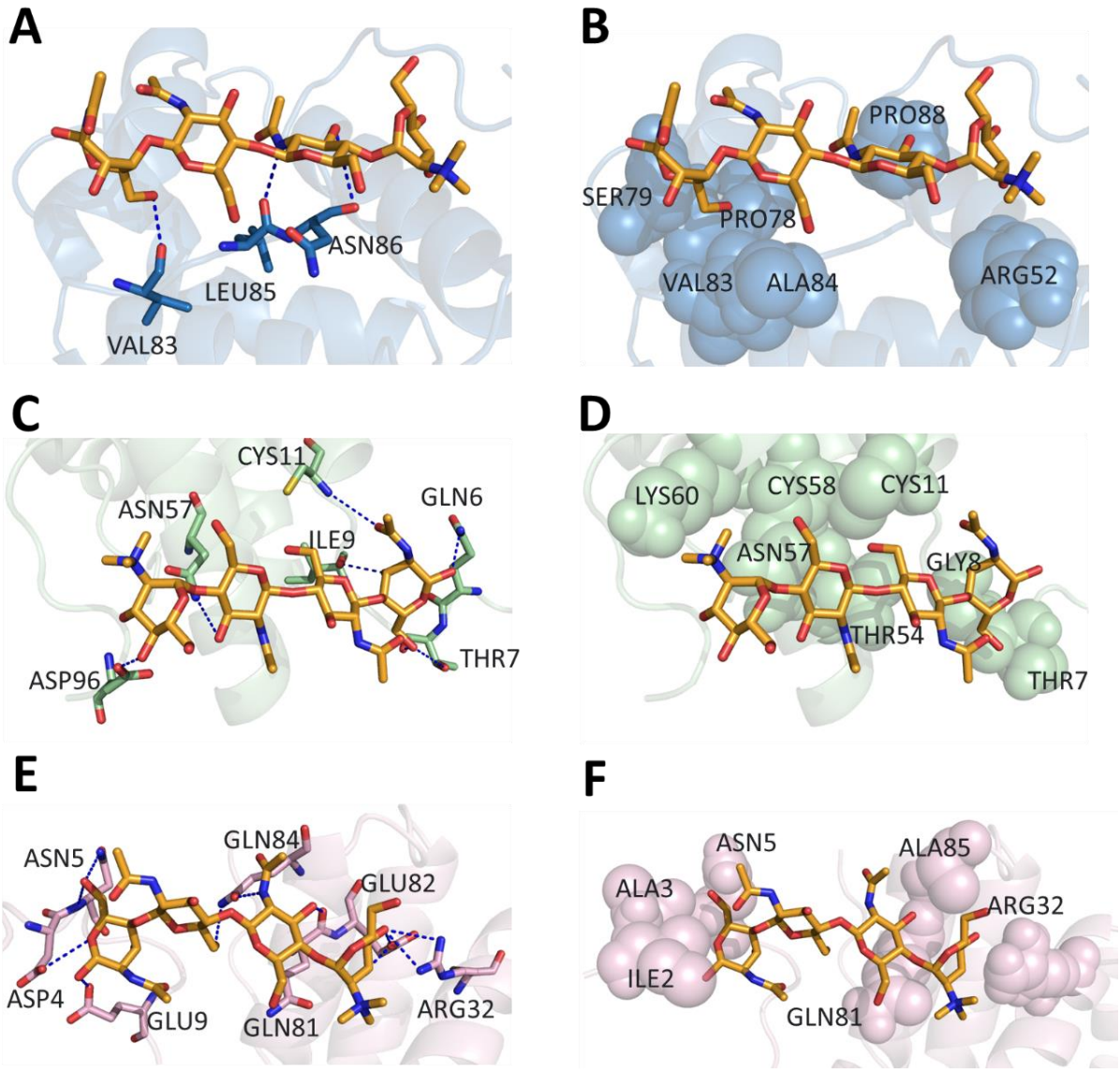

A
ASN86
LEU85
VAL83
B
PRO88
SER79
PRO78
VAL83
ALA84
ARG52
C
CYS11
GLN6
ASN57
ILE9
THR7
ASP96
D
LYS60
CYS58
CYS11
ASN57
GLY8
THR54
THR7
E
GLN84
ASN5
GLU82
ASP4
GLU9
GLN81
ARG32
F
ASN5
ALA85
ALA3
ARG32
ILE2
GLN81

FIGURE 7

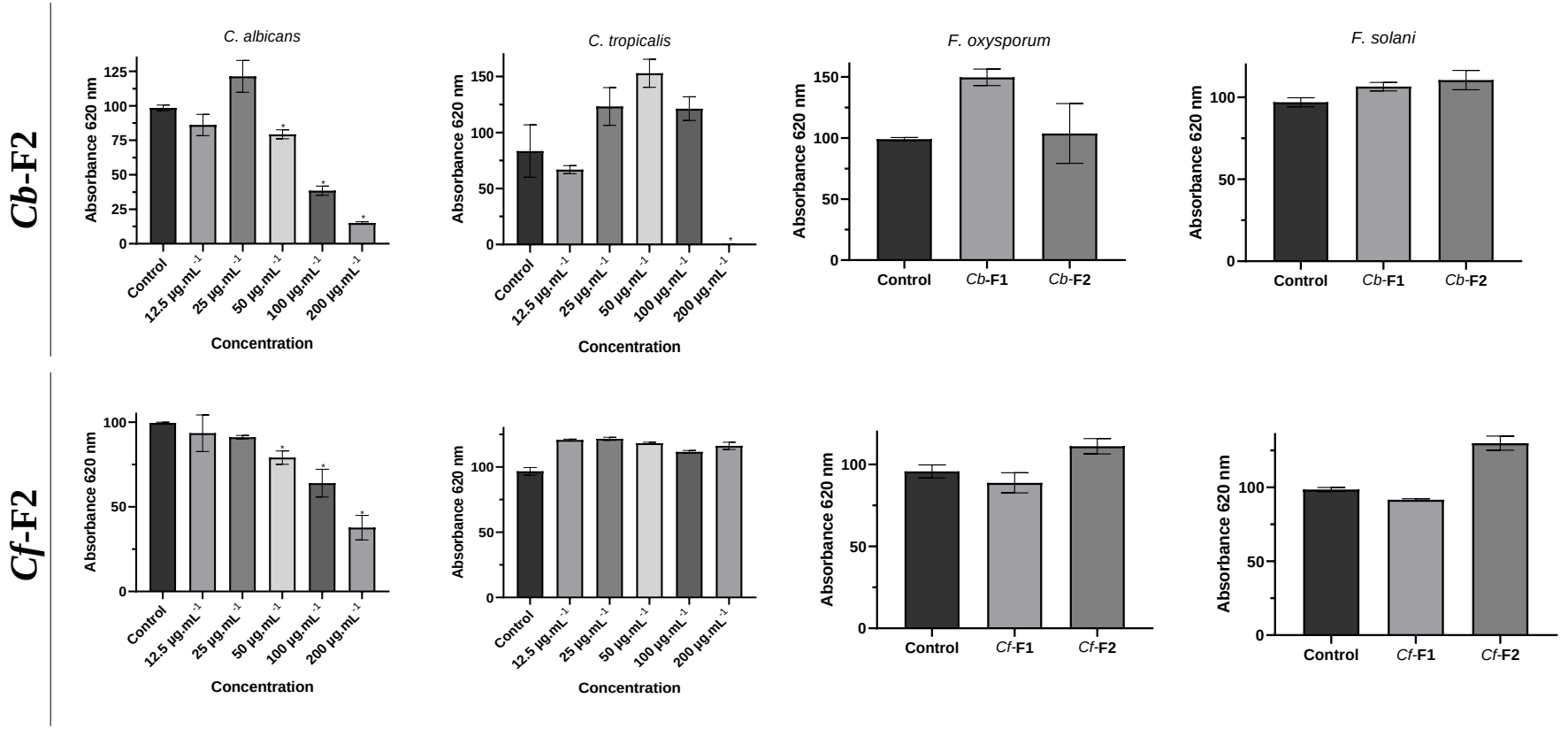
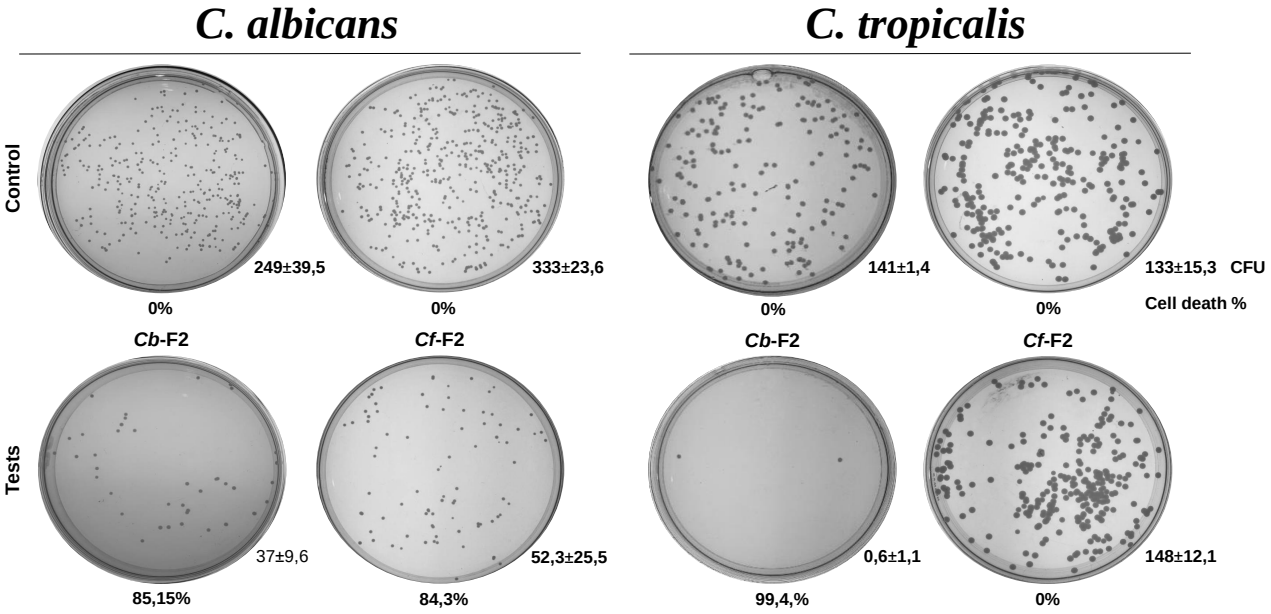
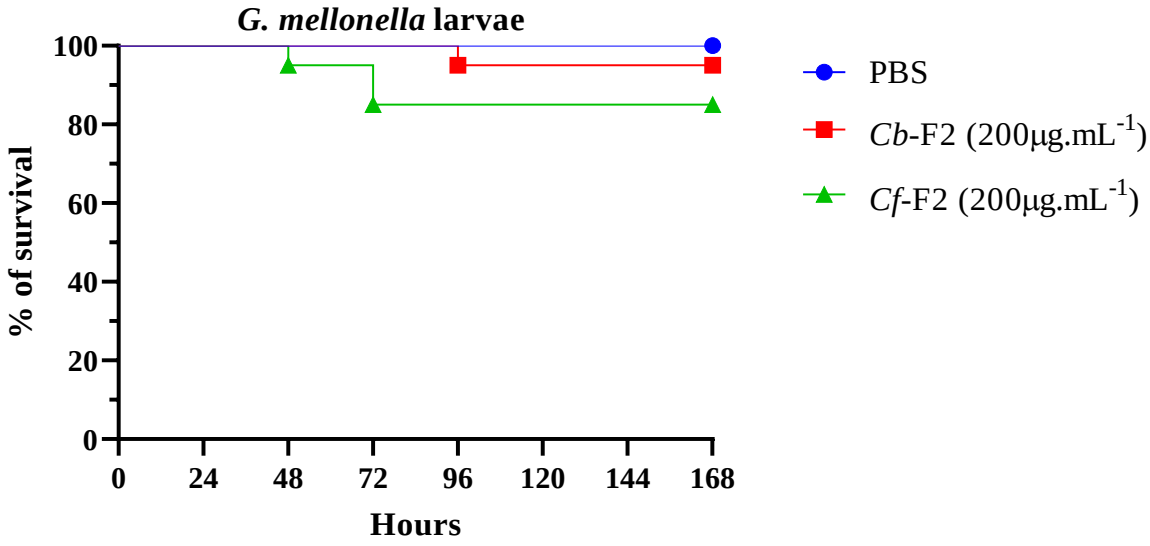

