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LUCAS PINHEIRO DIAS

PURIFICAÇÃO E CARACTERIZAÇÃO BIOQUÍMICA DE UM INIBIDOR DE TRIPSINA DE SEMENTES DE *CASSIA LEIANDRA* BENTH. E AVALIAÇÃO DE SUA ATIVIDADE INSETICIDA CONTRA *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

FORTALEZA
2016

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Orientadora: Profa. Dra. Ilka Maria Vasconcelos

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Ao Márcio,

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A vida é construída nos sonhos e concretizada no amor "

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RESUMO

A Organização Mundial da Saúde considera a dengue como um dos mais importantes problemas de saúde pública. *Aedes aegypti* (Diptera: Culicidae), um inseto sugador de sangue que se desenvolve em áreas tropicais e subtropicais, é o principal vetor do vírus da dengue, além de ser um importante transmissor das viroses Zica e Chikungunya. O controle químico através de inseticidas é bastante usado no combate do *Ae. aegypti*, mas sua aplicação indiscriminada e prolongada tem favorecido o surgimento de insetos resistentes. Isso tem motivado a busca por compostos naturais com atividade inseticida e as plantas têm se sobressaído como potenciais fontes de tais substâncias. Este estudo teve como objetivo a purificação e caracterização bioquímica de um inibidor de tripsina, denominado *Cl*TI, de sementes de *Cassia leiandra* Benth. (Fabaceae, Caesalpinoideae), uma espécie vegetal nativa da Amazônia. A ação do *Cl*TI sobre proteases intestinais do *Ae. aegypti* e seus efeitos no desenvolvimento e sobrevivência desse inseto foram também investigados. O *Cl*TI foi purificado por cromatografias em DEAE-Celulose e tripsina-Sepharose 4B, com índice de purificação de 15,5 vezes e rendimento proteico de 2,4%. O inibidor puro é composto de uma cadeia polipeptídica de 19.484 Da, como mostrado por espectrometria de massas com ionização por “electrospray” (ESI), não glicosilado e apresenta estrutura secundária constituída por 35% de folhas-β, 14% de β-voltas e 50% de estruturas desordenadas. O *Cl*TI é um inibidor incompetitivo de tripsina bovina (CI_{50} de $33,81 \times 10^{-8}$ M), com Ki de $6,25 \times 10^{-8}$ M e sequência de aminoácidos similar às de outros inibidores da família Kunitz. *Cl*TI se mostrou estável em ampla faixa de pH (2,2-10,0) e temperatura (30-70 °C), mas a incubação com ditiotreitol causou perda parcial de sua atividade inibitória. *In vitro*, *Cl*TI ($4,65 \times 10^{-6}$ M) promoveu redução de 50% na atividade das proteases intestinais de *Ae. aegypti*. I*n vivo*, esse inibidor apresentou toxicidade aguda dose-dependente para larvas de 3° estágio de *Ae. aegypti*, com uma CL_{50} de $2,28 \times 10^{-2}$ M. O tratamento crônico de *Ae. aegypti* com *Cl*TI ($1,54 \times 10^{-5}$ M concentração final) não interferiu na eclosão dos ovos, mas retardou o desenvolvimento larval do inseto em 24 h e causou mortalidade de 44%. Os resultados obtidos contribuem para um melhor entendimento da atividade inseticida dos inibidores de proteases e sugerem que *Cl*TI tem potencial biotecnológico para ser usado como estratégia alternativa no controle desse vetor de várias doenças, o qual pode ser usado sozinho ou em combinação com outros compostos inseticidas na perspectiva de potencializar a toxicidade.

Palavras-chaves: *Cassia leiandra*. Inibidor de Kunitz. Proteína inseticida. *Aedes aegypti*.

ABSTRACT

The World Health Organization (WHO) considers dengue virus infection one of the most important public health problems. *Aedes aegypti* (Diptera: Culicidae), a blood-sucking insect that lives and cohabits with the human population in tropical and subtropical areas, is the principal vector responsible for dengue virus transmission. *Ae. aegypti* is also the main transmitter of Chikungunya and Zika viruses. To eliminate dengue mosquitoes, chemical control with insecticides is widely used. However, prolonged and indiscriminate application of these compounds has favored the appearance of resistant insects. This issue has driven the search for natural compounds with insecticidal activity, and plants are potential sources of such constituents. This present study reports the purification and biochemical characterization of a trypsin inhibitor (*Cl*TI) from the seeds of *Cassia leiandra* Benth. (Fabaceae, Caesalpinioideae), a plant species native to the Amazon rainforest. In addition, the *Cl*TI action on *Ae. aegypti* midgut proteases and its effect on the development and survival of this mosquito are assessed. *Cl*TI was purified by DEAE-Cellulose and trypsin-Sepharose 4B chromatography, with a 15.5-fold purification and 2.4% yield. *Cl*TI is composed of one polypeptide chain of 19,484 Da, as revealed by ESI mass spectrometry, is non-glycosylated, and comprises 35% β-sheets, 14% β-turns, and 50% disordered structures. *Cl*TI is an uncompetitive inhibitor of bovine trypsin (IC_{50} of 33.81 x 10^{-8} M), with a Ki of 6.25 x 10^{-8} M, and has an amino acid sequence similar to other inhibitors of the Kunitz-type family. *Cl*TI was stable over a broad range of pH (2.2–10.0) and temperature (30–70 °C), but DTT incubation caused a partial loss of inhibitory activity. *Cl*TI (4.65 x 10^{-6} M) promoted 50% activity reduction of *Ae. aegypti* midgut proteases, showed a dose-dependent acute toxicity on *Ae. aegypti* 3rd instar larvae, with an LC_{50} of 2.28 x 10^{-2} M, and after ten days of exposure, caused a 24-h delay of larval development and 44% mortality. Our results are an important contribution to a better understanding of the insecticidal activity of protease inhibitors, suggesting that *Cl*TI has biotechnological potential as an alternative strategy to control this multiple disease vector, which may be used alone or in combination with other insecticidal compounds to produce enhanced toxicity.

Keywords: *Cassia leiandra*. Kunitz inhibitor. Insecticidal protein. *Aedes aegypti.*

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1 INTRODUÇÃO

O *Aedes aegypti* (Diptera, Culicidae) desenvolveu em sua trajetória evolutiva um comportamento estritamente sinantrópico e antropofílico, sendo reconhecido entre os culicídeos como a espécie mais associada ao homem. Como consequência, o mosquito está relacionado com a transmissão de várias doenças, incluindo a febre amarela e dengue. Além disso, o *A. aegypti* também é o transmissor do Zika vírus e do vírus causador da febre chikungunya (SCHAFFNER; MEDLOCK; VAN BORTEL, 2013; MEDEIROS-SOUSA *et al.*, 2015; WHO, 2016a).

A Organização Mundial da Saúde considera a dengue como um dos mais importantes problemas de saúde pública dos últimos anos. Estima-se que a doença se encontra disseminada em mais de 100 países, principalmente em regiões tropicais de clima quente e úmido. O número de casos notificados da doença aumentou de 2,2 milhões em 2010 para 3,2 milhões em 2015 (WHO, 2016b). A febre chikungunya é uma doença febril aguda, causada pelo vírus chikungunya (CHIKV), um vírus de RNA que pertence ao gênero *Alphavirus* da família Togaviridae. A doença já foi identificada em mais de 60 países e apresenta sintomas mais brandos que a dengue (WHO, 2016c). Já o vírus Zika é um flavivírus, transmitido principalmente pelo mosquito *A. aegypti*. A doença já foi relatada em 65 países e estima-se que, até o final de 2016, cerca de 4 milhões de pessoas nas Américas serão infectadas, sendo 1,5 milhões de casos no Brasil. Atualmente, a maior preocupação é a associação entre a infecção pelo vírus Zika e microcefalia e síndrome de Guillain-Barré (WHO, 2016d).

A principal estratégia para o controle dessas doenças é o combate ao vetor, que envolve ações de saneamento básico e educação ambiental, visando a eliminação dos criadouros do mosquito. O controle biológico também aparece como uma alternativa bastante promissora; nesse caso inclui o uso de vários predadores, invertebrados aquáticos (como *Toxorhynchites* ou *copépodos*) e peixes (*Gambusia* sp. e outros), os quais comem larvas e pupas (ZARA *et al.*, 2016). Entretanto, o controle químico, com inseticidas de origem orgânica ou inorgânica, é uma das metodologias mais adotadas na saúde pública, como parte do manejo integrado de vetores. Todavia, o uso prolongado e indiscriminado desses compostos tem favorecido o aparecimento de populações de insetos resistentes (ROCHA *et al.*, 2015). Além disso, muitos desses compostos se acumulam no meio ambiente e apresentam efeitos tóxicos para o homem e outros animais (BONALDO *et al.*, 2007; KOUTROS *et al.*, 2012; WAGGONER *et al.*, 2013).

Além das estratégias citadas acima, pesquisadores têm buscado na própria natureza moléculas com atividade inseticida (TENNYSON; RAVINDRAN; ARIVOLI; 2012; GOVINDARAJAN; BENELLI, 2016). Os vegetais produzem uma diversidade de compostos orgânicos, que parecem não ter função direta no seu crescimento e desenvolvimento. Alguns desses compostos são conhecidos como metabólitos secundários e, muitos deles, tais como terpenos, fenilpropanoides e rotenoides, apresentam atividade inseticida (MENEZES; JARED, 2002). Além dos metabólitos secundários, as plantas também sintetizam diversas proteínas que desempenham papel crucial na sua defesa contra o ataque de insetos (YE; NG, 2009; WONG *et al.*, 2012). Dentre as proteínas mais estudadas, estão as lectinas, ureases, proteínas inativadoras de ribossomos e os inibidores de proteases (CARLINI; GROSSI-DE-SÁ, 2002).

Os inibidores de proteases são proteínas ou peptídeos presentes em todos os seres vivos capazes de inibir a ação catalítica de proteases, através da formação de complexos estequiométricos com as enzimas alvo, bloqueando ou alterando seu sítio ativo (OLIVEIRA *et al.*, 2007; VOLPICELLA *et al.*, 2011; SHAMSI; PARVEEN; FATIMA, 2016). Essas moléculas podem formar complexos estáveis com proteases digestivas de insetos, diminuindo ou impedindo a digestão de proteínas da dieta, reduzindo, assim, o fornecimento de aminoácidos para o crescimento e desenvolvimento das larvas (STEVENS *et al.*, 2013). Há, por exemplo, vários relatos de inibidores de proteases que apresentam efeitos tóxicos contra larvas de *Ae. aegypti* (SILVA *et al.*, 2015; SASAKI *et al.*, 2015; ALMEIDA-FILHO *et al.*, 2016).

Análises preliminares conduzidas por nosso grupo de pesquisa demonstrou a presença de inibidores de tripsina em sementes de *Cassia leiandra* Benth., uma espécie nativa da Amazônia. Nesse contexto, o presente estudo foi proposto com intuito de responder os seguintes questionamentos:

- O inibidor de tripsina purificado das sementes de *C. leiandra* apresentam características bioquímicas e funcionais semelhantes àquelas de outros inibidores de proteases oriundos de sementes de leguminosas?
- Quais propriedades o inibidor de tripsina purificado de sementes de *C. leiandra* apresenta, que o creditaria a ser testado quanto à sua ação inseticida?
- Esse inibidor de tripsina de *C. leiandra* seria capaz de inibir, *in vitro*, proteases intestinais do inseto *A. aegypti*?
- Quais seriam os efeitos adversos desse inibidor de tripsina de *C. leiandra* para *A. aegypti*?

2 REVISÃO DE LITERATURA

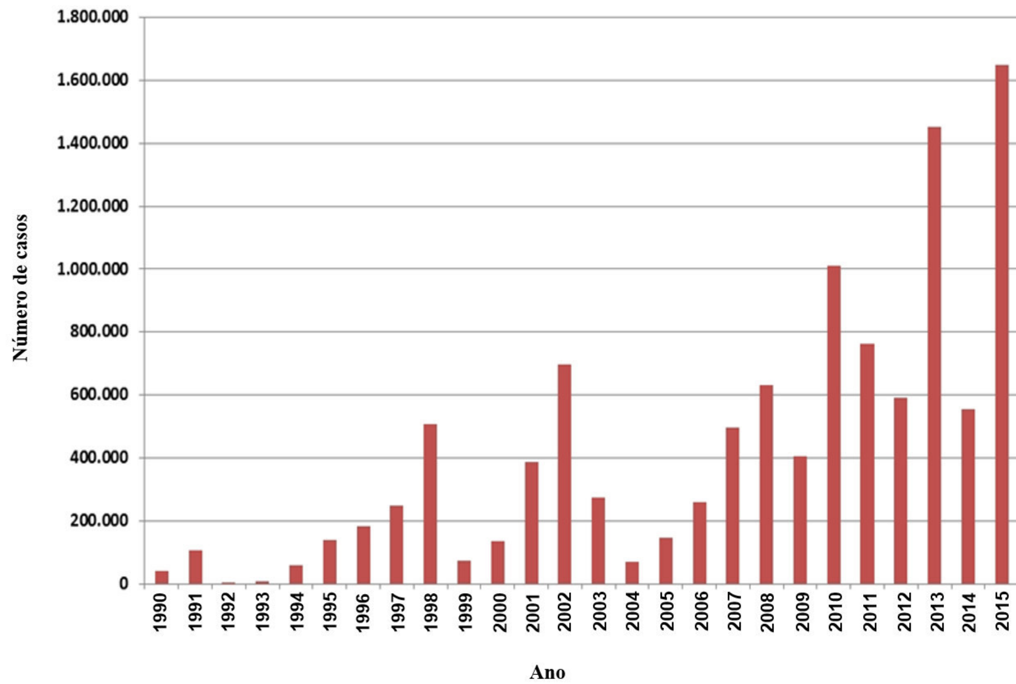
2.1 Dengue: um problema de saúde pública mundial

A dengue é uma doença infecciosa causada por qualquer um dos quatro sorotipos (DEN-1, DEN-2, DEN-3 e DEN-4) do vírus da dengue, um arbovírus do gênero *Flavivirus*, que pertence à família Flaviviridae (EBI; NEALON, 2016). Na apresentação clássica, a dengue é caracterizada por febre alta (39 a 40 ºC), associada à cefaleia, adinamia, mialgia, artralgia e dor retro-orbitária. As manchas vermelhas na pele, características da doença, geralmente surgem após o desaparecimento da febre. Entre o terceiro e o sétimo dia do início da doença podem surgir dor abdominal intensa e contínua, desconforto respiratório, sonolência ou irritabilidade excessiva, hipotermia, sangramento de mucosas, diminuição da sudorese e derrames cavitários (BRITO *et al.*, 2013).