Cb-F2
Cf-F2
C. albicans
C. tropicalis
F. oxysporum
F. solani
Absorbance 620 nm
Concentration
Control
12.5 µg.mL-1
25 µg.mL-1
50 µg.mL-1
100 µg.mL-1
200 µg.mL-1
Cb-F1
Cb-F2
Cf-F1
Cf-F2

FIGURE 8


C. albicans
C. tropicalis
Control
Tests
249±39,5
0%
333±23,6
0%
141±1,4
0%
133±15,3 CFU
0%
Cell death %
Cb-F2
Cf-F2
Cb-F2
Cf-F2
37±9,6
85,15%
52,3±25,5
84,3%
0,6±1,1
99,4,%
148±12,1
0%

.

FIGURE 9


G. mellonella larvae
% of survival
100
80
60
40
20
0
0
24
48
72
96
120
144
168
Hours
PBS
Cb-F2 (200µg.mL-1)
Cf-F2 (200µg.mL-1)

5 – CAPÍTULO 3

Purification, structural characterization, and anticandidal activity of a chitin-binding peptide with high similarity to hevein and endochitinase from pepper seeds

Chapter 3: Manuscript in preparation

Purification, structural characterization, and anticandidal activity of a chitin-binding peptide with high similarity to hevein and endochitinase isolated from pepper seeds

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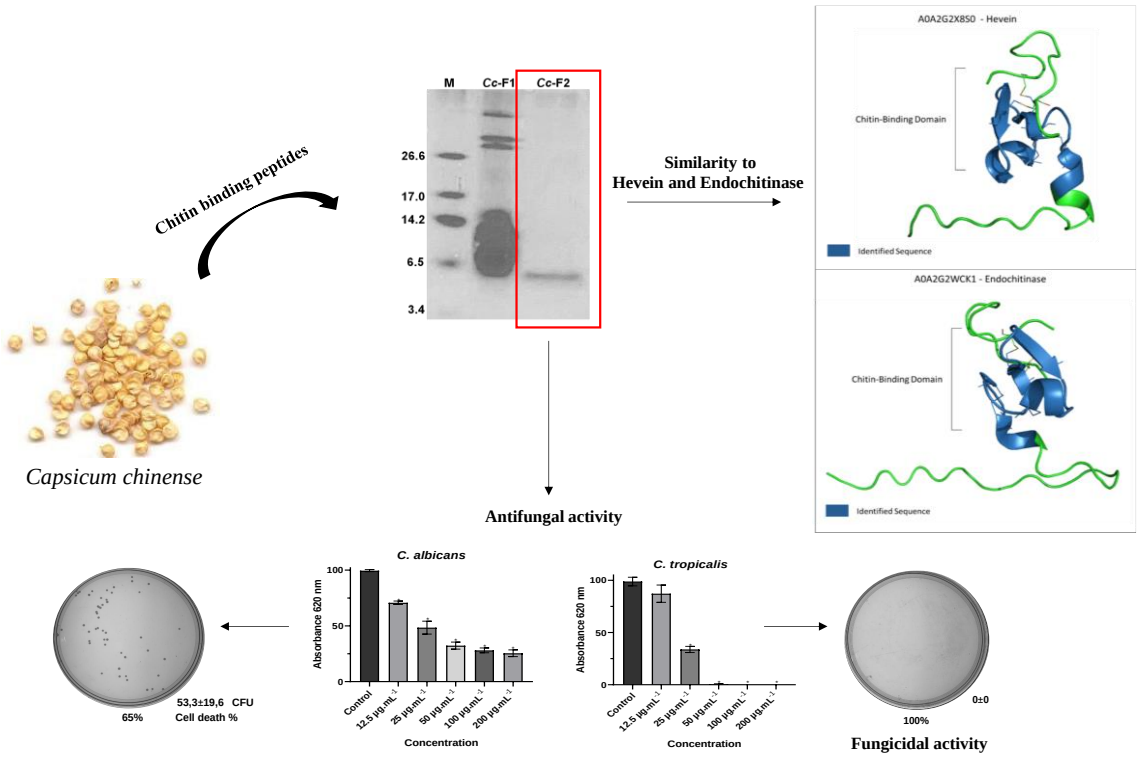
Graphical Abstract



Abstract

With the emergence of multidrug-resistant microorganisms, antimicrobial agents have become a serious global threat, affecting human health and various plants. Thus, new therapeutic alternatives, such as chitin-binding proteins with fungicidal activity, are necessary. Chitin is an essential component of the fungal cell wall, and chitin-binding proteins exhibit antifungal activity. Thus, in the present study, chitin-binding peptides isolated from *Capsicum chinense* seeds were characterized and evaluated for their in vitro antimicrobial effect against the growth of *Candida* and *Fusarium* fungi. First, the proteins were extracted in phosphate at pH 5.4, and a chitin column was equilibrated with sodium acetate (0.08 M, pH 4.5), in which 50 mg of the fraction rich in peptides was applied. Subsequently, the retained material was eluted with 0.1 M HCl. Tricine SDS–PAGE was used to visualize the peptides. Growth inhibition assays were performed to evaluate the ability of the peptides to inhibit microorganism growth. After chromatography, two fractions, *Cc*-F1 (not retained on the column) and *Cc*-F2 (retained on the column), were obtained. Electrophoresis revealed major protein bands between 6.5 and 26.6 kDa for *Cc*-F1 and only one ~6.5 kDa protein band for *Cc*-F2, which was subsequently subjected to mass spectrometry. The protein showed similarity with hevein-like and endochitinase and was subsequently named *Cc*-Hev. Then, we predicted the three-dimensional structure of the peptides and performed a peptide docking with $(NAG)_3$. *Cc*-Hev inhibited the growth of *C. albicans* and *C. tropicalis* and induced a 65% decrease in cell viability. Based on these results, new techniques to combat diseases caused by fungi could be developed through biotechnological applications; therefore, further studies are needed.

Keywords Antimicrobial peptides · *Capsicum chinense*, Fungi, *Candida albicans*, *Candida tropicalis*

Introduction

Antimicrobial peptides (AMPs) are a group of molecules that occur naturally in all living organisms and help them to defend against various pathogens, including fungi, bacteria and viruses (1). AMPs have a low molecular mass (~2-50 amino acids), are rich in hydrophobic amino acids and have a net positive charge (2). Some stable antimicrobial peptides can bind to chitin, an insoluble homopolymer present in several types of biological materials, such as the cell wall of fungi, insects and crustacean shells; however, chitin is not present in plants. Some peptides, including chitinases, vicilins, lectins, 2S albumins and hevein-like peptides, can bind to a matrix composed of chitin.

These peptides exhibit antimicrobial activity against bacteria and fungal pathogens, and they interact with fungal chitin through the chitin binding site, which plays a significant role in inhibiting fungal growth (3). The binding site shares structural similarity with hevein, an antimicrobial peptide from the latex of *Hevea brasiliensis* (Willd. exA. Juss.) Müll. Arg. The main element of all hevein-like peptides is that a conservative chitin-binding site is one of their essential structural modules (4, 5).

Hevein-like peptides are AMPs that are rich in cysteine, contain 29-45 amino acids and 5-7 residues contain glycine. A main feature of hevein-like peptides is a conservative chitin binding site with the amino acid sequence SXFGY/SXYGY, in which X is any amino acid residue (6). In addition, these peptides are present in the plant cysteine-rich antimicrobial peptide superfamily, are 2-6 kDa in weight, contain 6-10 Cys residues and contain three to five disulfide bridges (7). They also possess structural motifs composed of three antiparallel beta sheets and a short α-helix, which are stabilized by 3-5 disulfide bonds (8). Previously, hevein-like peptides have been reported to exhibit activity against several fungal microorganisms with or without chitin in their cell wall

(3). The mechanism underlying the activity of hevein-like peptides is mainly through depolarization of hyphae membrane (5).

Recently, several hevein-like peptides have been isolated from several plant species, such as wheat (8), quinoa (9) and moringa (10). These peptides exhibited activity against a range of microorganisms, such as *F. oxysporum*, *F. culmorum*, *Bipolaris sorokininana*, *Alternaria alternata* and *Cladosporium cucumerinum* (8). These findings have encouraged further studies on the application of these molecules against pathogenic fungi, as the molecules exhibit great activity and could be used to create new antifungal medications.

Peppers and chilies originate from the tropical Americas and belong to 22 families of *Solanaceae* and the genus *Capsicum*; 35 of these species have been domesticated, while the others remain semidomesticated and wild. The plants are dispersed worldwide, and in Brazil, they are cultivated mainly in the states of Rio de Janeiro, Minas Gerais, Bahia and Goiás; among the various locations, Rio de Janeiro is a special center of diversity (11). These plants are essential solanaceous vegetables with high world economic value. However, the growth, quality and yield of these plants are reduced by biotic (fungal, bactéria and virus infections and pests) and abiotic (drought, salinity and extreme temperatures) factors (12, 13).