Segundo um estudo conduzido na Universidade de Oxford, na Grã-Bretanha, a dengue atinge mundialmente cerca de 390 milhões de pessoas por ano, sendo que mais de 90 milhões desses casos são graves e o restante, leve ou assintomático (BHATT *et al.*, 2013). No período de 4 de janeiro de 2015 a 2 de janeiro de 2016, foram registrados 1.649.008 casos de dengue no Brasil. Nesse período, a região Sudeste registrou o maior número de casos (1.026.226 casos; 62,2%) em relação ao total do país, seguida das regiões Nordeste (311.519 casos; 18,9%), Centro Oeste (220.966 casos; 13,4%), Sul (56.187 casos; 3,4%) e Norte (34.110 casos; 2,1%). De acordo com dados do Ministério da Saúde, as regiões Centro-Oeste e Sudeste apresentaram as maiores incidências de dengue: 1.451,9 casos/100 mil habitantes e 1.205,7 casos/100 mil habitantes, respectivamente. Entre os estados, destacam-se Goiás (2.500,6 casos/100 mil habitantes) e São Paulo (1.665,7 casos/100 mil habitantes) (NARDI *et al.*, 2016).

O cenário que se descortina quanto à evolução da dengue no Brasil é preocupante (FIGURA 1). Diante disso, diversas políticas públicas têm sido propostas para o controle do avanço da doença. Estima-se que, no período de 2010 a 2014, o governo federal gastou cerca de R$ 4,2 bilhões em ações de prevenção e tratamento da dengue. No período analisado, o custo da dengue para a União aumentou 48%, passando de 613,4 milhões em 2010 para 911,8 milhões em 2014. Em dezembro de 2014, o Ministério da Saúde realizou um repasse adicional de R$ 150 milhões a todas as secretarias estaduais e municipais do país para reforço das atividades de vigilância, prevenção e controle da dengue em 2015 (NARDI *et al.*, 2016). Ainda assim, o número atual de pessoas acometidas pela dengue não deixa de ser alarmante.

Figura 1 - Avanço do número de casos de dengue registrados no Brasil no período de 1990 a 2015



Fonte: http://portalsaude.saude.gov.br/.

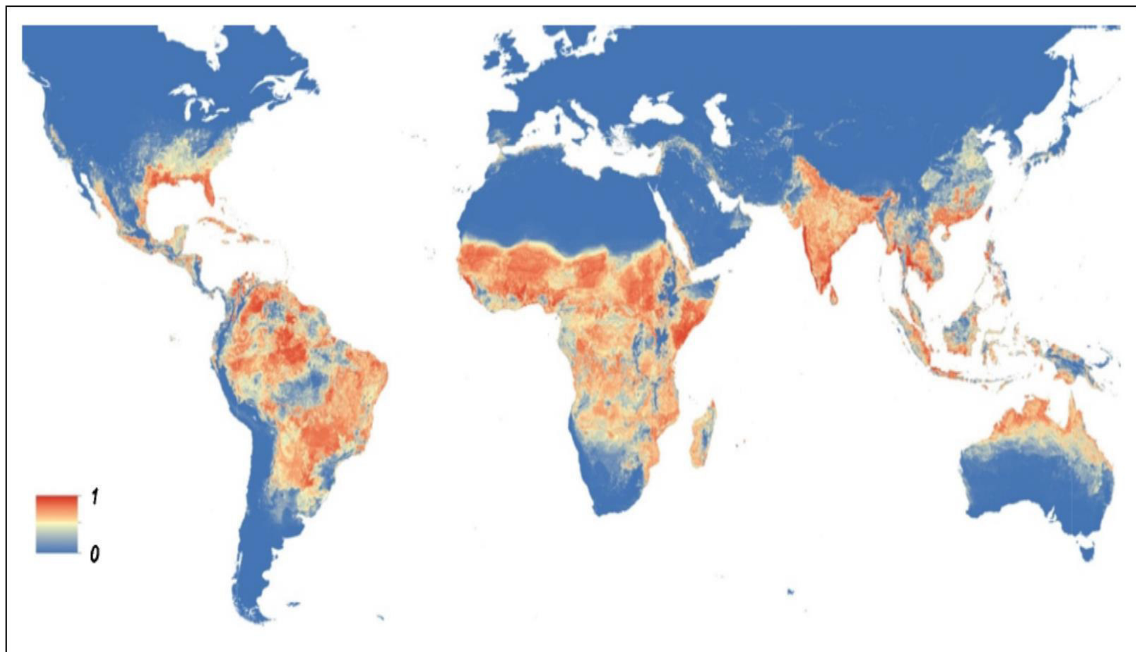
2.2 *Aedes aegypti*: biologia e importância epidemiológica

O *Ae. aegypti* é originário da África e foi, provavelmente, introduzido no Brasil na metade do século XIX, através de navios negreiros. Atualmente, esse inseto está distribuído por todas as regiões tropicais e subtropicais do mundo (FIGURA 2). O mosquito adulto mede menos de 1 cm e apresenta coloração preta com listras brancas no corpo e nas patas. São holometábolos e seu ciclo de vida compreende os estágios de ovo, larva, pupa e adulto (FIGURA 3) (NEVES, 2005; MARICOPA COUNTY ENVIRONMENTAL SERVICES, 2006; BRAGA; VALLE, 2007; WHO, 2016b).

Os ovos de *Ae. aegypti* medem aproximadamente 0,4 mm de comprimento e são capazes de sobreviver no meio ambiente, mesmo em condições adversas, durante longos períodos. Isso dificulta a erradicação do mosquito, além de favorecer sua dispersão, pois permite sua transferência para grandes distâncias em recipientes secos (SASAKI *et al*., 2015). A fase larval apresenta 4 instares (L1, L2, L3 e L4) e dura, em média, 7 dias. Em seguida, as larvas entram em estágio de pupa, no qual permanecem por 1 ou 2 dias. Após esse período, surge o mosquito adulto, que pode viver de 1 a 2 meses (ALMEIDA-FILHO *et al*., 2016). Geralmente, os mosquitos adultos alimentam-se de seiva vegetal, contudo, após o acasalamento, as fêmeas desenvolvem o hábito hematofágico. Isso ocorre porque as proteínas no sangue servem de precursoras do vitelo, sendo fundamentais para o desenvolvimento dos ovos. Mesmo na presença de outros hospedeiros, as fêmeas se alimentam preferencialmente de sangue humano, podendo picar várias vezes o mesmo indivíduo ou buscar novos hospedeiros, até completar o repasto. Essa característica aumenta a possibilidade de transmissão horizontal do vírus, aquela que ocorre entre mosquito-vetor e hospedeiros humanos. Além disso, se a fêmea estiver infectada pelo vírus quando realizar a postura de ovos, as larvas podem nascer infectadas (transmissão vertical através da via transovariana) (NATAL, 2002; MARICOPA COUNTY ENVIRONMENTAL SERVICES, 2006).

As enzimas digestivas identificadas no *Ae. aegypti* são tripsina, quimotripsina, aminopeptidases e carboxipeptidases. A tripsina e quimotripsina merecem destaque, pois são as principais enzimas atuantes na digestão do inseto, estando presentes em todos os estágios de seu desenvolvimento. Essas enzimas são bastante ativas na fase larval, sendo a tripsina mais abundante do que a quimotripsina (SOARES *et al*., 2011; SASAKI *et al*., 2015).

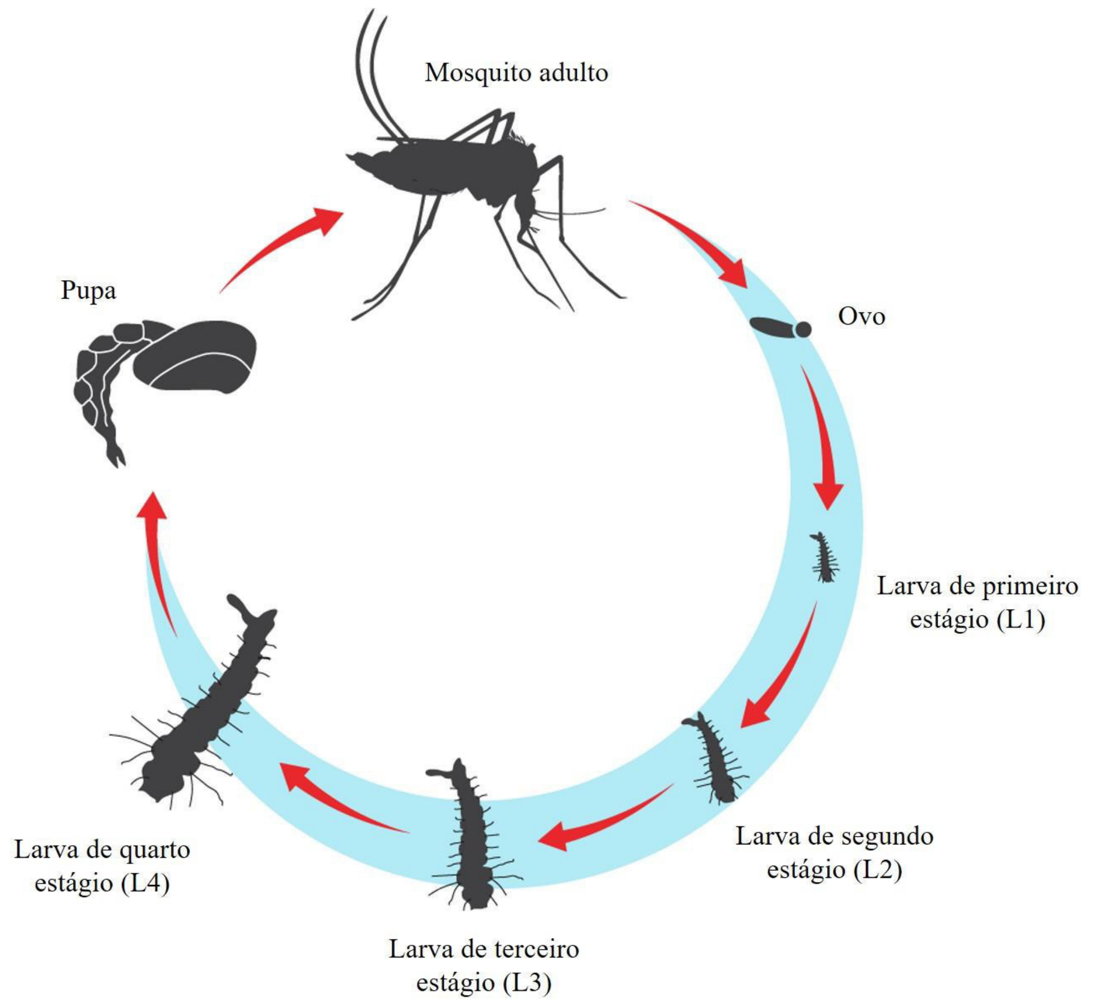
Figura 2 - Distribuição mundial do mosquito *Aedes aegypti*



Fonte: Tilak *et al*., 2016.

Regiões marcadas em vermelho apresentam probabilidade máxima de ocorrência do *Ae. aegypti*, enquanto aquelas marcadas em azul a probabilidade é mínima.

Figura 3 - Ciclo de vida do *Ae. aegypti*



Fonte: https://www.publico.pt/multimedia/infografia/zzzzzzzika-185, com modificações.

2.3 Estratégias para o controle do *Ae. Aegypti*

A melhor forma de prevenção da dengue e demais doenças transmitidas pelo o *Ae. aegypti* é através do controle da população do mosquito-vetor, o qual inclui a melhoria das condições de saneamento básico, além de campanhas para a conscientização da comunidade sobre a importância da eliminação dos criadouros domésticos do mosquito (ZARA *et al*., 2016). Porém, o controle biológico desponta como uma alternativa bastante vantajosa, uma vez que não leva à contaminação ambiental e é específica contra o organismo controlado, além do que a autodispersão facilita o controle das larvas em locais de difícil acesso. Para esse tipo de controle, são utilizados, geralmente, peixes larvívoros (*Gambusia affinis* e *Poecilia* spp.) e inseticidas biológicos (*Bacillus thurigiensis* var. *israelensis*, sorotipo H-14) (PAIVA *et al.*, 2014; BELLINI; ZELLER; BORTEL, 2014; MOHIDDIN; LASIM; ZUHARAH, 2016). Também, já foi demonstrado que larvas de Odonata e de mosquitos do gênero *Toxorhynchites* sp. são bons predadores de larvas de *Ae. aegypti* (WETERINGS; UMPONSTIRA; BUCKLEY, 2015). A principal limitação do controle biológico reside no fato de limitar-se às formas imaturas do vetor.

Adicionalmente às estratégias acimas citadas, inseticidas químicos também são bastante utilizados. Os principais grupos de inseticidas orgânicos usados são os organoclorados, organofosforados, carbamatos e piretroides; todos exercendo seus efeitos tóxicos sobre o sistema nervoso central dos insetos (MOREIRA; MANSUR; FIGUEIRA-MANSUR, 2012; BELLINI; ZELLER; BORTEL, 2014). Os organoclorados são inseticidas que possuem na sua constituição moléculas de carbono, hidrogênio e cloro. Eles permanecem, por longos períodos, no ambiente e podem se acumular em tecidos de animais e do homem. Nesse grupo, o diclorodifeniltricloroetano (DDT) é um dos compostos mais conhecidos devido à importância que tem no controle de vetores de doenças (LIU *et al.*, 2016). Os organofosforados são menos estáveis quando comparados com os organoclorados e, sendo assim, apresentam menor persistência no ambiente. Temephos® é o único do grupo aprovado pela Organização Mundial da Saúde para uso em água de consumo humano, por apresentar toxicidade aguda e persistência baixas no ambiente. O malation é outro organofosforado empregado no controle de mosquitos adultos e pragas urbanas, apresentando boa eficácia, sendo utilizado, na maioria das vezes, na forma de aerossol para pulverização de grandes áreas (BRAGA; VALLE, 2007; BASTOS *et al.*, 2016). Os compostos do grupo dos carbamatos são derivados do ácido carbâmico e têm sido utilizados no controle de insetos resistentes aos organoclorados. O propoxur é um dos

inseticidas desse grupo usado com mais frequência (MOREIRA; MANSUR; FIGUEIRA-MANSUR, 2012). Já os inseticidas do grupo dos piretroides são produzidos a partir do composto piretro. Tais inseticidas são compostos biodegradáveis e com baixa toxicidade para mamíferos, podendo ser utilizados como larvicidas e, também, no controle de mosquitos adultos. A deltametrina e a cipermetrina são dois exemplos de piretroides que têm sido muito usados no controle de vetores transmissores de enfermidades, como a dengue e malária (BRAGA; VALLE, 2007; ANTWI; REDDY, 2015).

Apesar da eficácia dos inseticidas químicos, o seu uso frequente e descontrolado tem levado ao surgimento de populações de insetos resistentes, comprometendo, assim, o controle do vetor. Resistência de insetos já foi detectada para todas as classes de inseticidas, o que tem resultado, inclusive, na elevada taxa de re-emergência das doenças transmitidas pelo *Ae. aegypti* (THONGWAT; BUNCHU, 2015; MARRIEL *et al.*, 2016). Dessa forma, novas alternativas precisam ser desenvolvidas para o controle do *Ae. Aegypti* e uma dessas seria a prospecção de compostos naturais com atividade inseticida.

Diversos extratos de plantas já mostraram efeitos tóxicos contra *Ae. aegypti* (MUNUSAMY *et al.*, 2016; SPINDOLA *et al.*, 2016; EL-SHEIKH; AL-FIFI; ALABBOUD, 2016). Esses extratos apresentam na sua composição compostos do metabolismo primário e secundário. Dentre as moléculas oriundas do metabolismo primário, estão as proteínas inseticidas, as quais incluem lectinas, ureases, proteínas inativadoras de ribossomos e, particularmente, os inibidores de proteases (CARLINI; GROSSI-DE-SÁ, 2002; LAWRENCE; KOUNDAL, 2002; STIRPE; 2013; OLIVEIRA *et al.*, 2016).

2.4 Inibidores de proteases: ferramentas alternativas para o controle de insetos

Inibidores de proteases são proteínas ou peptídeos, presentes em todos os seres vivos, capazes de inibir a ação catalítica de enzimas, através da formação de complexos estequiométricos com suas enzimas alvo, bloqueando ou alterando seu sítio ativo (OLIVEIRA *et al.*, 2007; VOLPICELLA *et al.*, 2011). Nos vegetais, os inibidores de proteases são encontrados, principalmente, em sementes e tubérculos, mas, também, podem estar presentes em partes aéreas das plantas (MACEDO *et al.*, 2006; WANG; LEE; SU, 2008).

A classificação dos inibidores de proteases pode ser baseada na especificidade e no mecanismo de ação. De acordo com esses parâmetros, os inibidores podem ser classificados em serínicos, cisteínicos, aspárticos e de metalo-proteinases (RYAN, 1990; RICHARDSON,

1991). Dentre essas classes, os mais frequentemente encontrados e caracterizados são os inibidores de proteases serínicas, classe na qual estão incluídos os inibidores de tripsina. Estes podem, ainda, ser subclassificados, de acordo com suas propriedades estruturais, número e posição de pontes dissulfeto e sítios reativos, nas subfamílias: Kunitz; Bowman-Birk; Batata I; Batata II; Abóbora e Cereal (RICHARDSON, 1991; OLIVA *et al*., 2010). Dentre estas subfamílias, as do tipo Kunitz e de Bowman-Birk são as mais estudadas (OLIVEIRA *et al*., 2007).

Os inibidores pertencentes à subfamília Kunitz apresentam massa molecular variando de 18 a 26 kDa. Normalmente, apresentam um único sítio ativo, possuidor de um resíduo de arginina (RICHARDSON, 1991). Entretanto, já há relatos da presença de, pelo menos, dois sítios ativos em inibidores de tripsina tipo Kunitz, associados à capacidade de inibir proteases pertencentes à mesma classe ou de classes diferentes. Por exemplo, o inibidor de tripsina do tipo Kunitz isolado de *Adenanthera pavonina* é capaz de inibir tripsina e papaína, proteases serínica e cisteínica, respectivamente (MIGLIOLO *et al*., 2010).

Os inibidores de proteases encontrados nas plantas apresentam várias funções. Alguns deles atuam como proteínas de armazenamento (CÂNDIDO *et al*., 2011), reguladores endógenos da atividade proteolítica (RYAN, 1990) ou, ainda, participam da morte celular programada (LIN; NG, 2008). Além disso, alguns deles têm se mostrado envolvidos com a defesa de plantas em resposta a estresses bióticos e/ou abióticos (MOSOLOV; VALUEVA, 2011).

O envolvimento dos inibidores de tripsina como estratégia de defesa das plantas contra a herbivoria tem sido, cada vez mais, investigado, na perspectiva de sua aplicação biotecnológica. Essas moléculas são capazes de se ligar às proteases intestinais de insetos de diferentes ordens. O inibidor de tripsina purificado de *Piptadenia moniliformis* inibiu as proteases intestinais de insetos das ordens Coleoptera (*Anthonomus grandis*, 90%), Lepidoptera (*Plodia interpuncptella*, 60%) e Diptera (*Ceratitis capitata*, 70%) (CRUZ *et al*., 2013). Proteases de outros insetos da ordem Lepidoptera, tais como *Anagasta kuehniella (89%), Spodoptera frugiperda (83%), Corcyra cephalonica (80%), Heliothis virescens (70%) e Helicoverpa zea (60%),* foram também inibidas pelo inibidor de Kunitz purificado de sementes de *Inga vera* (BEZERRA *et al*., 2016). O inibidor de tripsina purificado de sementes de *Clitoria fairchildiana* também se mostrou capaz de inibir proteases intestinais de insetos, dentre eles *A. kuehniella* (76%), *Diatraea saccharalis* (59%) and *H. virescens* (49%) (DANTZGER *et al*., 2015). A inibição de proteases intestinais dos insetos pode causar diversos efeitos deletérios,

incluindo retardo no desenvolvimento e aumento da mortalidade, além de ocasionar deformações e reduzir a fecundidade e fertilidade (PANDEY; JAMAL, 2014; EL-LATIF, 2014; DANTZGER *et al.*, 2015). O atraso no desenvolvimento e o aumento da mortalidade parecem estar relacionados com a depleção de aminoácidos essenciais.

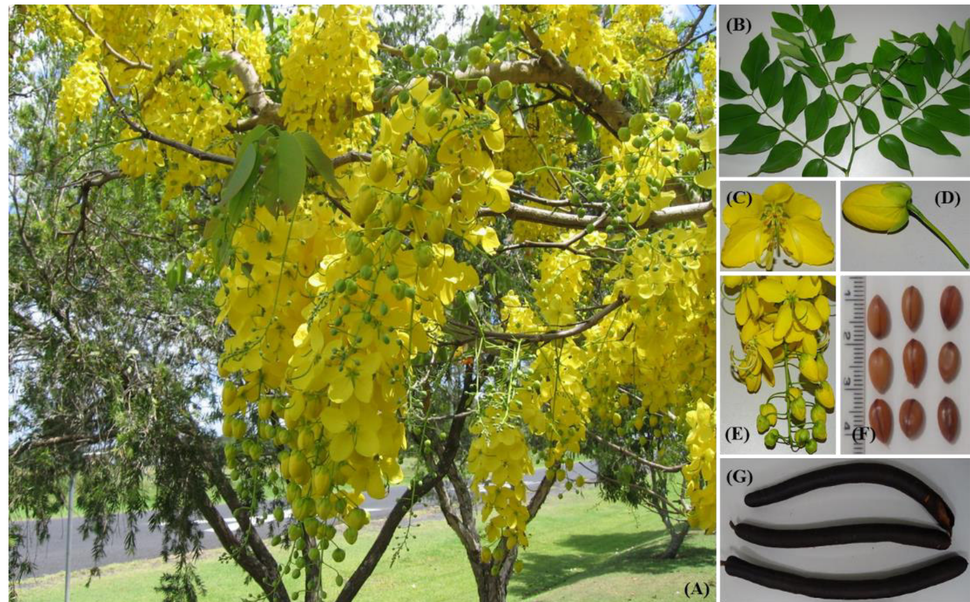
O efeito tóxico dos inibidores de proteases para insetos não se restringe apenas à inibição de suas proteases intestinais; estas moléculas podem apresentar domínios funcionais, que geram sinais distintos, capazes de induzir a morte de insetos (SUMIKAWA *et al*., 2010; ZHU-SALZMAN; ZENG, 2015). O inibidor de tripsina purificado das sementes de *Adenanthera pavonina* inibiu as proteases intestinais das larvas de *Ae. aegypti*. As larvas expostas a esse inibidor apresentaram redução no peso e na sobrevivência. Adicionalmente, as larvas apresentaram degeneração das microvilosidades das células epiteliais na região posterior do intestino e aumento no espaço ectoperitrófico (SASAKI *et al.*, 2015). De maneira similar, o inibidor de tripsina purificado das flores de *Moringa oleifera* também apresentou efeitos tóxicos para larvas de *Ae. aegypti*, retardando seu desenvolvimento (PONTUAL *et al.*, 2014).

2.5 *Cassia leiandra*: uma espécie nativa da Amazônia com potencial biotecnológico inexplorado

C. leiandra (FIGURA 4) é uma espécie nativa da Amazônia, encontrada principalmente nos estados do Pará e Amazonas. É uma leguminosa pertencente à subfamília Caesalpinoideae, apresentando altura média de 4-8 metros e copa ampla, porém muito rala. Seu tronco é geralmente tortuoso, com casca quase lisa e fina. Suas inflorescências são dispostas em rácemos terminais pendentes, com flores amarelas muito vistosas e seu fruto é do tipo legume lenhoso, cilíndrico, com muitas sementes imersas numa polpa suculenta e agridoce (CAVALCANTE, 1976; VIANA *et al*., 2011).

Para nosso conhecimento, não há na literatura estudos envolvendo proteínas purificadas de *C. leiandra.* Porém, estudos preliminares desenvolvidos por nosso grupo de pesquisa mostraram que o extrato proteico das sementes de *C. leiandra* apresentava atividade inibitória de tripsina. Diante disso, e das várias outras informações apresentadas nessa Revisão de Literatura, estudos, como o que está aqui descrito, se justificam, face à contribuição a ser dada ao conhecimento do potencial biotecnológico de espécies nativas da flora brasileira, particularmente se considerada a utilização de suas biomoléculas como ferramentas alternativas para controle do *Ae. aegypti*, um vetor de múltiplas doenças.

Figura 4 - *Cassia leiandra*



Fonte: Próprio autor.