Several AMPs have been isolated from different organs of plants within the *Capsicum* genus (14). For example, a hevein-like peptide was identified from *C. annuum* leaves; this peptide is approximately 4.2 kDa and was named HEVCAN. In addition, the peptide belongs to the chitin-binding domain family and exhibits antimicrobial activity (14). Our research group has previously isolated and identified several other antimicrobial peptides, such as defensin (15), LTP (16), protease inhibitors (17) and chitin-binding peptides (18), from *Capsicum* seeds. In this study, we used a chitin affinity column to

purify peptide fractions from *C. chinense* seeds, and these fractions exhibited chitin-binding capacity and activity against yeasts.

Materials and Methods

Plant Material

The seeds of *C. chinense* (Access UENF 1755) were provided by the Laboratório de Melhoramento Genético Vegetal (LMGV), Centro de Ciências e Tecnologias Agropecuárias (CCTA), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes – Rio de Janeiro (RJ). The seeds of *C. chinense* were sown in 72-cell polystyrene trays with substrate fertilized with the formulation (4 N/14P/8K) (Vivatto®). The trays were kept in a growth chamber at 28°C and irrigated once a day. After the emergence of two pairs of definitive leaves, the plants were transplanted individually into plastic pots (5 L) containing a mixture of soil and substrate (at a 2:1 ratio). The plants were subsequently transferred to an acclimatized greenhouse. The plants were irrigated once a day until the ripe fruits were harvested.

Microorganisms

The yeast species *C. albicans* (CE022) and *C. tropicalis* (CE017) and the filamentous fungi *F. oxysporum* and *F. solani* were cultivated in Sabouraud agar (10 g/L peptone, 40 g/L D(+)glucose, 15 g/L agar) (Merck) and kept in a refrigerator at 4°C in the Laboratório de Fisiologia e Bioquímica de Microrganismos (LFBM) of the Centro de Biociências e Biotecnologia (CBB) from UENF.

Protein Extraction and Precipitation with Ammonium Sulfate

First, 5 g of seeds was ground with the aid of a mortar, pestle and liquid nitrogen to form very fine-grained flour. After the flour was obtained, the proteins were extracted immediately (19). Initially, the proteins were extracted in phosphate buffer (0.01 M Na_2HPO_4, 0.015 M NaH_2PO_4, 0.1 M KCl, 1.5% EDTA), pH 5.4, at a ratio of 1:10 (5 g flour: 50 mL buffer) for 3 h under constant stirring at 4°C. The homogenate was subjected to centrifugation (15.000 × *g* for 30 min), the residue was discarded, and ammonium sulfate was added to the supernatant for precipitation at 0-90% saturation. The solution was kept overnight at 4 °C. After this process, the suspension was centrifuged again at 15.000 × g for 30 min. Proteins precipitated were recovered by resuspension in 10 mL of distilled water and heated at 80 °C for 15 min. Then, the mixture was subjected to the last centrifugation (10.000 × *g* for 10 min). The supernatant was dialyzed against distilled water (avg. flat width 32 mm, 1.27 in.) at 4 °C for 3 days and finally lyophilized.

Protein Quantification

Quantitative protein determinations were performed using the bicinchoninic acid method (20), with ovalbumin (Sigma) used as the standard protein.

Isolation of Proteins and Peptides with Affinity for Chitin

The chitin (poly-(1→4)-β-N-acetyl-D-glucosamine) used for this study was obtained from practical grade, powder and commercially available shrimp shells from Sigma–Aldrich. Chitin was chemically treated (21), where 25 g of industrial chitin was acidified with 500 mL of 100 mM HCl. This mixture was incubated for 24 h at 4 °C with periodic

shaking. After 24 h, all the HCl was removed, and 250 mL of 100 mM NaOH was added to the resulting precipitate. This mixture was heated at 100 °C for 16 h. After heating, the NaOH was removed, and another 250 mL of NaOH was added to the precipitate for another 16 h of heating. This step was repeated once again, resulting in three 16-h warm-ups. After the last heating, all the NaOH was removed, and 200 mM HCl was added to the precipitate. The HCl was then removed from the mixture, and distilled water was added to the precipitate for storage. A chitin column was equilibrated with sodium acetate buffer (0.1 M, pH 5.5), and a 50 mg peptide-rich heated fraction solubilized in the same sodium acetate buffer was applied to the column. Chromatography was initially performed with equilibrium buffer. The retained fraction was eluted with 0.05 M HCl solution, and the absorbance of the desorbed proteins was monitored at 280 nm. The flow rate used was 1 $mL.min^{-1,}$ and the volume used was 3 mL per tube. Protein peaks were collected and recovered after dialysis and lyophilization.

Tricine Gel Electrophoresis in the Presence of SDS

For gel assembly, 8 × 10 cm and 7 × 10 cm glass plates and 0.75 mm spacers were used. The separating gel was prepared at a 16.4% acrylamide/bis-acrylamide concentration, and the concentration was 3.9%. The samples were heated for 5 min at 100 °C and centrifuged at 15,000 × *g* for 5 min. After these procedures, 20 $\mu g.mL^{-1}$ of sample was added to the gel. The reaction was performed at a constant voltage of 24 V for approximately 16 h. Ultra-low Range Molecular Weight Marker (Sigma–Aldrich) was used as a protein molecular weight marker. The molecular masses of the proteins are shown in Da (26.600; 17.000; 14.200; 6.500; 3.496; 1.060) (22).

In-Gel Digestion

Using a scalpel, the gel bands were cut into slices and then into tiny cubes of ~1mm^3. Each cubed slice was placed in a separate 1.5 mL tube, and 1000 µL of destaining solution (50 mM AmBic/50% ACN - 1:1) was added to each sample. The tubes were gently agitated on a Thermomixer at room temperature overnight. After removing the solution, 200 µL of fresh destaining solution was added for 1 h before removal. The gel bands were then dehydrated by adding 500 µL of 100% ACN to each tube for 1 min, followed by a repeated step. For protein reduction, 200 µL of a solution containing 10 mM DTT/100 mM AmBic was added to each tube and incubated at 55 °C for 30 min with gentle agitation on a thermometer. Subsequently, 500 µL of 100% ACN was added to each tube for dehydration, followed by the addition of 200 µL of alkylation solution (55 mM iodoacetamide-IAA/100 mM AmBic). The tubes were kept in the dark on a thermomixer at room temperature for 30 min.

For protein digestion, 200 µL of cold trypsin solution (digestion solution containing trypsin in 10 mM AmBic/10% ACN) was used. The tubes were kept at 4 °C for 30 min and then transferred to a Thermomixer at 37 °C overnight for complete digestion. Afterwards, 200 µL of extraction buffer (containing 1:2 of 5% formic acid to 100% ACN) was added, and the mixture was incubated at 37 °C for 30 min in a thermomixer. The samples were then evaporated in a SpeedVac until completely dry. Prior to mass spectrometry analysis, the samples were resuspended in 50 µL of 0.1% formic acid in 50 mM AmBic (23).

Mass Spectrometry Analysis

A nanoAcquity UPLC coupled to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, United Kingdom) was used for ESI–LC–MS/MS. During separation, the samples were loaded onto a nanoAcquity UPLC 5 µm C18 trap column (180 µm × 20 mm) at 5 µL.min for 3 min and then onto a nanoAcquity HSS T31 1.8 µm analytical reversed-phase column (75 µm × 150 mm) at 400 nL/min, with a column temperature of 45 °C. For peptide elution, a binary gradient was used, with mobile phase A (water and 0.1% formic acid) and mobile phase B (acetonitrile and 0.1% formic acid). The gradient elution started at 7% B, then ramped from 7% B to 40% B until 92.72 min, then remained at 99.9% until 106.00 min, decreased to 7% B until 106.1 min, and finally remained at 7% B until the end of the experiment at 120 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35.000 FWHM, with ion mobility and independent-data acquisition mode ($HDMS^E$). The ion mobility wave was adjusted to a velocity of 600 m s^{-1}; the transfer collision energy was ramped from 19 V to 55 V in high-energy mode; the cone and capillary voltages were 30 V and 2750 V, respectively; and the source temperature was 70 °C. For the TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass ranging from 50 to 2000 Da. Human [Glu1]-fibrinopeptide B (Sigma–Aldrich) at 100 fmol.μL^{-1} was used as an external calibrator, and lock mass acquisition was performed every 30 s. Mass spectra were acquired with MassLynx v4.0 software (24). The calculation of the percentages of positive and identical residues was carried out considering only the residues obtained by mass spectrometry.

Proteomic Data Analysis

For spectral processing and database searching, the ProteinLynx Global Server (PLGS; version 3.0.2) (Waters, USA) and ISSOQuant workflow software were used (25, 26). The PLGS was processed using a low-energy threshold of 150 (counts), an elevated-energy threshold of 50 and an intensity threshold of 750. In addition, the analysis was performed using the following parameters: two missed cleavages, a minimum fragment ion per peptide equal to 3, a minimum fragment ion per protein equal to 7, a minimum peptide per protein equal to 2, fixed modifications of carbamidomethyl and variable modifications of oxidation and phosphoryl groups. The false discovery rate was set to a maximum of 1%. The proteomics data were processed against *C. chinense* (https://www.uniprot.org/proteomes/UP000224522) for *C. chinense* samples.

Protein Structure Analysis

The predicted structural model for the hevein was generated using the FASTA sequences in the AlphaFold Protein Structure Database. AlphaFold is an AI system that predicts a protein's 3D structure from its amino acid sequence. The obtained models were further edited using PyMOL software to remove signal peptides when necessary.

Docking of Proteins with the Trimer of N-acetylglucosamine $(NAG)_3$

Docking of hevein with $(NAG)_3$ and of endochitinase with $(NAG)_3$ was performed using the HTP SurflexDock 0.6 program (27). Docking was performed using a search space defined by a 15 Å radius in the central region of the hevein active site (residue 21). The $(NAG)_3$ model was created from PyMol with the Azahar plug-in to design

oligosaccharides. All rotations of the ligand were free to rotate while the atoms of the protein were kept rigid. The best complex was evaluated using the affinity energy value and submitted to the PLIP Web Tool (28) and the LigPlot+ v program 2.2.4 (29). Noncovalent interactions between hevein-$(NAG)_3$ and endochitinase-$(NAG)_3$ were identified by these programs.