(A) Árvores com cachos de flores pendentes, característicos da espécie. (B) Folhas. (C) Flor. (D) Botão floral. (E) Inflorescência. (F) Sementes. (G) Vagem.

3 OBJETIVOS

3.1 Objetivo geral

Purificar e caracterizar bioquimicamente um inibidor de tripsina de sementes de *Cassia leiandra* e avaliar sua ação inseticida contra *Aedes aegypti*.

3.2 Objetivos específicos

- Estabelecer um procedimento experimental que resulte na obtenção do inibidor de tripsina puro, presente em sementes de *C. leiandra* (*Cl*TI);
- Caracterizar o *Cl*TI quanto à massa molecular, número de subunidades, presença de carboidratos em sua estrutura, sequência aminoacídica, composição de estruturas secundárias e estabilidade estrutural;
- Definir as propriedades cinéticas do *Cl*TI, com ênfase na determinação de sua especificidade enzimática, mecanismo e constante de inibição e estabilidade da atividade inibitória,
- Avaliar a capacidade do *Cl*TI de inibir, *in vitro*, proteases intestinais do *Ae. aegypti*;
- Avaliar os efeitos do *Cl*TI na eclosão dos ovos, bem como na sobrevivência e desenvolvimento larval do *Ae. aegypti*.

4 ARTIGO DA TESE

*Cl*TI, a trypsin inhibitor purified from *Cassia leiandra* seeds, has insecticidal activity against *Aedes aegypti* and potential to control the insect population

*Cl*TI, a trypsin inhibitor purified from *Cassia leiandra* seeds, has insecticidal activity against *Aedes aegypti* and potential to control the insect population

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Abstract

A trypsin inhibitor from *C. leiandra* seeds, named *Cl*TI, was purified, characterized, and evaluated for its insecticidal activity. *Cl*TI was purified by DEAE-Cellulose and trypsin-Sepharose 4B chromatography, with a 15.5-fold purification and 2.4% yield. *Cl*TI is composed of one polypeptide chain of 19,484 Da, as revealed by ESI mass spectrometry, is non-glycosylated, and comprises 35% β-sheets, 14% β-turns, and 50% disordered structures. *Cl*TI is an uncompetitive inhibitor of bovine trypsin (IC_{50} of 33.81 x 10^{-8} M), with a Ki of 6.25 x 10^{-8} M, and has an amino acid sequence similar to other inhibitors of the Kunitz-type family. *Cl*TI was stable over a broad range of pH (2.2–10.0) and temperature (30–70 °C), but DTT incubation caused a partial loss of inhibitory activity. *Cl*TI (4.65 x 10^{-6} M) promoted 50% activity reduction of *Ae. aegypti* midgut proteases, showed a dose-dependent acute toxicity on *Ae. aegypti* 3rd instar larvae, with an LC_{50} of 2.28 x 10^{-2} M, and after ten days of exposure, caused a 24-h delay of larval development and 44% mortality. These results suggest that *Cl*TI has potential to be utilized as a control strategy for *Ae. Aegypti*, a vector of several diseases.

Keywords: *Cassia leiandra*, protease inhibitor, Kunitz inhibitor, insecticidal activity, *Aedes aegypti*, biocontrol

1. Introduction

The World Health Organization (WHO) considers dengue virus infection one of the most important public health problems. Studies estimate that the disease affects approximately 390 million people annually and more than 90 million of these cases are serious. To further aggravate the problem, approximately 3.9 billion people in 128 countries live in risk areas of dengue infection [1,2]. *Aedes aegypti* (Diptera: Culicidae), a blood-sucking insect that lives and cohabits with the human population in tropical and subtropical areas, is the principal vector responsible for dengue virus transmission [3]. *Ae. aegypti* is also the main transmitter of Chikungunya and Zika viruses. Chikungunya has been identified in more than 60 countries. Its symptoms are sometimes milder than those of the dengue, and it is rarely fatal. Zika virus has been reported in 65 countries, and it is estimated that by the end of 2016, approximately 4 million people in the Americas will be infected, with 1.5 million cases in Brazil. Presently, the major concern is the association between Zika virus infection and microcephaly and Guillain-Barré syndrome [2].

As there are no antiviral drugs or vaccines available to prevent transmission and no specific medicine to treat patients with the viruses transmitted by *Ae. aegypti*, the main strategy for disease control is to combat the vector through improvement of basic sanitation and environmental practices to eliminate all sites where mosquitoes can lay eggs [4]. To eliminate dengue mosquitoes, chemical control with insecticides (organophosphates, carbamates, and pyrethroids) is widely used. However, prolonged and indiscriminate application of these compounds has favored the appearance of resistant insects [5]. This issue has driven the search for natural compounds with insecticidal activity, and plants are potential sources of such constituents. Indeed, the extracts and various compounds isolated from different plant parts have shown activity against *Ae. aegypti* [6-8].

Several plant proteins, including protease inhibitors (PIs), are among these compounds with insecticidal activity. PIs are proteins or peptides present in all living beings that are capable of inhibiting the catalytic action of proteases through the formation of stoichiometric complexes with their target enzymes, blocking or altering the active site [9]. PIs can form stable complexes with insect digestive proteases to decrease or block dietary protein digestion, which provides amino acids for larval growth and development [10]. Plant protease inhibitors (PPIs) are present in storage tissues as seeds and tubers and also in leaves, flowers, and fruits [11,12], where they function as storage proteins and/or endogenous regulators of proteolytic activity [13]. Additionally, PPIs are involved in programmed cell death [14] and other events related to the protection of plants against pests and pathogens [15]. These PPIs can be categorized as serine, cysteine, aspartic, and metalloproteinase inhibitors, according to their target proteases [16]. PPIs have also been grouped into families according to their sequence relationships and structural properties [17,18]. The Kunitz-type PPI family is one of the most studied and characterized. The Kunitz-type PPI family generally consists of a single polypeptide chain with a molecular mass of approximately 18–22 kDa and possesses two disulfide bridges and a single reactive site [19].

Several Kunitz inhibitors purified from legume seeds have insecticidal activity. Their mode of action involves interference with insect digestive processes by reducing digestion, decreasing nutrient assimilation, and impairing essential amino acid absorption [20-22]. PPIs do inhibit *in vitro* the hydrolytic activity of *Ae. aegypti* midgut proteases [23]. This present study reports the purification and biochemical characterization of a trypsin inhibitor (*Cl*TI) from the seeds of *Cassia leiandra* Benth. (Fabaceae, Caesalpinioideae), a plant species native to the Amazon rainforest. In addition, the *Cl*TI action on *Ae. aegypti* midgut proteases and its effect on the development and survival of this mosquito are assessed. The results suggest that *Cl*TI is

a strong candidate as a biologically active molecule to control *Ae. aegypti* and could be potentially exploited for future biotechnological applications.

2. Materials and methods

2.1. General

C. leiandra mature seeds were collected at the campus of the Federal University of Mato Grosso do Sul (UFMS), Ivinhema City, Mato Grosso do Sul State, Brazil (Authorization and Information System on Biodiversity - SISBIO # 47978-1). The plant species was identified by Professor Almeida G. M., UFMS. *Aedes aegypti* (Rockefeller strain) larvae and eggs were obtained from NUVET/SESA (Center for Vector Control –Healthy Secretary of Ceara, Brazil). Molecular mass markers, chromatographic matrices and immobilized pH gradient gel strips were obtained from GE Healthcare Life Science (New York, USA). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, USA).

2.2. Protein determination

The total protein concentration was estimated following the method described by Bradford [24]. Bovine serum albumin (BSA) was used as a protein standard.

2.3. Trypsin inhibitory activity assay

The inhibitory activity assay was performed according to Erlanger et al. [25] by measuring the residual hydrolytic activity of bovine trypsin on *N*-*α*-benzoyl-dl-arginine-*p*-nitroanilide (BApNA) as a substrate. Different sample concentrations, dissolved in 0.05 M Tris-HCl, pH 8.0, were incubated with 20 µL trypsin (0.3 mg mL^{-1} in 0.001 M HCl) at 37 ºC for 10 min. Then, 500 µL of 1.25 x 10^{-3} M BApNA (prepared in 100% dimethyl sulfoxide and 0.05

M Tris-HCl buffer, pH 8.0) was added to the mixture. After 15 min, the reaction was stopped by adding 250 µL of 30% (v/v) glacial acetic acid. BApNA hydrolysis was monitored at 410 nm. One trypsin inhibitor activity unit (TIU) was defined as a decrease of 0.01 units of absorbance at 410 nm in relation to the control sample (without inhibitor).

2.4. Purification of C. leiandra trypsin inhibitor (ClTI)

C. leiandra mature seeds were ground in a coffee mill to obtain a fine flour, which was defatted with *n*-hexane (1:10, m/v). The defatted flour was air-dried at room temperature (23 ± 2 °C) and stored at -20 °C until use. For protein extraction, the fine flour was brought into contact with 0.05 M sodium phosphate buffer, pH 7.5 (1:10, m/v) and was subjected to moderate stirring (3 h, 4 °C). The suspension was filtered through a fine-screen cloth, and the filtrate was centrifuged at 10,000*g* for 30 min at 4 °C. The precipitate was discarded, and the supernatant was dialyzed against 0.05 M sodium phosphate buffer, pH 7.5 and designated as the soluble protein extract (SPE). Ten milliliters of SPE (80 mg protein) was loaded on a DEAE-Cellulose column (1.5 x 16 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, at a 45 mL h^{-1} flow rate. The non-retained proteins were eluted with the equilibration buffer, and the fraction with trypsin inhibitory activity was eluted with 0.1 M NaCl prepared in the equilibration buffer. Fractions of 2 mL were collected, and the absorbance was read at 280 nm (Spectrophotometer Biochrom Libra S21, Cambridge, England). The fractions with antitrypsin activity were pooled, dialyzed against distilled water at 4 °C, and freeze-dried. The lyophilized sample (10 mg) was dissolved in 2 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. After centrifugation (10,000*g*, 4 °C, 15 min), the resulting supernatant was applied to a trypsin-Sepharose 4B column (2.5 x 5 cm) that was pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl, at a 45 mL h^{-1} flow rate. Protein fractions of 2 mL were collected, and the absorbance was read at 280 nm, as above. The non-retained

proteins were eluted with equilibration buffer. The fractions containing trypsin inhibitor were eluted with 0.1 M HCl, pooled, dialyzed against distilled water at 4 °C, and lyophilized for further analysis. The purified trypsin inhibitor was named *Cl*TI (*C. leiandra* trypsin inhibitor).

2.5. Characterization of *Cl*TI

2.5.1. Molecular mass determination

*Cl*TI was subjected to polyacrylamide gel electrophoresis (PAGE) in the absence (native-PAGE) and presence of sodium dodecyl sulfate (SDS-PAGE) in a vertical system [26]. The samples (10 µg) were heated at 100 °C for 10 min in the presence or absence of 5% (v/v) β-mercaptoethanol and loaded on 12.5% (m/v) polyacrylamide gel (8.5 x 8.0 cm) that was prepared in 0.025 M Tris-HCl buffer, pH 8.9, containing 1% SDS (only in the SDS gel), at a constant 20 mA current. Protein bands were stained with 0.1% (m/v) Coomassie Brilliant Blue R-250. Phosphorylase B (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.2 kDa) were used as molecular mass markers.

The native molecular mass of *Cl*TI was determined using a HiLoad® 16/600 Superdex® 75 pg column (1.6 × 60 cm) that was pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.4 M NaCl. *Cl*TI (1 mg) was solubilized in the column equilibration buffer, and the protein was eluted at a 0.5 mL min^{-1} flow rate. The elution profile was monitored at 280 nm. Alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), and cytochrome c (12.4 kDa) were used as molecular mass standards.

The intact mass of *Cl*TI (1 mg mL^{-1} in water/acetonitrile [1:1, v/v]) was determined using a Synapt G1 HDMS Acquity UPLC instrument (Waters Co.). The spectrometer was

operated in the 'V' mode at a minimum resolution of 12,000 *m/z*. The analysis was performed using nanoelectrospray ionization in positive ion mode (ESI+) and a NanoLockSpray source. Data were collected, processed, and analyzed using MassLynx 4.1 and 2.4 ProteinLynx software (Waters Co.).

2.5.2. Reverse zymography

Visualization of the in-gel inhibition profile of *Cl*TI was performed according to Prasad [27]. *Cl*TI (50 µg) was applied to 12.5% (m/v) SDS-PAGE containing 0.1% (m/v) gelatin. After the electrophoretic run, the gel was incubated in 2.5% (v/v) Triton X-100 for 20 min at 37 °C, washed with distilled water, and incubated with a trypsin solution (0.06 mg mL^{-1} in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.02 M $CaCl_2$) for 2 h at 37 °C. Staining was performed with a solution containing 0.1% (m/v) Coomassie Brilliant Blue R-250 in methanol, acetic acid and water (4:1:5 v/v/v) for 4 h. Excess dye was removed from the gel using a solution of methanol, acetic acid, and water (4:1:5, v/v/v). Kunitz soybean trypsin inhibitor (SBTI) was also analyzed under the same conditions.

2.5.3. N-terminal amino acid sequencing

N-terminal amino acid sequencing of *Cl*TI was performed on a PPSQ 23A automated protein sequencer (Shimadzu) by Edman degradation. The phenylthiohydantoin-amino acid derivatives were detected at 269 nm after separation on a reversed-phase C18 column (4.6 x 2.5 mm) under isocratic conditions according to the manufacturer's instructions. The amino acid sequence was subjected to automatic alignment using the NCBI-BLAST system [28].

2.5.4. Mass spectrometry analysis

Capillary liquid chromatography/nanoelectrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was performed using a Synapt G1 HDMS Q-ToF mass spectrometer (Waters Co.) coupled to a Waters ultra-high-performance liquid chromatography (UPLC) unit. *Cl*TI (50 µg) was digested with 1 µg trypsin (Promega®) at 37 °C for 16 h. The tryptic peptides were separated using a nanoACQUITY UPLC System (Waters) equipped with a C18 HSS T3 reversed-phase column. After elution, the peptides were ionized in a nanoESI source through a one-spray cone-shaped 22-µm silica capillary. The spectrometer was operated under the same conditions described in *Section 2.5.1*. The data were processed using Protein Lynx Global Server software (Waters Co.) and subjected to a database search using the Mascot search engine [29]. The searches were performed with the assumptions that there was a maximum of one missed trypsin cleavage and that the experimental masses of the peptides were monoisotopic. Furthermore, carbamidomethylation of cysteine was included as a fixed modification, and oxidation of methionine was included as a variable modification. MS/MS ion searches were performed against the NCBI non-redundant database (last accessed on September 04, 2016) using a significance threshold of $P < 0.05$. The peptide mass tolerance and fragment mass tolerance were both initially set to ± 0.1 Da for MS/MS ion searching. Nevertheless, candidate peptide IDs were only accepted if the m/z values were within 0.1 Da (typically less than 0.05 Da) of the theoretical mass of the candidate ID, as determined by manually reviewing the MASCOT search results.

2.5.5. Circular dichroism (CD) measurement

CD spectra measurements were recorded on a JASCO J-715 spectropolarimeter (Jasco Instruments) under a nitrogen atmosphere at 25 °C. *Cl*TI (50 µg) was solubilized in 500 µL 0.01 M sodium phosphate buffer, pH 7.5, and subjected to analysis. Measurements were recorded as an average of 8 successive scans performed at a 20 nm min^{-1} scan rate with 4 s

response time in the 190–250 nm region using a circular quartz cuvette with a 1-mm optical path. Three methods were used to quantify the secondary structure elements using CDPro software (CDSSTR CONTINLL, and SELCON3). CD spectroscopy was also used to assess the *Cl*TI thermal stability. *Cl*TI (200 µg mL^{-1} in 0.01 M sodium phosphate buffer, pH 7.5) was incubated at temperatures ranging from 25 to 90 °C for 20 min in a TC-100 circulating water bath (Jasco Instruments), and the spectra were recorded using the above parameters. To evaluate the structural stability as a function of pH, *Cl*TI (200 µg mL^{-1}) was incubated for 30 min in 0.02 M acetate/phosphate/borate buffer at different pH values (2.5, 4.0, 6.0, 8.0 and 10.0) at room temperature (23 ± 2 °C) before recording the CD spectra.

2.5.6. Carbohydrate determination

The glycoprotein nature of *Cl*TI was evaluated by periodic acid-Schiff staining, as described previously [30]. Briefly, *Cl*TI (20 µg) electrophoresis was performed in SDS-PAGE, as above, and was then fixed in 7.5% (v/v) acetic acid solution for 2 h, immersed in 0.2% (v/v) periodic acid solution at 4 °C for 45 min, followed by immersion in the Schiff reagent at 4 °C for 45 min. To reveal the glycoprotein nature of the protein, the gel was immersed in a 0.5% (m/v) potassium metabisulfite solution prepared in 0.05 M HCl.

2.5.7. IC_{50} and kinetic analysis

The *Cl*TI concentration capable of reducing 50% of the trypsin activity (IC_{50}) was determined, as described in *Section 2.3*, using the inhibitor in the concentration range of 45.6 x 10^{-8} M to 4.5 x 10^{-8} M. The kinetic measurements of trypsin inhibition by *Cl*TI were conducted according to Costa et al. [31]. *Cl*TI was prepared in 0.05 M sodium phosphate buffer, pH 7.5, at different concentrations (9.12 x 10^{-8} M, 18.24 x 10^{-8} M, and 27.36 x 10^{-8} M) and was incubated with 20 µL trypsin (0.3 mg mL^{-1} in 0.001 M HCl) at 37 °C. The reaction was initiated

by adding 500 µL of BApNA at different concentrations (0.8 x 10^{-3} to 1.6 x 10^{-3} M) and was stopped after 15 min by the addition of 120 µL 30% (v/v) acetic acid. The liberated *p*-nitroaniline was measured at 410 nm. A Lineweaver–Burk plot was obtained by the reciprocal of the rate of the enzyme reaction (1/v) versus the reciprocal of the substrate concentration (1/[S]) in the absence and presence of *Cl*TI. The inhibition constant (Ki) was determined according to Dixon [32]. Ki was obtained by the intersection of the three lines at the x-axis, corresponding to the substrate concentrations (0.40 x 10^{-3} M, 0.80 x 10^{-3} M, and 1.25 x 10^{-3} M).

2.5.8. Enzyme specificity

The ability of *Cl*TI to inhibit chymotrypsin was evaluated according to Erlanger et al. [25] using azocasein as the substrate. Twenty microliters of bovine chymotrypsin (0.1 mg mL^{-1} in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.02 M $CaCl_2$) was incubated with 100 µL of *Cl*TI (10 µg mL^{-1}) for 15 min at 37 °C. A 200 µL aliquot of 1% (m/v) azocasein in 0.05 M Tris-HCl buffer, pH 7.5, was added, and after 30 min, the reaction was stopped by adding 300 µL of 20% (v/v) trichloroacetic acid. The mixture was centrifuged (10,000*g*, 10 min), aliquots were withdrawn from the supernatants, 2 M NaOH was added at a ratio of 1:1 (v/v) to intensify the color of the cleavage product, and the absorbance was measured at 440 nm. The papain inhibitory assay was performed as described by Abe et al. [33] using *N*α-benzoyl-DL-arginine β-naphthylamide hydrochloride (BANA) as the substrate. To 60 µL of papain solution (0.02 mg mL^{-1} in 0.25 M sodium phosphate buffer, pH 6.0), 40 µL of the activating solution (0.25 M sodium phosphate buffer, pH 6.0, 2 x 10^{-3} M ethylenediaminetetraacetic acid [EDTA], 3 x 10^{-3} M dithiothreitol [DTT]), 200 µL 0.25 M sodium phosphate buffer, pH 6.0, and 200 µL of *Cl*TI (10 µg/mL) were added. The mixture was incubated at 37 °C for 10 min, and 200 µL of 1 x 10^{-3} M BANA (solubilized in DMSO and 0.25 M sodium phosphate buffer, pH 6.0) were added. After 20 min, the reaction was stopped by the addition of 500 µL 2% (v/v) HCl in 95% (v/v)

ethanol. Next, 500 µL of 0.06% (m/v) 4-(dimethylamino)-cinnamaldehyde (DMACA, dissolved in 95% [v/v] ethanol) were added and incubated for 30 min, and the absorbance was measured at 540 nm. The porcine pancreatic α-amylase inhibition assay was performed using 3,5-dinitrosalicylic acid (DNS), as previously described [34]. The enzyme (1.0 mg) was solubilized in 0.02 M sodium phosphate buffer, pH 6.9, containing 0.006 M NaCl (0.1 mg mL^{-1}). The reaction medium was prepared by combining 20 µL of α-amylase, 60 µL of *Cl*TI (0.2 mg mL^{-1}), and 170 µL of the above buffer. The reaction mixture was incubated at 37 °C for 10 min, and the reaction was initiated by the addition of 250 µL of 1% (m/v) starch solution and was stopped after 15 min by the addition of 500 µL of DNS (1% in 1 M NaOH and 25% sodium potassium tartrate). The mixture was boiled in a water bath for 10 min, diluted with 2.5 mL of distilled water, and cooled to room temperature (23 ± 2 °C), and the absorbance was read at 540 nm. In all assays, the inhibitory activity was calculated by comparing the residual enzyme activity in the presence and absence (control) of *Cl*TI.

2.5.9. Stability of the inhibitory activity against bovine trypsin

The thermal stability of *Cl*TI was evaluated according to Jamal [35]. *Cl*TI (6 µg mL^{-1}) was dissolved in 0.05 M sodium phosphate buffer, pH 7.5, and 500 µL aliquots were incubated in a water bath at different temperatures (30, 40, 50, 60, 70, 80, 90, and 100 °C) for 20 min. The samples were cooled to room temperature (23 ± 2 °C) before testing the residual inhibitory activity. The *Cl*TI stability at different pH was evaluated according to Klomklao et al. [36]. *Cl*TI (6 µg mL^{-1}) was dissolved in different buffers: 0.05 M glycine-HCl, pH 2.2; 0.05 M sodium acetate, pH 5.2; 0.1 M sodium phosphate, pH 7.8; 0.1 M Tris-HCl, pH 8.0, or 0.1 M borate, pH 10.0. After 30 min incubation in each buffer at room temperature (23 ± 2 °C), the residual trypsin inhibitory activity was evaluated, as previously described. The effect of reducing agent on the activity of the trypsin inhibitor was evaluated according to Bezerra et al.

[6]. *Cl*TI samples (6 mg mL^{-1}) were incubated with different DTT concentrations (0.001 to 0.1 M) for different amounts of time (15, 30, 60, and 120 min). The reaction was terminated by adding iodoacetamide at twice the DTT concentration, and the residual inhibitory activity was determined.

2.6. Activity of ClTI against Ae. aegypti

2.6.1. Mosquitoes

Ae. aegypti larvae and eggs were maintained at 27 ± 2 ºC, 70 ± 10% relative humidity, and 12-h light and 12-h dark photoperiod, and were fed a diet for turtles (ReptoLife Alcon Club®). Third instar larvae were collected with a Pasteur pipette and transferred to a beaker containing distilled water until the bioassays were conducted.

2.6.2. Inhibition assay of Ae. aegypti gut proteases

Homogenates of the larval midgut were prepared according to Almeida Filho et al. [37], with minor modifications. For the assay, *Ae. aegypti* larvae were cold-immobilized, and their midguts were surgically removed, macerated in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$, and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatants were used immediately as the source of proteases. Aliquots (60 µL) of the supernatant were incubated with 100 µL of *Cl*TI (100 µg mL^{-1}) and 640 µL of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ for 10 min at 37 °C, and then 500 µL of 1.25 x 10^{-3} M BApNA was added. After 15 min, the reaction was stopped by adding 120 µL of 30% (v/v) acetic acid, and the absorbance was measured at 410 nm. The activity of midgut samples without *Cl*TI was considered as 100% activity. To determine the *Cl*TI concentration (IC_{50}) required to reduce 50% of the activity of the intestinal proteases of *Ae. Aegypti*, the assay was performed under the same conditions

described above, using increasing concentrations of *Cl*TI (25–175 μg mL^{-1} or 1.28 x 10^{-3}–8.98 x 10^{-3} M).

2.6.3. Egg hatching and larval development assay

The effect of *Cl*TI on the hatching of *Ae. aegypti* eggs was assessed as described by Almeida Filho et al. [37]. Ten *Ae. aegypti* eggs were placed in plastic tubes containing 5 mL of 1.54 x 10^{-5} M *Cl*TI in distilled water (0.3 mg mL^{-1}), with 5 replicates. BSA and distilled water were also tested under the same conditions. After 48 h incubation, the hatched larva number in each treatment was recorded. The number of individuals at the different stages of development and the survival rate were monitored for 10 days. To prevent microbial growth, larvae were transferred to a new solution containing turtle feed (0.2 mg per larva) every 48 h. The experimental results were taken as the average of three independent bioassays.

2.6.4. LC_{50} Bioassay

The median lethal concentration (LC_{50}) was determined as previously described [38,39]. *Ae. aegypti* larvae (3rd instar) were exposed to different concentrations (1000–31 μg mL^{-1} or 5.13 x 10^{-2} M–1.60 x 10^{-3} M) of *Cl*TI for 48 h, with 5 replicates (n = 50). BSA and water were tested under the same conditions.

2.7. Statistical analysis

All analyses were conducted at least in triplicate and were reported as the means with the standard deviation. For each set of results, analysis of variance (ANOVA) was applied, followed by the Dunnett or Tukey test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification of ClTI

The SPE obtained from *C. leiandra* seeds presented trypsin inhibitory activity (specific activity 15.40 TIU mg^{-1} protein). Purification of *Cl*TI from the SPE required two chromatography steps. SPE was separated into three protein fractions after DEAE-Cellulose, one of which, eluted with 0.1 M NaCl (Fig. 1A), displayed inhibitory activity against trypsin. At this stage, the trypsin inhibitor was purified to 6.0-fold with a protein yield of 8.2% and specific activity of 93.1 TIU mg^{-1} protein (Table 1). This fraction was further purified by trypsin-Sepharose 4B affinity chromatography, and the unique retained fraction (Fig. 1B), which exhibited antitryptic activity, presented a single protein band in the native-PAGE (Fig. 2A), as well as when examined by SDS-PAGE under non-reducing conditions in the presence of 5% β-mercaptoethanol (Fig. 2B). A single antitryptic activity band was also visualized by reverse zymography, which showed a dark blue band on a light background (Fig. 2C). The purity of *Cl*TI was also evaluated using a HiLoad 16/600 Superdex 75 pg column, which produced a prominent single peak (Fig. 1B, inset). The purification strategy adopted for *Cl*TI resulted in 15.5-fold purification and 2.4% protein yield (Table 1).