Effect of Peptides on Fungal Growth

For yeasts, aliquots of different species were taken from plates containing grown colonies and placed in new Petri dishes containing Sabouraud agar (10 g/L peptone, 40 g/L D(+)glucose, 15 g/L agar) (Merck). These new plates were kept in an oven at 30 °C for 24 h. For filamentous fungi, the inoculums were transferred from the stock and grown in Petri dishes containing Sabouraud agar (Merck) for 11 days at 30 °C. After growth, the cells/spores were removed and homogenized in 10 mL of Sabouraud broth (10 g/L peptone, 20 g/L D(+)glucose) (Merck) for quantification in a Neubauer chamber (LaborOptik) with the aid of an optical microscope (Axiovison A2, Zeiss). Subsequently, the yeast (1×10^4 cel.mL^{-1}) and fungal spore cells (1×10^3 cel.mL^{-1}) were incubated in Sabouraud broth added with different concentrations of the fractions obtained from the seeds. The assay was performed in cell culture microplates (96 wells) at 30 °C for a period of 24 h for yeast and 36 h for filamentous fungi. Cell growth was determined by optical density, which was monitored every 6 h in a microplate reader at a wavelength of 620 nm. Each test was performed in triplicate. The entire procedure was performed under aseptic conditions in a laminar extractor according to the methodology adapted from Broekaert *et al*. (30). Cell growth was also determined without the addition of proteins.

Cell Viability Analysis

After 24 h of incubation, the control cells (without *Cc*-Hev) and the test cells (with *Cc*-Hev) were diluted 1000×. A 60 µL aliquot of the dilution was spread with a Drigalski loop over the surface from a Petri dish containing Sabouraud agar and cultivated at 30 °C for 36 h. At the end of this period, the colony forming units (CFU) were determined, and the Petri dishes were photographed (31). The experiments were performed in triplicate, and the results were presented assuming that the control represents 100% cell viability.

Statistical Analysis

The data from the yeast and filamentous fungal growth inhibition assays were evaluated via one-way ANOVA. Differences in means of $p < 0.05$ were considered significant. All the statistical analyses were performed using GraphPad Prism software (version 5.0 for Windows).

Results

Chitin Affinity Chromatography and Electrophoretic Profiling

Chitin-binding proteins were obtained from na aqueous extracts of *C. chinense* by chitin affinity chromatography. The chromatogram showed that the extract was fractionated into two major peaks called *Cc*-F1 and *Cc*-F2; *Cc*-F1 was the nonretained fraction in the column and *Cc*-F2 was the fraction retained in the column (Fig. 1a). These results indicate that proteins and peptides with chitin-binding ability were present. The electrophoretic profile of the fractions obtained via chromatography is shown in Fig. 1b. The bands in the *Cc*-F1 fraction had molecular masses in the marker range between 6.5 and 26.6 kDa (Fig. 1b); moreover, bands were observed in the *Cc*-F1 with molecular masses above the

26.6 kDa band. In addition, only one band with a molecular mass of ~6.5 kDa was observed in *Cc*-F2.

Mass Spectrometry

To identify peptides obtained from *Cc*-F2, we performed mass spectrometry with the band obtained by electrophoresis (20 μg). The spectra were interpreted by PLGS software, and fragments of peptide residue sequences were obtained. The UniProt BLASTP database was used to identify similar proteins for all residues. The obtained peptides LCCSQYGYCGSTRAYCGVGCQSNCGR and AYCGVGCQSNCG were similar to hevein-like and endochitinase (Fig. 2b). After that, we named *Cc*-F2 in *Cc*-Hev.

The type 1 chitin-binding domain has a signature profile composed of a repeating pattern with 8 cysteine residues, which establish disulfide bonds between themselves, as illustrated in Fig. 2a. In this pattern, C1 bridges with C4, while C2 bridges with C5. C3 bridges with C6 and finally, C7 bridges with C8. In the sequence identified in this work, it was possible to observe 6 cysteines, 4 of which follow this same pattern: C2 forms a disulfide bridge with C9 and C20 forms a bridge with C24 (number corresponding to the amino acid residue). For the *C. chinense* sequences, these residues correspond to C37, which forms a disulfide bridge with C44, and C55, which forms a disulfide bridge with C59, respectively.

Three-dimensional Structure

Two peptides were identified in this work, pro-hevein and endochitinase. A search was performed on the UniProt database using the amino acid sequences, and we predicted the

three-dimensional structure of the peptides (Fig. 3). For both proteins, the area highlighted in blue represents the region identified via mass spectrometry. Both models show that cysteine residues form sulfur bridges in the chitin-binding domain. These cysteine residues are represented by lines.

Docking of the Peptide Sequences with $(NAG)_3$

The three-dimensional structure of the protein (UniProt code: A0A2G2X8S0) was subjected to molecular docking with $(NAG)_3$, and the best model showed negative affinity energy values (-7.62 kcal/mol), indicating spontaneous binding (Fig. 4a). Based on the results obtained from docking experiments, the hevein amino acids Ser19 and Tyr21 form hydrogen bonds with (NAG)3 (Fig. 4a, b). The hydrophobic interactions between hevein residues Ser19, Tyr21, Tyr23, Ala29 and Tyr30 with $(NAG)_3$ are shown in Fig. 4b.

For the endochitinase (UniProt code: A0A2G2 WCK1), the three-dimensional structure was subjected to molecular docking with $(NAG)_3$, and the best model showed negative affinity energy values (-7.18 kcal/mol), indicating spontaneous binding (Fig. 5a). As revealed by the results of docking experiments, the hevein amino acids Ser19 and Tyr30 form hydrogen bonds with $(NAG)_3$ (Fig. 5a, b). The hydrophobic interactions of hevein residues Gln1, Ser19, Tyr21, Phe23, Ala29 and Tyr30 with $(NAG)_3$ are shown in Fig. 5a, b.

Effect of *Cc*-Hev on Fungal Growth

Initially, an antimicrobial assay was performed to evaluate the effect of 12.5, 25, 50, 100 and 200 $\mu g.mL^{-1}$ *Cc*-Hev on the growth of *C. albicans* and *C. tropicalis* after 24 h (Fig. 6). *Cc*-Hev inhibited the growth of *C. albicans* at all concentrations tested, with the

highest inhibition percentages being 68%, 72% and 75% for 50, 100 and 200 μg.mL^{-1}, respectively. For *C. tropicalis*, *Cc*-Hev inhibited growth at concentrations of 25, 50, 100 and 200 μg.mL^{-1}, and at the last three concentrations mentioned above, 100% of the growth of the microorganism was inhibited. For the two fungi that were tested, *F. oxysporum* and *F. solani*, the *Cc*-Hev obtained by chitin affinity chromatography did not inhibit the growth of the fungus at 200 μg.mL^{-1} (Fig. 6). *Cc*-F1 did not show activity against the microorganisms tested.

Cell Viability

Based on the results obtained for the antimicrobial activity of *Cc*-Hev, the viability of the yeasts *C. albicans* and *C. tropicalis* was analyzed (Fig. 7). *Cc*-Hev at 200 μg.mL^{-1} caused a significant reduction in the number of CFU of *C. albicans* after 36 h of incubation, indicating a 65% loss of viability. For *C. tropicalis*, *Cc*-Hev reduced the number of CFUs by 100%, indicating that over this species the peptide has a fungicidal effect.

Discussion

In this work, a peptide isolated from *C. chinense* seeds (accession 1755) was purified and identified. After the extract was heated, fractions rich in peptides were generated and purified by chitin affinity chromatography. After this purification, two fractions were obtained, called *Cc*-F1 (which does not bind to chitin) and *Cc*-F2 (which binds to chitin), in which the peptide eluted from the column was obtained (Fig. 1a). The electrophoretic profile of the fractions eluted from the chitin column by SDS–PAGE-Tricine showed bands corresponding to low-molecular-weight proteins and peptides (Fig. 1b).

Chitin-binding peptides have been identified in several species, such as *Solanum lycopersicum* (32), *Ficus microcarpa* (33) and *Vigna unguiculata* (34), has already been reported in the literature. For example, the Iu-CBP protein was identified and characterized from *Iberis umbellata* seeds. The purified protein presented an approximately 11 kDa band via SDS–PAGE, which was similar to *Moringa oleifera* chitin-binding protein (Mo-CBP$_3$-1) and showed activity against several microorganisms (35). In the case of *Capsicum* plants, Ali *et al*. (36) reported that the CaChiIV1 gene, which was isolated from *C. annuum* plants, regulates responses to the fungus *Phytophthora capsici* and to abiotic stresses. Gonçalves *et al*. (18) studied the chitin-binding fraction of *C. annuum* seeds. The fraction inhibited the fungal growth of *C. albicans* and *C. tropicalis*, was not deleterious when tested on *Galleria mellonella* larvae and neither to mammalian cells. Researchers also found that the protein band was similar to that 2S albumin.

After mass spectrometry was performed with the *Cc*-F2 band, amino acids were identified that showed similarity to hevein-like and endochitinase proteins (Fig. 2). An essential structural module of all hevein-like peptides is a conservative chitin-binding site (37). This chitin-binding site is common for hevein-like AMPs and other chitin-binding proteins, such as class I and IV chitinases and other wound-inducing proteins (6). Hevein and hevein-like proteins, as well as chitinases I and IV, contain six, eight or ten cysteine residues that form a typical cysteine motif. This motif specifically binds to chitin and exhibits antifungal activity against several pathogens with or without chitin (5).

The sequence of the hevein was previously identified and is very similar to that of the chitin-binding agglutinin *Urtica diocica* L. (UDA) from (nettle), which is an antifungal agent against chitin-containing fungal strains (30, 38, 39). Based on the characterization of hevein, the protein contains a characteristic coil-β-β-coil-β motif, in

which the central antiparallel region comprises a β sheet surrounded by spirals and stabilized with disulfide bridges (5).

Our results demonstrated that the interaction between hevein-$(NAG)_3$ and endochitinase-$(NAG)_3$ was negative, indicating that spontaneous docking was facilitated by intermolecular interactions, such as hydrogen bonds and hydrophobic interactions. The highly conserved disulfide bridges between heveins leads to the exposure of aromatic residues and potentially participates in N-acetylglucosamine recognition (40, 41). Previous studies have indicated that aromatic residues at relative positions 21, 23, and 30 in the hevein domains are essential for carbohydrate binding because the residues stabilize the complexes. Furthermore, hydrogen bonds occur at residues Ser19 and Tyr30 (42, 43). In this work, docking experiments revealed that aromatic residues, such as tyrosine and tryptophan, directly participate in binding with the N-acetylglucosamine trimer.

After the chitin-binding protein fraction was obtained, growth inhibition assays were performed with the yeasts *C. albicans* and *C. tropicalis* and the phytopathogenic fungi *F. oxysporum* and *F. solani*. *Cc*-Hev at different concentrations exhibited antimicrobial effects on both yeast strains; however, for phytopathogenic fungi, *Cc*-Hev did not inhibit fungal growth at the tested concentration. *Cc*-Hev exhibited a fungicidal effect on *C. tropicalis* yeast cells. The discovery of new biomolecules can increase the effectiveness of modern industrial fungicides and may increase the variety of natural sensitizers available for fungal infection, contributing to the management of various fungal pathogens (44).

As previously reported, hevein-like peptides exert antimicrobial effects on various pathogens, such as bacteria and fungi (3). According to the work of Loo *et al*. (9), the CB-HLPs peptide exhibited activity against four phytopathogenic fungi, *A. alternata*, *Curvularia lunata*, *Rhizoctonia solani* and *F. oxysporum* with IC_{50} values of

approximately 9, 5, 3 and 0,3 μM, respectively. In another study, two chemically synthesized antimicrobial peptides from *Pharabitis nil*, called Pn-AMP1 and Pn-AMP2, were reported. Pn-AMP1 demonstrated inhibitory activity against the yeasts *K. lactis, Z. bailii, D. hansenii* and *S. cerevisiae*, with minimum inhibitory concentrations (MICs) of 400-500 $\mu g.mL^{-1}$, 400-500 $\mu g.mL^{-1}$, 1000 $\mu g.mL^{-1}$ and 500 $\mu g.mL^{-1}$, respectively; this activity was revealed by the mechanism of action and the permeabilization of the yeast membranes. The Pn-AMP2 peptide showed activity only against the yeasts *K. lactis, Z. bailii* and *S. cerevisiae,* with MIC values ranging from 500-1000 $\mu g.mL^{-1}$, 1000 μg.mL and 1000 $\mu g.mL^{-1}$, respectively. The yeast *Z. rouxii* was not sensitive to any concentration tested for the two peptides. According to the MIC found, only Pn-AMP1 was characterized for its potential application as a food preservative (45). In the previous study, the concentrations used were high even at minimum inhibitory concentrations, 50 $\mu g.mL^{-1}$ for *C. albicans* and 200 $\mu g.mL^{-1}$ for *C. tropicalis*. In this study, the MICs were 12.5 $\mu g.mL^{-1}$ for *C. albicans* and 25 $\mu g.mL^{-1}$ for *C. tropicalis*; the concentration used to inhibit 100% of *C. tropicalis* growth was 50 $\mu g.mL^{-1}$.

Transgenic plants with hevein-like peptides are generated due to their strong activity against pathogens, as observed in tobacco and tomato (Pn-AMP2). The SmAMP1 and SmAMP2 peptides from tobacco demonstrated resistance to phytopathogenic fungi. These studies provide opportunities to develop transgenics based on peptides with activity against fungal pathogens; in addition, the studies provide a foundation for new studies on an important family of peptides (5).

Conclusions

Characterizing and investigating this type of molecule can help researchers control pathogenic and phytopathogenic diseases. *Cc*-Hev is a low-molecular-weight protein

purified from *C. chinense* seeds that is similar to hevein-like and endochitinase. In addition, the protein exhibits antimicrobial activity against the yeasts *C. albicans* and *C. tropicalis* but does not show inhibitory activity against the fungi *F. oxysporum* and *F. solani*. Although promising, these results are initial, and further studies are needed to determine the mechanism of action of the peptide; however, these peptides are great candidates for the development of new drugs to control fungal diseases.

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

Conflict of interest The authors declare no conflicts of interest.

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

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Legends

Fig. 1 a: Chromatogram obtained after chitin affinity chromatography was performed with the *C. chinense* fraction. Protein elution was monitored by absorbance at 280 nm. The flow rate was 0.5 $mL \cdot min^{-1}$. **b:** Electropheheretic visualization by SDS–tricine–PAGE of peptide-enriched fractions. *Cc*-F1 (1) and *Cc*-F2 (2) represent *C. chinense*. M: low-mass molecular weight marker (kDa)

Fig. 2 a: The type 1 chitin-binding domain has a signature profile composed of a repeating pattern with 8 cysteine residues, which establish disulfide bonds between themselves, as illustrated. The sequences identified in the present work and the aligned sequences have a conserved cysteine residue profile that involves disulfide bonds, this matches the signature of the chitin-binding type-1 domain. **b:** Alignment analysis between the sequences identified in this work and their orthologs with the highest percentage of identity, which was performed to identify the conserved domains. For the A0A2G3BFR5 protein, the sequences used were CHI1 from *Oryza sativa*, mO1 from *Moringa oleifera*, HEVL from *Arabidopsis thaliana* and the Hevein sequence from *C. chinense*. For the A0A2G3BZ09 protein, the sequences used were PN-AMP1 from *C. annuum*, AMP2 from *Fagopirum esculentum*, CBD1 from *Panicum virgatum* and endochitinase from *C. chinense*. Gray highlights represent regions of similarity, while black highlights represent identical regions. I indicates the percentage of identical residues highlighted in black, and P indicates the percentage of positive residues, that is, those that have the same biochemical characteristics

Fig. 3 Three-dimensional structure of the proteins identified in this work. A0A2G2X8S0 refers to the pro-hevein protein, while A0A2G2 WCK1 refers to the endochitinase protein; both of these proteins were deposited in the UniProt database for *C. baccatum*.

The peptide sequences identified in this work are highlighted in blue. Cysteine residues that form sulfur bridges within the chitin-binding domain are represented by the line

Fig. 4 Docking of hevein with the trimer of N-acetylglucosamine $(NAG)_3$ **a:** Three-dimensional structure of hevein (UniProt code: A0A2G2X8S0) interacting with $(NAG)_3$. The blue lines = hydrogen bonds, the dark blue rods = nitrogen atoms, and the red rods = oxygen atoms. **b:** 2D diagram representing the interactions between amino acid residues of hevein and (NAG)3 (LigPlot + program v 2.2.4). Red arcs = hydrophobic interactions, blue dotted lines = hydrogen bonds, black spheres = carbon atoms, blue spheres = nitrogen atoms, red spheres = oxygen atoms

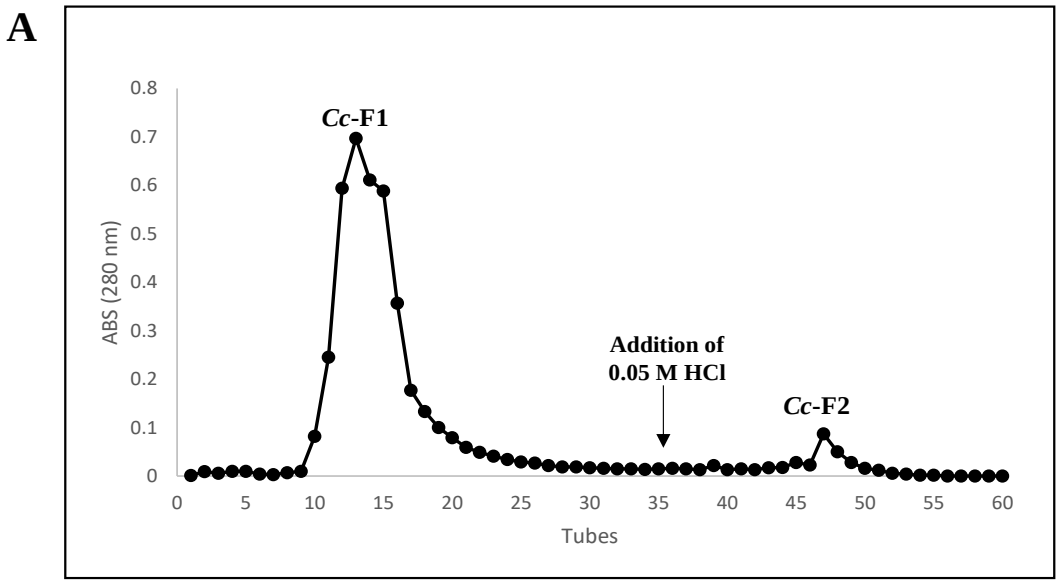
Fig. 5 Docking of endochitinase with the trimer of N-acetylglucosamine $(NAG)_3$ **a:** Three-dimensional structure of endochitinase (UniProt code: A0A2G2 WCK1) interacting with $(NAG)_3$. The blue lines = hydrogen bonds, the dashed gray lines = hydrophobic interactions, the dark blue rods = nitrogen atoms, and the red rods = oxygen atoms. **b:** 2D diagram representing the interactions between amino acid residues of hevein and (NAG)3 (LigPlot + program v 2.2.4). Red arcs = hydrophobic interactions, black spheres = carbon atoms, blue spheres = nitrogen atoms, red spheres = oxygen atoms

Fig. 6 Effect of the *Cc*-F2 fraction from *C. chinense* on the growth of *C. albicans* and *C. tropicalis* at concentrations of 12.5, 25, 50, 100 and 200 $\mu g.mL^{-1}$ for 24 h and of *F. oxysporum* and *F. solani* at a concentration of 200 $\mu g.mL^{-1}$ for 36 h. The values are presented as the means (± SDs) of triplicate samples. Asterisks indicate significant differences ($p < 0.05$) between treatments and controls. The values above the bars indicate the percentage of growth inhibition

Fig. 7 Cell viability of the yeasts *C. albicans* **a** and *C. tropicalis* **b**. Petri dish images showing the growth of colonies in the control (without the *Cc*-F2 fraction) and after treatment with 200 $\mu g.mL^{-1}$ of the *Cc*-F2 fraction from *C. chinense* for 36 h. The

percentage of cell death was calculated in relation to that of the control, untreated cells (cell viability – 100%). The experiments were performed in triplicate. CFU = colony-forming units. The numbers under the images indicate the percentage of cell death

Fig. 1


A
ABS (280 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Tubes
Cc-F1
Addition of
0.05 M HCl
Cc-F2

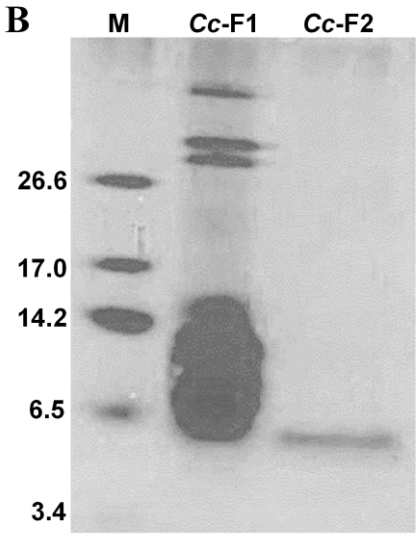
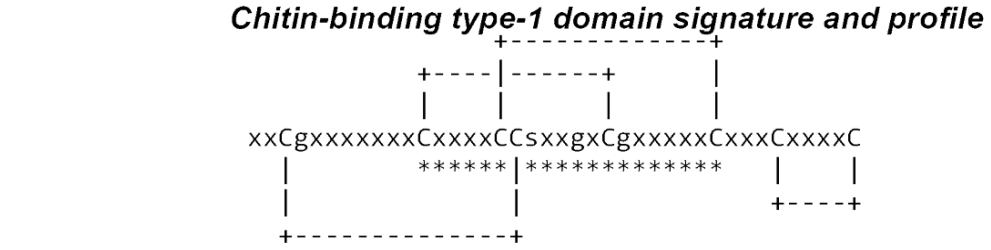