3.2. Characterization of ClTI

3.2.1. Molecular mass and assessment of the glycoprotein nature

SDS-PAGE (12.5%) showed that *Cl*TI has an apparent molecular mass of 20,000 Da, regardless of the presence of β-mercaptoethanol (Fig. 2B). Native gel filtration chromatography of *Cl*TI using a HiLoad 16/600 Superdex 75 pg column gave a molecular mass of 21,900 Da (Fig. 1B, inset). ESI mass spectrometry under native conditions revealed a major peak at 19,484 Da (Fig. 3), which is very close to the molecular mass of *Cl*TI found by SDS-PAGE and gel

filtration. *Cl*TI was not stained by periodate-Schiff's reagent on SDS-PAGE, suggesting that it is a non-glycosylated protein.

3.2.2. Protein identification

The 40 N-terminal amino acid sequence of *Cl*TI was identified as SVELDSDGEPIRNGGGLYYILPVVQGKGGGLELAKTGSQS (UniProt accession number C0HK48). Alignment of this N-terminal sequence with known sequences deposited in the NCBI non-redundant database showed that *Cl*TI has identity with trypsin inhibitors purified from the *Caesalpinioideae*, *Papilionoideae*, and *Mimosoideae* subfamilies (Table 2). The highest degree of N-terminal sequence identity was found with Kunitz-type inhibitors from *Prosopis juliflora* (73%), *Enterolobium contortisiliquum* (68%), *Copaifera langsdorffii* (65%), and *Acacia confusa* (65%). In addition, peptide sequences were obtained by mass spectrometry analysis, which confirmed the identity of *Cl*TI with trypsin inhibitors (Table 3).

3.2.3. Secondary structure content

The CD spectrum of *Cl*TI in 0.01 M sodium phosphate buffer, pH 7.5, at room temperature, exhibited a very weak positive ellipticity maximum at 227 nm and a strong negative minimum at 197.2 nm (Fig. 4). Deconvolution of this CD spectrum using the CDPro program (CDSSTR method) showed that *Cl*TI is composed of 35% β-sheets, 14% β-turns, and 50% disordered structures.

The conformational stability of *Cl*TI to pH and temperature variation was investigated by CD spectra analysis. Heating at different temperatures resulted in a progressive loss of the conformational pattern of *Cl*TI (Fig. 8B). Temperatures greater than 70 °C for 20 min caused complete loss of the secondary structure. These conformational changes were reverted when

*Cl*TI was cooled to 25 °C from 90 °C. However, incubation of *Cl*TI at pH extremes (2.5 and 10.0) did not cause significant changes in its secondary structure (Fig. 4C).

3.2.4. Enzyme specificity, IC_{50}, inhibition mechanism and Ki determination

*Cl*TI specifically inhibited bovine trypsin, and did not show inhibitory activity to chymotrypsin, papain, and porcine pancreatic α-amylase. The IC_{50} of *Cl*TI on trypsin was 33.81 x 10^{-8} M (Fig. 5A). The Lineweaver–Burk (Fig. 5B) and Dixon (Fig. 5B, inset) diagrams revealed that *Cl*TI is an uncompetitive inhibitor, with a Ki of 6.25 x 10^{-8} M.

3.2.5. Temperature, pH, and DTT stability

The ability of *Cl*TI to inhibit trypsin was stable up to 70 °C, but higher temperatures caused steady decreases in the inhibitory activity (Fig. 6A). At 100 °C for 20 min, approximately 48% of the activity was lost. However, the trypsin inhibitory activity remained constant in the pH range of 2.2 to 10.0 (Fig. 6B). The effect of DTT (0.001 M, 0.01 M, and 0.1 M) on the *Cl*TI activity was studied (Fig. 6C). The *Cl*TI activity decreased with increasing DTT concentration. When incubated with 0.1 M DTT for 2 h, a loss of 40% of the inhibitory activity of *Cl*TI was observed.

3.3. Insecticidal activity of ClTI against Ae. aegypti

Increased *Cl*TI concentrations promoted gradual inhibition of *Ae. aegypti* midgut digestive proteases, reaching 50% reduction at 4.65 x 10^{-6} M (Fig. 7). Based on this result, the bioinsecticidal potential of *Cl*TI on *Ae. aegypti* egg hatching and larval development was evaluated 10 days after 48 h egg exposure to this protein (chronic test). *Cl*TI at a final concentration of 1.54 x 10^{-5} M (0.3 mg mL^{-1}) did not inhibit egg hatching. However, *Ae. aegypti*

larvae were negatively affected; they did not develop properly or survive longer then controls (Table 4). *Cl*TI delayed adult emergence by 24 h and caused 44% mortality.

Acute toxicity of *Cl*TI on 3rdinstar *Ae. aegypti* larvae was dose-dependent, with an LC_{50} value of 445 µg mL^{-1}, which is equivalent to 2.28 x 10^{-2} M (Fig. 8).

4. Discussion

Amazonian traditional communities use the leaves, fruits and entire *C. leiandra* plants for human and animal feed, medicinal and ornamental purposes, and as fishing lure [40]. However, its potential as a source of new molecules of biotechnological interest has not been exploited, especially with regard to bioactive proteins, such as PIs. In this study, a new trypsin inhibitor, named *Cl*TI, was purified from *C. leiandra* seeds, its biochemical characterization was conducted, and its potential insecticidal activity toward *Ae. aegypti* was evaluated.

*Cl*TI was purified by anion exchange chromatography, followed by affinity chromatography. These two steps constituted a simple and effective purification strategy, which achieved a 2.4% protein yield (Table 1). The *Cl*TI recovery was 15 to 16 times higher than recorded for *Trigonella foenum-graecum* (0.16%) [41] and *Piptadenia moniliformis* (0.15%) [42] trypsin inhibitors but similar to that of *Vigna radiata* (2.2%) [36]. PIs represent approximately 1% to 10% of the constitutive proteins in plants and are consistently more abundant in seeds than in tubers, leaves, and flowers [13]. The *de novo* PI synthesis can be induced by jasmonates, which are hormone signals involved in plant defense against herbivores, in addition to mechanical wounding [43,44].

The molecular mass of *Cl*TI was assessed by mass spectrometry (19,484 Da) (Fig. 3), SDS-PAGE (20,000 Da) (Fig. 2B), and size exclusion chromatography (21,900 Da) (Fig. 1, inset), which produced similar results. *Cl*TI is composed of a single polypeptide chain (Fig. 2A,

B and C). The trypsin inhibitors from *Dimorphandra mollis* [45] and *Senna tora* [46] have a molecular mass of 20,000 Da and are also composed of a single polypeptide chain. Thus, based on these biochemical characteristics, the properties of *Cl*TI are similar to those of other classic PIs purified from plants belonging to the Caesalpinioideae subfamily. Further, *Cl*TI has no covalently linked carbohydrates, as indicated by the periodic acid-Schiff staining assay. Indeed, most trypsin inhibitors are non-glycoproteins, such as those from *Sapindus trifoliatus* seeds [47] and *R. communis* cake [23]. However, some Kunitz-type inhibitors are glycoproteins, such as those from *Peltophorum dubium*, *Acacia victoriae,* and *Solanum tuberosum* [48-50].

After the tryptic digestion of *Cl*TI, the amino acid sequences of seven derived peptides were identified. One of these sequences overlapped with the *Cl*TI N-terminal amino acid sequence determined by Edman degradation. Through these analyses, 103 amino acid residues were identified, corresponding to 58% of the entire *Cl*TI primary sequence. Alignment of the amino acid sequence of *Cl*TI with other proteins revealed strong identity with Kunitz-type PIs present in species of all the subfamilies in the Fabaceae family, particularly those belonging to the Mimosoideae subfamily (Table 2). Different PPI types have evolved during the evolution of the Fabaceae family, and the Kunitz-type PIs are mainly present in the species of the most primitive Caesalpinioideae and Mimosoideae subfamilies, whereas species of the Papilionoideae subfamily, the most advanced, typically contain Bowman-Birk inhibitors [42]. Thus, PIs from the most primitive subfamilies might be closely related. In addition, *Cl*TI has an asparagine residue at position 13 (Asn^{13}), which is conserved among members of the Kunitz-type inhibitor family and is crucial to the hydrogen bond stabilization of the primary binding loop and contributes to the inhibitory activity [51]. These findings suggest that *Cl*TI is a new member of the Kunitz-type inhibitors.

*Cl*TI presents disordered structures and β-sheets but no α-helix structure (Fig. 4A). Kunitz-type inhibitors typically possess few α-helix structures and 12 antiparallel β-strands

connected by long loops [18]. Disordered structures may provide flexibility to certain inhibitors to inhibit enzymes of different classes [41]. For example, some PIs are highly specific for trypsin [52], but others inhibit trypsin and chymotrypsin [41,53] and even different protease classes, such as trypsin and papain [42,54]. *Cl*TI promoted the uncompetitive inhibition of bovine trypsin (Fig. 5B) but was inactive against chymotrypsin, papain, and α-amylase.

Uncompetitive inhibition is unusual among Kunitz-type inhibitors, although it was previously reported for the trypsin inhibitor from *Moringa oleifera* leaves [55]. *Cl*TI had a Ki of 6.25 x 10^{-8} M (Fig. 5C), similar in order of magnitude to those determined for the *Albizia amara* (1.24 x 10^{-8} M) [56] and *Ricinus communis* (1.90 x 10^{-8} M) [23] inhibitors. The Ki is an important parameter to indicate the degree of interaction between an enzyme and its corresponding inhibitor [55]. The low Ki value of *Cl*TI indicates its high affinity to trypsin. Indeed, the IC_{50} value of *Cl*TI (33.81 x 10^{-8} M) (Fig. 5A) for trypsin was lower than that (60.00 x 10^{-8} M) calculated for the *M. oleifera* inhibitor [55].

Generally, Kunitz-type trypsin inhibitors maintain their activity after exposure to high temperatures and large pH variation. *Cl*TI was heat stable (Fig. 6A), similarly to the trypsin inhibitors from *Poincianella pyramidalis* [57] and *Adenanthera pavonina* [58] seeds, which maintained their inhibitory activity after incubation in wide temperature range (37–70 °C and 25–70 °C, respectively) for 30 min. Moreover, a trypsin inhibitor obtained from *Pithecellobium dumosum* seeds maintained approximately 90% of its activity after exposure to temperatures varying from 37 to 100 °C for 30 min. [59]. The high thermal stability of trypsin inhibitors from species of the Fabaceae family is associated with the presence of intramolecular disulfide bridges. Kunitz-type inhibitors have four cysteine residues involved in two disulfide bridges [17]. Oliva et al. [22] reported that the most important disulfide bridge for the stability of Kunitz-type inhibitors is that established between cysteine residues 39 and 86, which is also responsible for stabilizing the loop involved with the inhibitor reactive site. In addition, *Cl*TI

was also stable over a wide range of pH (Fig. 6B), similarly to trypsin inhibitors from *P. dumosum* [59] and *Entada acaciifolia* [60] seeds, which maintained their functional stability at extreme pH conditions. In general, PPIs remain active in a wide pH range (2.0–10.0). Maintenance of the functional stability of trypsin inhibitors after exposure to a large range of temperature and pH might be associated with the rigidity of their tridimensional structure, which allows only slight conformational changes under adverse conditions and/or their capacity to renature as soon as they are brought to appropriate physiological conditions. Nevertheless, such stability is an important feature of bioactive molecules with the potential to be employed in biotechnology-based processes [55].

The conformational stability of *Cl*TI was confirmed by circular dichroism (Fig. 4B and C), which showed only slight changes in its tridimensional structure when the inhibitor was incubated at extreme pH (2.5 and 10.0). A similar result was reported for the *Cajanus cajan* trypsin inhibitor, which also showed small changes in its CD spectra when incubated at extreme pH [61]. In contrast, significant changes in the spectra were observed when *Cl*TI was subjected to thermal treatment. Alterations in the secondary structure of *Cl*TI were detected at 70 °C and became more evident as the temperature increased until complete loss of the structural pattern at 90 °C. The inhibitory activity of *Cl*TI was recovered after heat treatment, which suggested that it has the ability to renature, within a given limit, under the physiological conditions of the assay.