B
M
Cc-F1
Cc-F2
26.6
17.0
14.2
6.5
3.4

Fig. 2

A



B

```
                    *         20        *         40        *         60        *          I(%)  P(%)
A0A2G3BFR5 : MKF-QVVILVLLALLLTTTSAQQCGRQAGGRACANRLCCSQYGYCGSTRAYCG--VGCQSNCGRSA-TGEGE :  68  100   100
CHI1_ORYSJ : MRA-LAVVVVATAFAVVAVRGEQCGSQAGGALCPNCLCCSQYGWCGSTSAYCG--SGCQSQCSGSC-GGGGP :  68   77    80
MO1_MOROL  : MAK-LSFLSLFLLCLVATATAQNCGRQAGNRACANQLCCSQYGFCGSTSEYCSRANGCQSNCRGGG-GADGA :  70   68    71
HEVL_ARATH : MKIRLSITIILLSYTVATVAGQQCGRQGGGRTCPGNICCSQYGYCGTTADYCSPTNNCQSNCWGSGPSGPGE :  72   61    67
HEVEIN_CAP : ------------------------------------LCCSQYGYCGSTRAYCG--VGCQSNCGR-------- :  26
             m                        cg q g   c   6CCSQYG5CG3T  YC    gCQSnC        g

                   80        *        100        *        120        *        140          I(%)  P(%)
A0A2G3BZ09 : ----------------------------QQCGRQAGGRACANRLCCSQYGFCGTTRAYCGVGCQSNCG----- :  60   100   100
PN-AMP1_CA : ----------------------------QNCGRQAGGRACANRLCCSQYGYCGSTRAYCGVGCQSNCG----- :  65   100   100
AMP2_FAGES : ----------------------------AQCGAQGGGATCPGGLCCSQWGWCGSTPKYCGAGCQSNCR----- :  40    75    75
CBD1_PANVG : GCQSNCNEGPAPAPTPSLASVELVKVGEQCGIQAGGATCANNLCCSQFGFCRLGAQYCGVGCQSNCHGSPTI : 144    83    83
ENDOCHI_CA : ------------------------------------------------------AYCGVGCQSNCG----- :  12
                                       cg q gg  c   lccsq g c     YCGvGCQSNC
```

Fig. 3

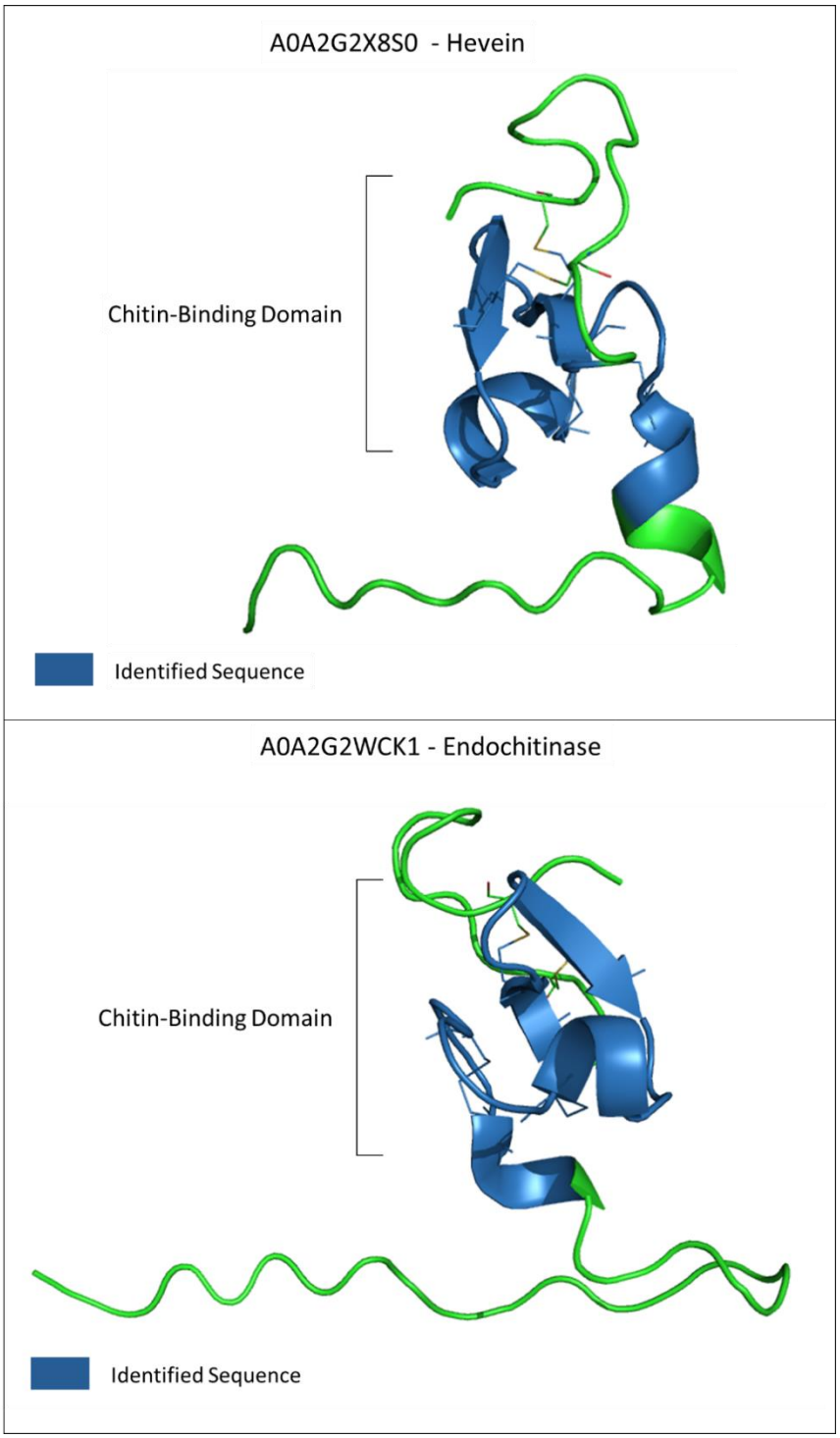

A0A2G2X8S0 - Hevein
Chitin-Binding Domain
Identified Sequence
A0A2G2WCK1 - Endochitinase
Chitin-Binding Domain
Identified Sequence

Fig. 4

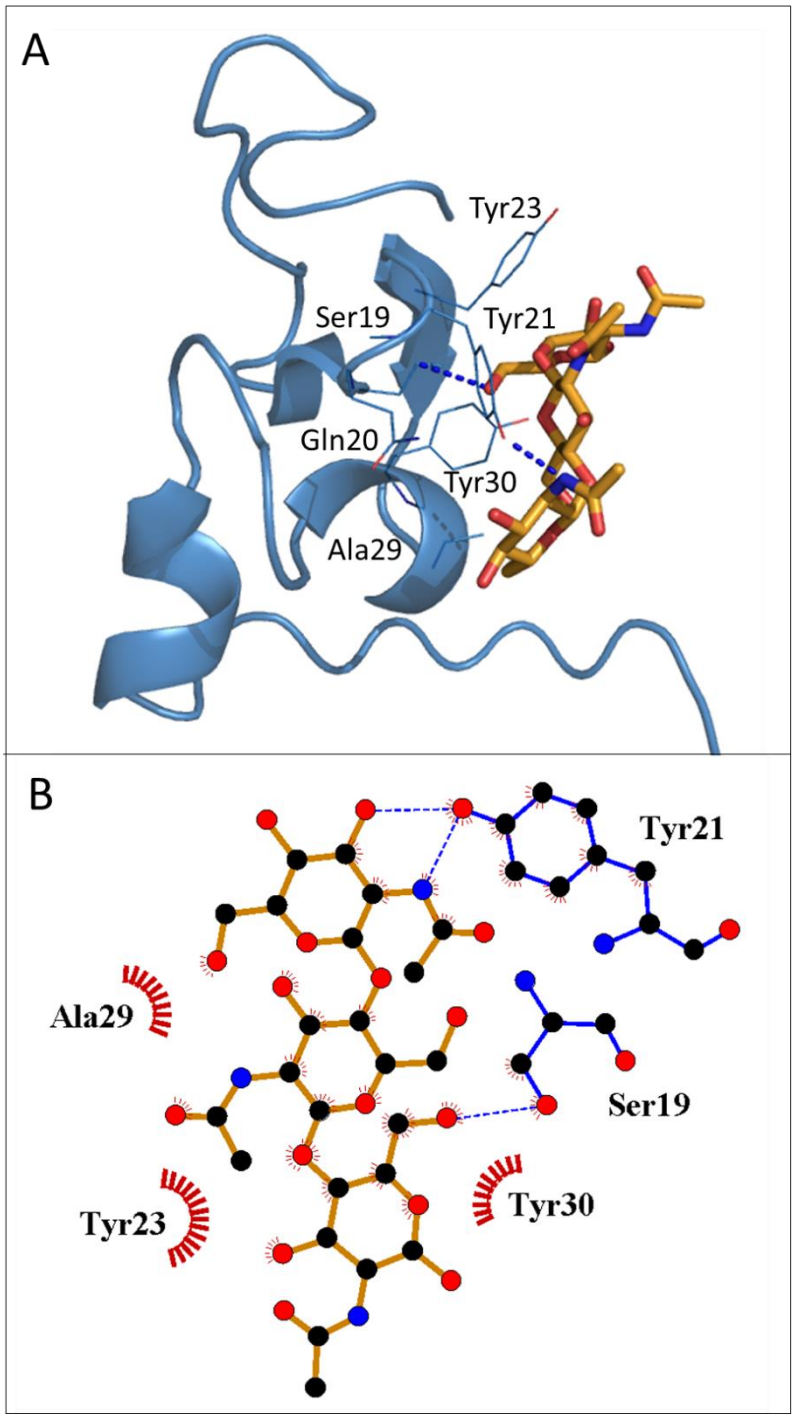

A
Tyr23
Ser19
Tyr21
Gln20
Tyr30
Ala29
B
Tyr21
Ala29
Ser19
Tyr23
Tyr30

Fig. 5

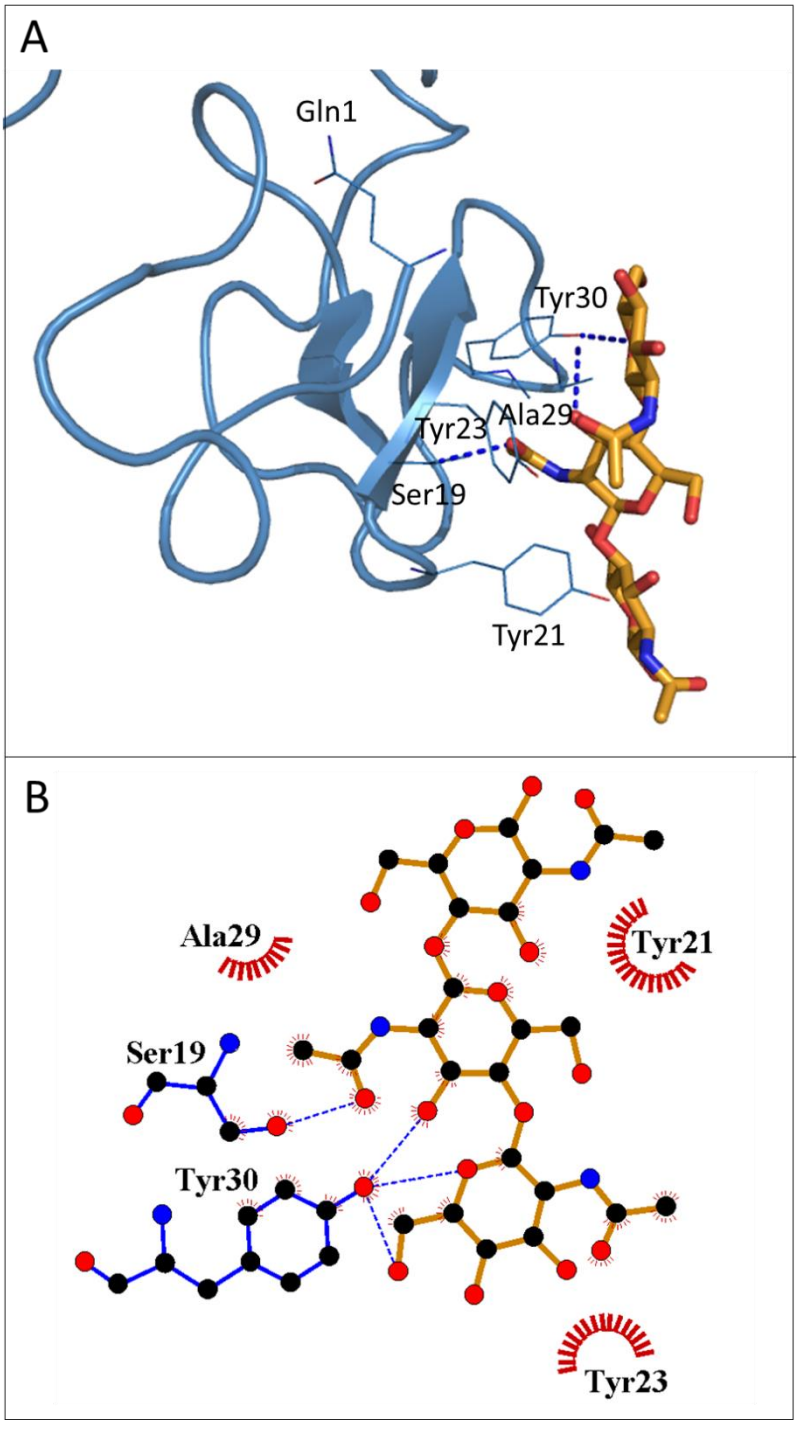

A
Gln1
Tyr30
Ala29
Tyr23
Ser19
Tyr21
B
Ala29
Tyr21
Ser19
Tyr30
Tyr23

Fig. 6

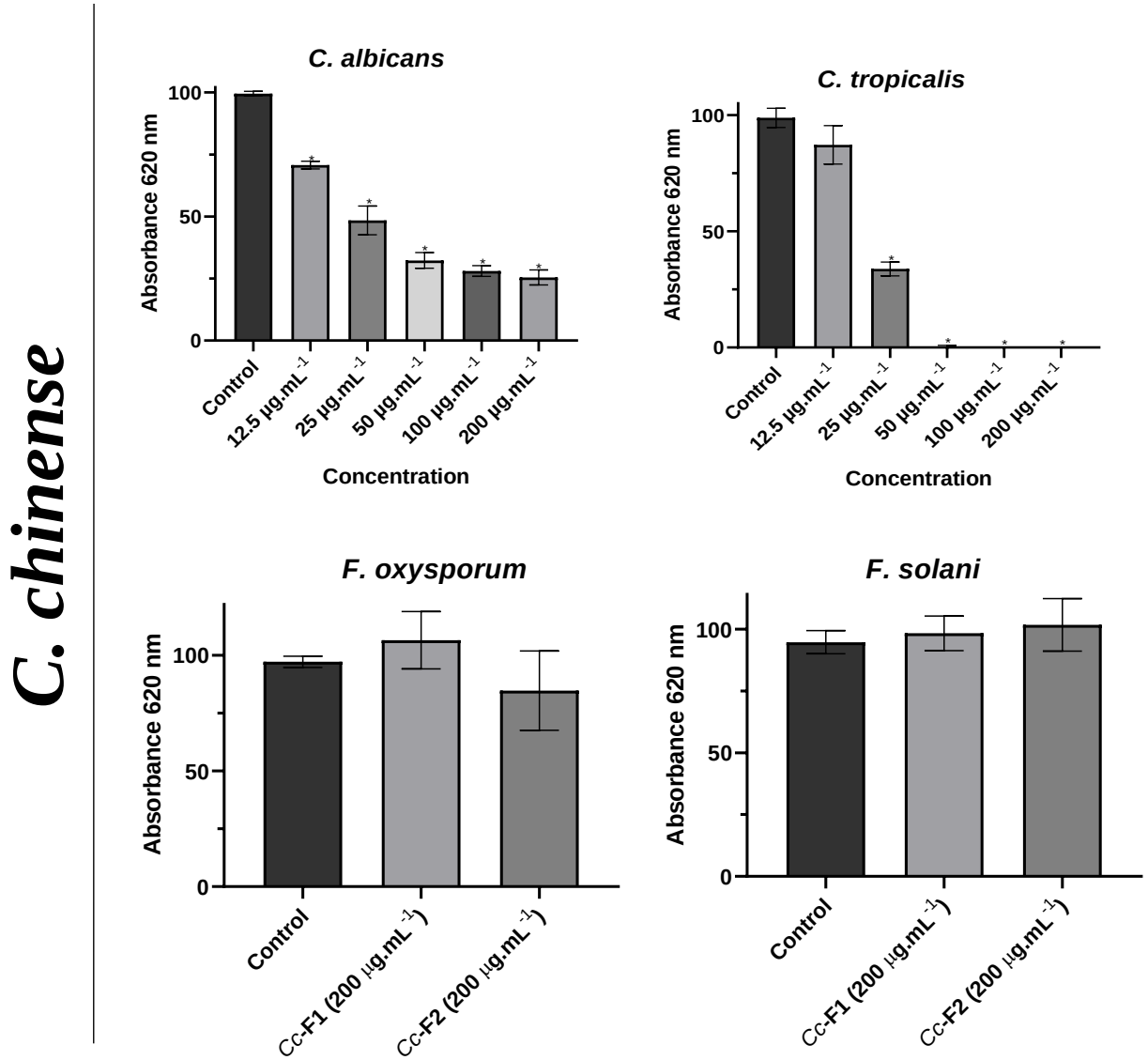
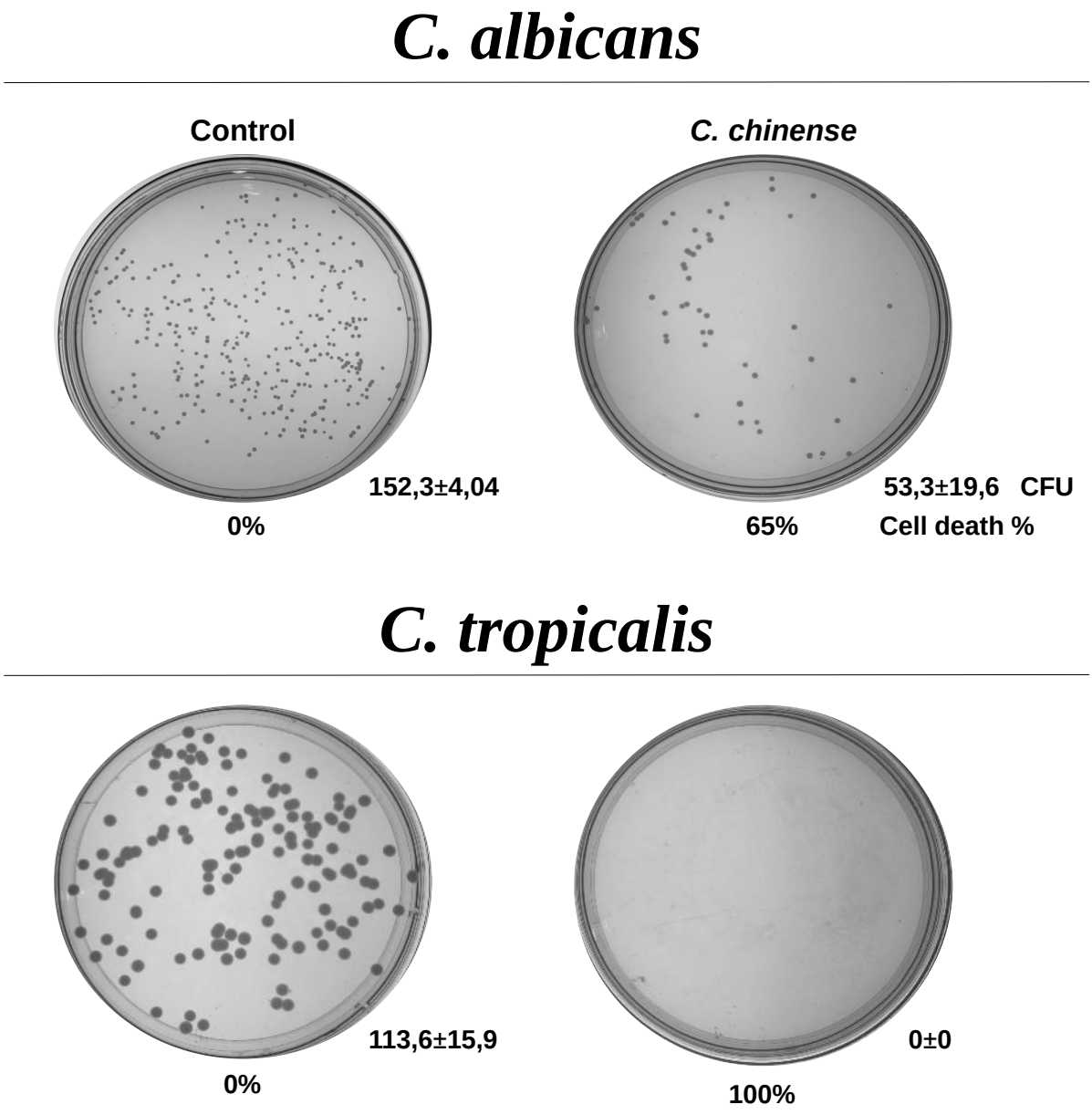