The inhibitory activity of *Cl*TI was also evaluated after incubation with DTT. The partial loss of inhibitory activity of *Cl*TI after incubation with 0.1 M DTT for 120 min (Fig. 6C) suggests the importance of the disulfide bridges for the maintenance of its structure and functionality, a characteristic presented by other PPIs. For instance, incubation of the trypsin inhibitor of *Inga vera* with 0.1 M DTT for 120 min reduced its inhibitory activity by approximately 30% [6]. A trypsin inhibitor from *P. dubium* lost 90% of its activity after 45 min

of 0.1 M DTT incubation [48]. Most Kunitz inhibitors have two disulfide bridges that are accessible to reducing agents, such as DTT. However, these bridges, in some cases, are not directly involved in the functionality of trypsin inhibitors, as the reactive site structures are maintained predominantly by weak interactions, such as hydrogen bonds, Van der Waals forces, and hydrophobic interactions. In contrast, the loss of activity of some inhibitors by reducing agents indicates that the disulfide bridges might, in this case, be involved in the maintenance of their reactive site architecture and are thus fundamental to the preservation of the structural conformation and functionality [62,63].

Most PIs were found to be able to bind to the gut proteases of insects of different orders [64-66]. Trypsin inhibitors from *I. vera* [6], *P. moniliformis* [42], and *Clitoria fairchildiana* [65] inhibited the proteases of coleopterans, lepidopterans, and/or dipterans and caused various deleterious effects, such as retarded growth and development, increased mortality, and deformations. *Cl*TI inhibited the gut proteases of *Ae. aegypti* [Fig. 7], as did the *R. communis* inhibitor [23]. Trypsin and chymotrypsin are the main enzymes involved in the dietary protein digestion by *Ae. aegypti* throughout the entire insect developmental stages. Both enzymes are very active during the larval stage, but trypsin is the most abundant [67,68]. Because *Cl*TI is a trypsin inhibitor, interaction with *Ae. aegypti* intestinal proteases might act like an antimetabolic agent, which could interfere with insect physiology and development. Chronic treatment of *Ae. aegypti* with *Cl*TI delayed larval development and increased mortality (Table 4), which might be related to the diminished uptake of dietary essential amino acids, resulting in starvation and death [69]. In addition to the protease inhibitor activity, the deleterious effect of PIs against insects is thought to come from other functional domains that can elicit signals leading to insect death [70]. Other inhibitors showed similar effects to *Cl*TI. *Ap*TI, a trypsin inhibitor from *A. pavonina* seeds, in addition to reducing the proteolytic activity of *Ae. aegypti* midgut enzymes, diminished larval weight and survival. Furthermore, larvae fed on *Ap*TI-containing diets

showed microvillus degeneration at the posterior region of the midgut epithelial cells, hypertrophy of the gastric caeca cells, and increased ectoperitrophic space [71]. The inhibitor of *M. oleifera* flowers delayed the development rate of *Ae. aegypti* first-instar larvae, although did not affect pupa survival [72]. Similar negative effects of PIs on the life cycle of herbivores belonging to different insect orders were previously reported. The *Cassia fistula* inhibitor (CFTI-1) not only prolonged the *Helicoverpa armigera* life cycle but also increased larval mortality and decreased larval weight [73]. *Ap*TI caused deleterious effects on the larvae and pupae of *Diatraea saccharalis* [74]. The *Bauhinia rufa* inhibitor negatively affected *Callosobruchus maculatus* development [70]. One strategy to decrease insect populations is the interruption or delay of its life cycle [75]. Within this context, and according to the findings described above and elsewhere, PIs represent promising tools for insect control [42], with the advantage of offering low potential risk for insect populations to evolve resistance to these natural insecticides [73].

5. Conclusions

In the present study, a novel trypsin inhibitor from *C. leiandra* seeds (*Cl*TI) was purified, characterized, and evaluated for its insecticidal activity. *Cl*TI showed similar structural features to Kunitz-type inhibitors and activity against *Ae. Aegypti* by inhibiting its midgut proteases and causing delayed larval development and mortality. Our results are an important contribution to a better understanding of the insecticidal activity of PIs, indicating that *Cl*TI has biotechnological potential as an alternative strategy to control this multiple disease vector, which may be used alone or in combination with other insecticidal compounds to produce enhanced toxicity.

Authors contributions

Study conception and design: LPD, JTAO, IMV. Acquisition of data: LPD, LCBRB, DOBS, HPSC, NMSA, PMST, MDPL, FBMBM, JLSL, IMV. Analysis and interpretation of data: LPD, JTAO, LCBRB, AFUC, ACOMM, BAMR, LMB, IMV, Drafting of manuscript: LPD, JTAO, AFUC, IMV. Critical revision: JTAO, IMV.

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Figure legends

Fig. 1. *Cl*TI purification. (A) Soluble protein extract (SPE, 80 mg protein) was loaded on a DEAE-cellulose column that was previously equilibrated with 0.05 M sodium phosphate, pH 7.5. The fraction with trypsin inhibitory activity was eluted with equilibration buffer containing 0.1 M NaCl. (B) The pooled fractions with antitrypsin activity (10 mg) were applied to a trypsin-Sepharose 4B column that was equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. *Cl*TI was eluted with 0.1 M HCl. In both chromatographic steps, 2 mL fractions were collected at a 45 mL h^{-1} flow rate. Inset: Gel filtration of *Cl*TI (1 mg) on a HiLoad® 16/600 Superdex 75 column, pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.4 M NaCl at a 0.5 mL min^{-1} flow rate, showing a single peak at 21,900 kDa. Horizontal bars indicate fractions exhibiting trypsin inhibitory activity

Fig. 2. Polyacrylamide gel electrophoresis (12.5%, m/v) and zymography of *Cl*TI. (A) Native-PAGE. (B) SDS-PAGE. Lane 1: Molecular mass markers; lanes 2 and 3: *Cl*TI (10 µg) in the absence or presence of 5% (v/v) β-mercaptoethanol. (C) lanes 4 and 5: Activity of SBTI (50 µg) and *Cl*TI (50 µg), respectively, on gelatin SDS-PAGE.

Fig. 3. Mass spectrometry of *Cl*TI. ESI-MS of *Cl*TI (1 mg mL^{-1} in water/acetonitrile [1:1, v/v]) under native conditions revealed a major peak at 19,484 Da.

Fig. 4. Circular dichroism spectra of *Cl*TI. Far-UV CD spectra of *Cl*TI (5.1×10^{-3} M) in 0.01 M sodium phosphate buffer, pH 7.5, using a circular quartz cuvette with a 1-mm optical path.

Fig. 5. Inhibition kinetics of *Cl*TI. (A) Effect of *Cl*TI against bovine trypsin. The IC_{50} of *Cl*TI on trypsin was 33.81 x 10^{-8} M. (B) Lineweaver–Burk plot analysis of the inhibition of trypsin by *Cl*TI. The parallel lines represent different *Cl*TI concentrations and are typical of uncompetitive inhibitors. (C) Dixon plot for the determination of the dissociation constant (Ki) of *Cl*TI at three BApNA concentrations. The Ki value for *Cl*TI was 6.25 x 10^{-8} M. The standard deviation was less than 10%.

Fig. 6. Stability of *Cl*TI. (A) Temperature stability of *Cl*TI after 20 min incubation at different temperatures. (B) pH stability of *Cl*TI after incubation at different pH for 30 min at 37 °C. (C) Residual trypsin inhibitory activity of *Cl*TI in the presence of different DTT concentrations and incubation times at 37 °C. Bars indicate the standard deviation from triplicate measurements.

Fig. 7. Inhibitory activity of *Cl*TI against *Ae. aegypti* midgut digestive proteases. Intestinal homogenates of *Ae. aegypti* were incubated with increasing concentrations of *Cl*TI. After 15 min incubation with BApNA, the reaction was stopped, and the inhibition percentage was calculated and compared with the activity of the homogenate in the absence of the inhibitor. The IC_{50} value for *Cl*TI was 4.65 x 10^{-6} M.

Fig. 8. Effect of *Cl*TI on *Ae. aegypti* survival. Larvae (3rd instar) were exposed to different concentrations (1000–31.0 µg mL^{-1} or 5.13 x 10^{-2} M–1.60 x 10^{-3} M) of *Cl*TI for 48 h. *Cl*TI presented an LC_{50} of 2.28 x 10^{-2} M. The values correspond to the mean of 5 replicates (n = 50). Different letters indicate significant differences ($P < 0.05$).

Table 1
Purification steps of *C. leiandra* trypsin inhibitor (*Cl*TI)

Steps	Total protein[a] (mg)	Total activity[b] (TIU)	Specific activity (TIU mg^{-1} protein)	Yield[c] (%)	Purification index[d]
Soluble protein extract (SPE)	1279.0	19701.0	15.4	100	1.0
DEAE-Cellulose	105.1	9780.0	93.1	8.2	6.0
Trypsin-Sepharose 4B	31.0	7404.0	239.0	2.4	15.5

Results are presented as the means of six similar runs.

[a]The total amount of protein recovered from 10 g of defatted flour from *C. leiandra* seeds.

[b]One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 unit of absorbance at 410 nm.

[c]The recovery of protein at each purification step (SPE, 100%).

[d]Purification index is calculated as the ratio between the specific activity obtained at each purification step and that of the SPE taken as 1.0.

Table 2
Identity of the N-terminal sequence of *Cl*TI with other protease inhibitors from *Fabaceae* seeds (NCBI databases)

Subfamily	Species	Sequence	Accession number (NCBI)	Identity (%)
	Cassia leiandra	01 SVELDSDGEPIRNGGGLYYILPVVQGKGGGLELAKTGSQS 40	C0HK48	
Caesalpinioideae	*Copaifera langsdorffii*	03 VDTDGKPIENDGAEYYILPSVRGKGGGLVLAKSG 36	1R8O_A	68
	Phanera variegata	04 LDTDGEVVRNNGGPYYIIPAFRGNGGGLTLTRVGSET 40	P83595.1	57
	Bauhinia rufa	01 SVVLDTKGQPVRNAADAYYLEPVARG-DGGLALAKVGNEA 39	P84882.1	50
	Bauhinia bauhinioides	01 SVILDTKGEPVSNAADAYYLVPVSHGE-GGLALAKVGNEA 39	P83051.2	50
Papilionoideae	*Glycine max*	27 IVFDTEGNPIRNGG-TYYVLPVIRGKGGGIEFAKTETET 64	NP_001236275.1	56
	Cajanus cajan	30 LDTDGKLLRNGGS-YYVVPVKRGSGGGIELAATGNET 65	KYP60026.1	57
	Erythrina caffra	01 VLLDGNGEVVQNGGT-YYLLPQVWAQGGGVQLAKTGEET 38	P09943.1	54
Mimosoidae	*Prosopis juliflora*	04 LDVDGEILRNGGS-YYILPAFRGKGGGLELAKT 35	P32733.1	76
	Enterolobium contortisiliquum	04 LDVDGEILRNGGS-YYILPAFRGKGGGLELAKT 35	P32733.1	70
	Acacia confusa	04 LDADGDILRNGGA-YYILPALRGKGGGLTLAKTGDES 39	AAB26177.1	68
	Adenanthera pavonina	04 LDVDGNFLRNGGS-YYIVPAFRGKGGGLELARTGSET 39	P09941.1	65

Conserved residues are shaded in gray.

Table 3
Amino acid sequences of tryptic peptides from *Cl*TI identified by LC-ESI-MS/MS

Peptide Sequence[1]	Mass (Da)		Species/trypsin inhibitor with identity	GenBank accession number	Identity (%)
	Experimental	Calculated			
$_{26}$GKGGGLELAK$_{35}$	928.5388	928.5342	*Enterolobium contortisiliquum*	ABQ42566	100
$_{162}$DGDPLAVRFVK$_{172}$	1215.6104	1215.6612	*Enterolobium contortisiliquum*	4J2K_A	100
$_{148}$VSDDEFNNYK$_{157}$	1229.5258	1229.5200	*Glycine max*	BAD04941.1	100
$_{13}$NGGLYYILPVFR$_{25}$	1410.5182	1410.7660	*Psophocarpus tetragonolobus*	P32877.1	88
$_{194}$SSNPLVVQFVK$_{205}$	1216.4697	1216.6815	*Cajanus cajan*	KYP60029.1	78
$_{1}$LPVFDSDGEPLR$_{12}$	1343.4414	1343.6721	*Solanum tuberosum*	AIT42210.1	77

[1]The numbers before and after each sequence indicate the predicted residue positions relative to Ser[1], which is the N-terminal residue of the mature *Cl*TI.

Table 4
Survival and development of *Ae. aegypti* larvae arising from eggs treated with *Cl*TI

Treatment	*Ae. Aegypti*							
	Total number of individuals 10 days after egg treatment	Number of individuals who reached each developmental stage[a]						Mortality (%)
		Larvae instar				Pupae	Mosquito	
		L1	L2	L3	L4			
BSA	39	0	0	0	2	20	17	22.0 ± 4.5[a]
*Cl*TI	28	0	0	3	8	9	8	44.0 ± 5.7[b]
Control	40	0	0	0	3	19	18	20.0 ± 6.9[a]

[a] Survival and development of *Ae. aegypti* were determined after ten days of egg exposure to *Cl*TI (1.54 x 10^{-5} M or 0.3 mg mL^{-1}, final concentration). The results are the means of three independent experiments. Different letters indicate significant differences ($P < 0.05$) based on the Tukey test.