C. chinense
C. albicans
Absorbance 620 nm
100
50
0
Control
12.5 µg.mL -1
25 µg.mL -1
50 µg.mL -1
100 µg.mL -1
200 µg.mL -1
Concentration
C. tropicalis
Absorbance 620 nm
100
50
0
Control
12.5 µg.mL -1
25 µg.mL -1
50 µg.mL -1
100 µg.mL -1
200 µg.mL -1
Concentration
F. oxysporum
Absorbance 620 nm
100
50
0
Control
Cc-F1 (200 µg.mL -1)
Cc-F2 (200 µg.mL -1)
F. solani
Absorbance 620 nm
100
50
0
Control
Cc-F1 (200 µg.mL -1)
Cc-F2 (200 µg.mL -1)

Fig. 7


C. albicans
Control
C. chinense
152,3±4,04
53,3±19,6 CFU
0%
65%
Cell death %
C. tropicalis
113,6±15,9
0±0
0%
100%

6 – CONSIDERAÇÕES FINAIS E CONCLUSÕES

Considerações Finais

Os peptídeos antimicrobianos, como parte dos antibióticos naturais, estão cada vez mais ganhando atenção como antimicrobianos emergentes de amplo espectro de próxima geração. Eles apresentam diversidade estrutural e funcional, o que oferece ilimitadas possibilidades terapêuticas. Um dos grandes candidatos a medicamentos, os peptídeos sintéticos curtos bioinspirados em peptídeos naturais, apresentam apenas a parte ativa da molécula. Estes podem ser modificados com suas propriedades melhoradas, ter melhor eficácia terapêutica e produção mais barata em larga escala (Slezina; Odintsova 2023).

Mesmo esses peptídeos representando uma fonte potencial de agentes antifúngicos com novos e promissores mecanismos de ação, seu uso é limitado por uma série de desafios, incluindo propriedades físico-químicas e biológicas. A estabilidade biológica dos peptídeos para utilização sistêmica vem sendo uma barreira à comercialização. Eles são suscetíveis a degradação proteolítica, o que pode diminuir sua atividade e encurtar sua meia-vida (Konakbayeva; Karlsson 2023).

Para solucionar algumas dessas interações, esses peptídeos poderiam ser planejados e melhorados para atingir componentes específicos de interesse da membrana celular fúngica, e que não são componentes de células de mamíferos (Rautenbach *et al*., 2016). O peptídeo antifúngico histatina 5, um peptídeo encontrado na saliva humana com forte propriedade fungicida, pode ser aplicado no tratamento da candidíase oral, constituindo-se em uma grande inspiração para novos desenhos de drogas e alvos específicos em fungos (Puri; Edgerton 2014). Algumas defensinas estão atualmente em fase de desenvolvimento e testes para uso clínico no tratamento de diversas infecções fúngicas, bacterianas e virais. No entanto, até o momento, apenas um peptídeo derivado de uma defensina de planta está nessa lista (Pezadeftida ou HXP124). Sendo assim, existe a necessidade do avanço nessa área de pesquisa a fim de aumentar o número de antifúngicos eficazes disponíveis (Hein *et al*., 2022).

A partir destes estudos, proteínas antimicrobianas com amplo espectro de atividade vêm recebendo bastante atenção, já que podem ser utilizadas como ferramenta para o desenvolvimento de novos produtos para controle de doenças e pragas agrícolas e para a produção de novos antibióticos para o tratamento de inúmeras doenças humanas (Souza, 2020). Sua alta eficiência se deve a características como baixa massa molecular, sequência primária determinada, carga, conformação, ligações dissulfeto e razão hidrofóbica. Além disso, são proteínas catiônicas que podem interagir e desorganizar a membrana celular dos

microrganismos (Souza *et al*., 2016). Para alcançar uma maior eficácia dessas e outras proteínas especificas da parede ou membrana celular fúngica, o estudo direcionado às proteínas e/ou peptídeos antimicrobianos de plantas poderia levar a novas moléculas e mecanismos de ação distintos, em comparação com os peptídeos e agentes antifúngicos conhecidos (Konakbayeva; Karlsson, 2023).

As LTPs também podem ter diversas aplicações, incluindo a saúde humana, considerando sua ação antimicrobiana. Alguns efeitos adicionais foram previstos, incluindo atividade antinociceptiva, pró-apoptótica, indução de sinalização celular sob estresse, eliminação de ROS (antioxidante), efeitos antiproliferativos em células cancerosas (hepatoma, câncer de mama e leucemia promielocítica), além da inibição da α-amilase (com potencial uso no controle da obesidade). Além disso, elas podem auxiliar na estabilidade dos peptídeos, proporcionando estabilidade química e física a compostos farmacológicos (Amador *et al*., 2021).

Dois peptídeos estudados neste trabalho apresentam similaridade com proteínas alergênicas (albumina 2S e LTP), e suas estruturas primárias foram identificadas através de espectrometria de massas. Modificações como substituição com aminoácidos naturais ou não naturais, carga, hidrofobicidade e estrutura, são estratégias que poderiam não só auxiliar no problema da alergenicidade, como também ajudar a melhorar a atividade e a estabilidade dos peptídeos encontrados. Com base nesses resultados, orientaríamos a pesquisa para as próximas etapas, com o objetivo de destacar os peptídeos encontrados como candidatos promissores para futuros ensaios clínicos. Confiando na atividade desses peptídeos, a albumina 2S e LTP poderiam ser utilizadas no controle a patógenos humanos.

As quitinases possuem diversas aplicações em vários campos, incluindo a indústria de alimentos, e são reconhecidas como substâncias antifúngicas com potencial uso como defensivo agrícola. Uma ampla variedade de cepas de fungos teve seu crescimento inibido, provando que as quitinases são agentes antifúngicos que podem ser aplicados como possíveis agentes de biocontrole, substituindo fungicidas químicos em larga escala no manejo de doenças fúngicas de plantas. Sua estabilidade em temperaturas moderadas (30 °C e 50 °C), as tornam uma enzima potencialmente útil no bioprocessamento para produzir quitooligossacarídeos para várias aplicações nos setores de alimentação, saúde e agricultura. Em breve, uma tecnologia de baixo custo que atinja o interesse público em geral, especialmente o setor agrícola, é essencial para dar a esse amplo campo de pesquisa uma direção frutífera (Li *et al*., 2019; Malik, 2019). O

primeiro transgênico expressando uma heveina-símile foi desenvolvido em mostarda, e posteriormente, diversos outros transgênicos foram criados, incluindo o Pn-AMP2 em tabaco e tomate, além do SmAMP1 e SmAMP2 em tabaco. Eles apresentaram significativo aumento na resistência a fungos fitopatogênicos. Estes estudos abrem caminho para o desenvolvimento de transgênicos baseados em peptídeos ativos contra diferentes grupos de fungos fitopatogênicos (Azmi *et al*., 2021).

Ainda em relação à perspectiva agronômica, o uso de genótipos resistentes é uma das principais abordagens genéticas utilizadas (Holaskova *et al*., 2015), mas isso se restringe a poucas culturas e fitopatógenos. As proteínas da família das heveínas ou quitinases poderiam ser mais direcionadas para aplicações na agricultura, podendo atuar como agentes contra fitopatógenos, bem como poderiam ser utilizadas para superexpressão em plantas visando o aumento da resistência contra fungos fitopatogênicos. Além disso, poderiam ser realizados ensaios em sinergia com drogas utilizadas comercialmente. Desta forma, a tentativa de controle de tais patógenos, de modo ecologicamente correto, se apresenta como importante e prioritária, sendo a prospecção de proteínas e/ou peptídeos antifúngicos e a transferência de genes que confiram resistência contra esses organismos de grande interesse. Essas famílias de proteínas e/ou peptídeos podem desempenhar um papel essencial no desenvolvimento de novas moléculas antimicrobianas, aumentando as chances de superar o desafio imposto pela resistência microbiana.

Conclusões Gerais

Os resultados obtidos neste trabalho, permitem concluir que:

- Todas as espécies de *Capsicum* usadas neste trabalho apresentaram frações ligantes à quitina, as quais apresentaram proteínas de baixa massa molecular, variando de 3,0 a 26,6 kDa;
- Todas as frações isoladas de ligação à quitina apresentaram atividade inibitória contra as leveduras testadas em diferentes concentrações, com exceção de *Cf*-F2 de *C. frutescens* que não apresentou atividade sobre *C. tropicalis*;
- Todas as frações testadas diminuíram a viabilidade celular de *C. albicans* e apenas *Cf*-F2 de *C. frutescens* não causou redução na viabilidade celular de *C. tropicalis*;
- Nenhuma fração de ligação à quitina apresentou atividade inibitória sobre os fungos *F. oxysporum* e *F. solani*;
- A fração *Ca*-F2 apresentou baixa hemotoxicidade, em diferentes concentrações, quando testada contra eritrócitos de carneiro;
- A fração *Ca*-F2 foi capaz de promover alterações morfológicas nas células e causar permeabilização na membrana plasmática da levedura *C. albicans*, na concentração de 100 $\mu g.mL^{-1}$;
- A fração *Ca*-F2 foi a única fração ligante à quitina que demonstrou ser capaz de inibir a enzima tripsina;
- *Ca*-F2, *Cb*-F2 e *Cf*-F2 demonstraram baixa toxicidade quando testadas *in vivo* em larvas de *G. mellonella*, na concentração de 200 $\mu g.mL^{-1}$;
- A banda proteica de aproximadamente 6,5 kDa da fração *Ca*-F2 foi submetida ao sequenciamento de aminoácidos por espectrometria de massas e apresentou similaridade com albumina 2S;
- As bandas proteicas de aproximadamente 6,5 kDa das frações *Cb*-F2 e *Cf*-F2 foram submetidas ao sequenciamento de aminoácidos por espectrometria de massas e após análise demonstraram similaridade com LTP;
- A banda proteica de aproximadamente 6,5 kDa de *Cc*-F2 foi submetida ao sequenciamento de aminoácidos por espectrometria de massas e exibiu similaridade com heveína e endoquitinase, sendo denominada como *Cc*-Hev.

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