Fig. 1

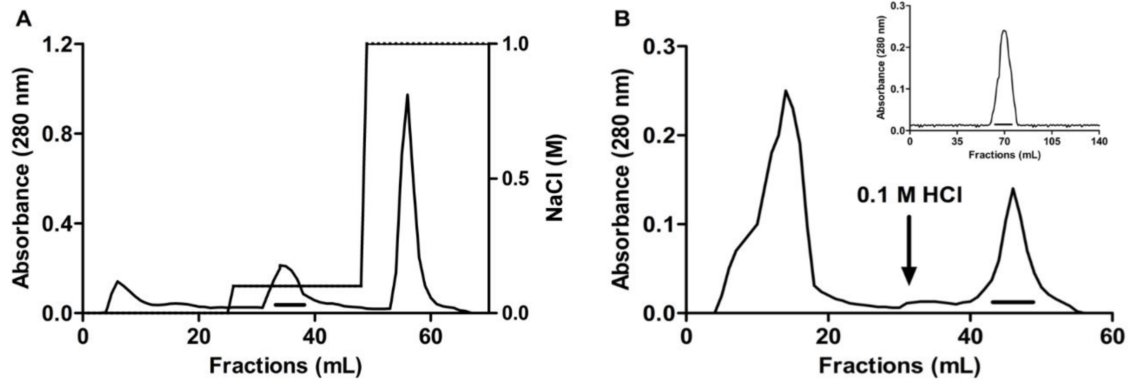


Fig. 2

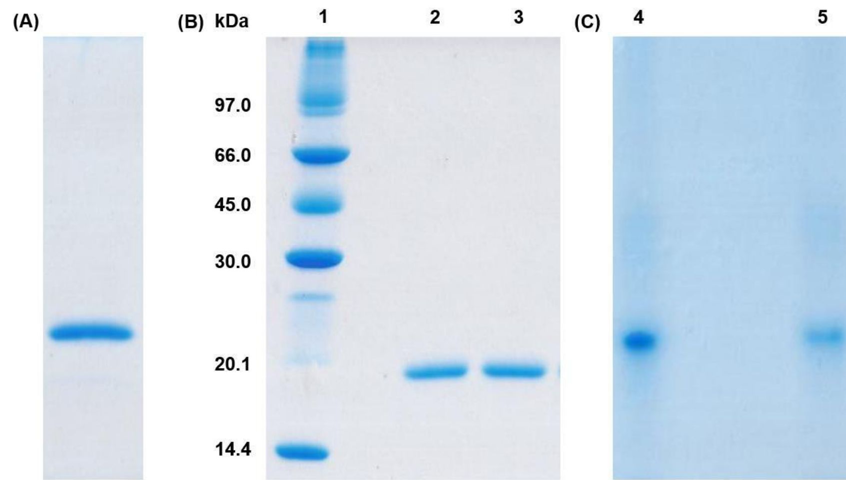


Fig. 3

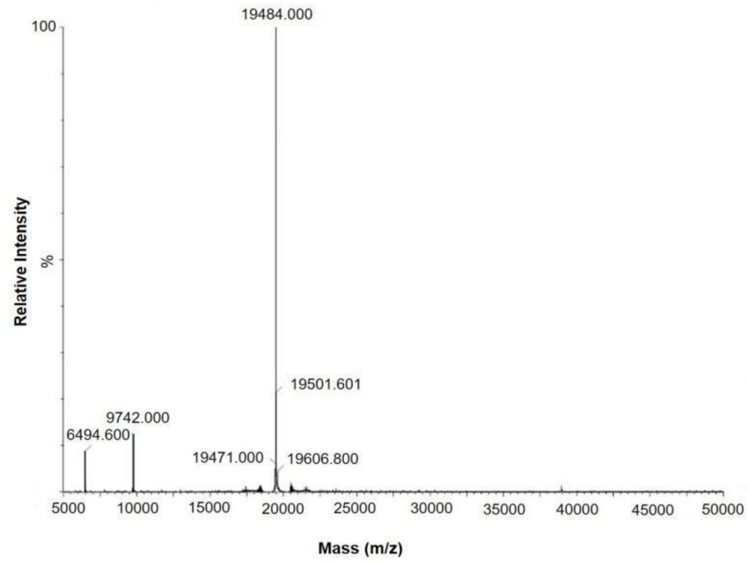


Fig. 4

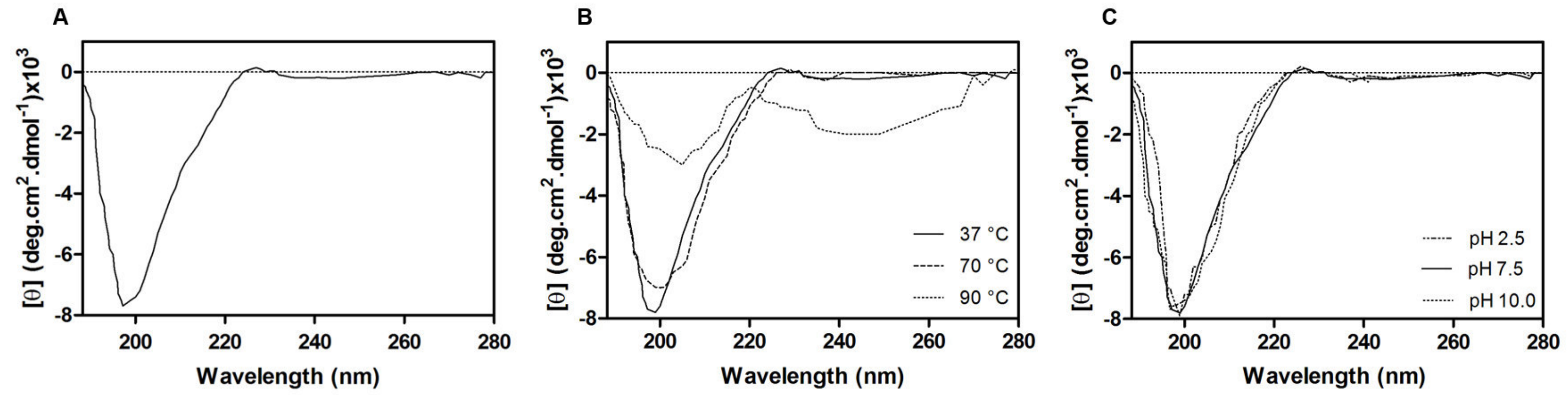


Fig. 5

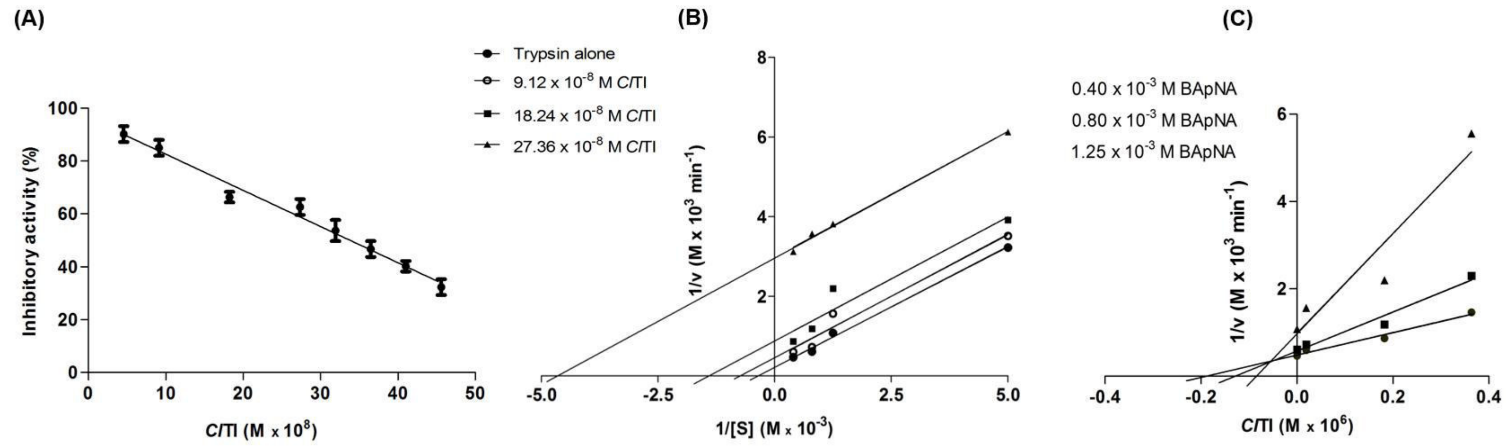


Fig. 6

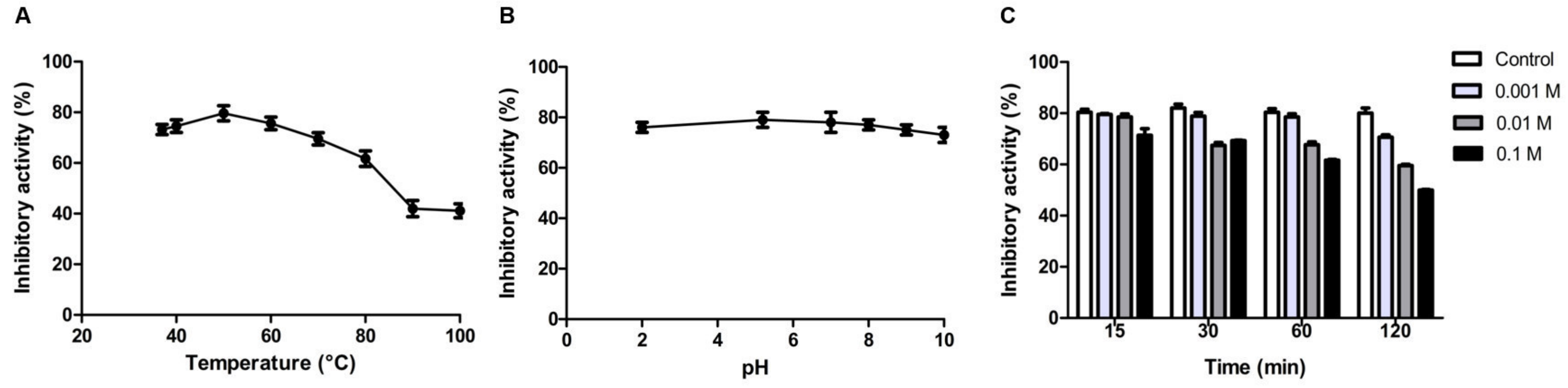


Fig. 7

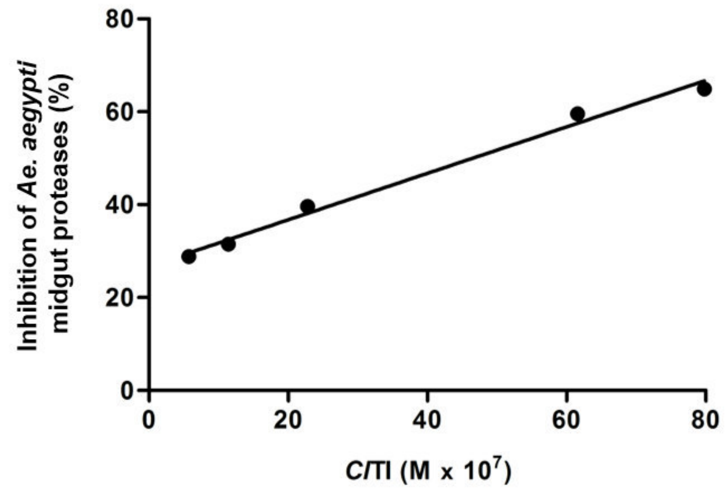
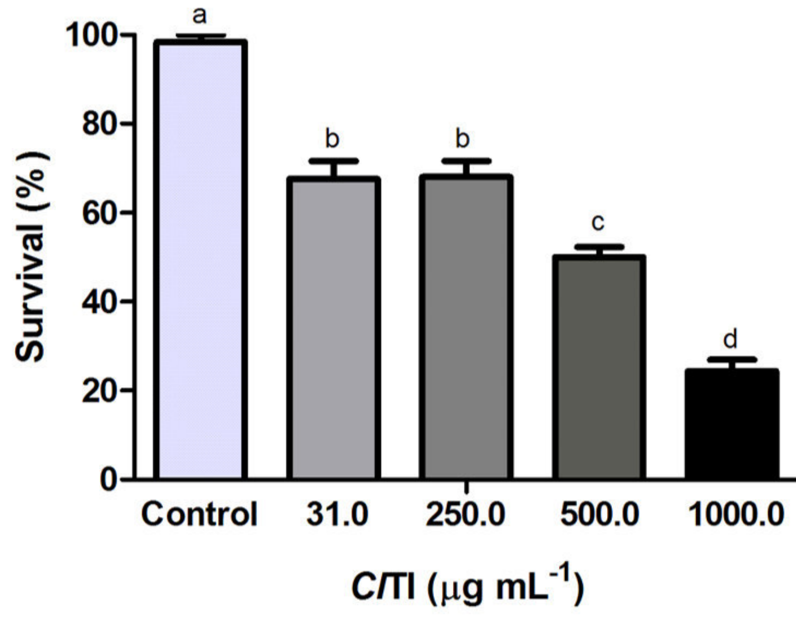


Fig. 8



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