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# Identificação e caracterização de compostos de sementes de *Clitoria fairchildiana* com atividade inseticida contra *Callosobruchus maculatus* e *Aedes aegypti*

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CAMPOS DOS GOYTACAZES – RJ

FEVEREIRO DE 2022

# Agradecimentos

- Agradeço a Deus por ter me mantido forte para superar todos os obstáculos pelo caminho para chegar até aqui.

- À minha querida orientadora Drª Kátia Valevski Sales Fernandes, por todo estímulo, paciência, carinho e atenção durante a minha formação. Agradeço também pela sua valiosa amizade e pelos momentos felizes que vivemos.

- À Drª Antônia Elenir Amâncio de Oliveira, por todo o tempo, suporte e atenção dispensados a mim. Sua atenção foi de grande valia na minha formação.

- Ao Drº Ivo José Curcino Vieira, pela oportunidade de aprender novas técnicas no LCQUI e por todo incentivo, apoio e colaboração neste trabalho.

- Ao Drº Francisco José Alves Lemos, pela oportunidade de trabalhar no insetário do LBT e por todo incentivo, apoio e colaboração neste trabalho.

- Aos Drº André T. S. Ferreira e Jonas Perales da FIOCRUZ, pela sua valiosa contribuição e colaboração neste trabalho.

- À Drª. Olga Lima Tavares Machado, por ter aceitado revisar essa tese, e por suas valiosíssimas sugestões a esse trabalho.

- Às Drª. Marílvia Dansa de Alencar e Érica de Oliveira Mello, e ao Drº. José Roberto da Silva, por aceitarem fazer parte da banca examinadora e contribuir para enriquecer meu trabalho.

- Aos professores, alunos e técnicos do LQFPP, do LCQUI, do LBT e do LBCT, pela boa vontade e presteza em me auxiliar em tudo que precisei.

- Aos órgãos de fomento FAPERJ, CAPES e CNPq, que possibilitaram o desenvolvimento desta pesquisa.

- Aos meus pais Marlene e Sebastião pelo amor, apoio e incentivo de sempre. E por me ensinarem a correr sempre atrás dos meus objetivos e a escolher sempre o caminho certo.

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## Abreviaturas

BCA - bicinchoninic acid method

BLAST - Basic Local Alignment Search Tool

CFD – Dichloromethane *Clitoria fairchildiana*

CFD-R – Rotenoids *Clitoria fairchildiana* dichloromethane partition

*Cf*vic – Vicilin *Clitoria fairchildiana*

ChBS - Chitin binding sites

CID – Collision-induced dissociation

HCl – Hydrochloric acid

HRESI-MS – High-resolution electrospray ionisation mass spectrometry

IITA - International Institute of Tropical Agriculture

MS/MS - Tandem Mass Spectrometry

NAG - N-acetyl D-glucosamine

NCBI - National Center for Biotechnology Information

NMR – Nuclear magnetic resonance

PDB – Protein Data Bank

PPM – Parts per million

ROS – Oxigen-reactive species

TFA – Trifluoroacetic acid

## Resumo

O desenvolvimento tecnológico revela a cada dia que os produtos naturais possuem muitas potencialidades a diferentes setores industriais, tais como: na produção de alimentos, fármacos, cosméticos, aromatizantes, inseticidas e muitos outros. Isso também trouxe à tona a importância do estudo da biodiversidade brasileira, mostrando o valor agregado de nossas riquezas naturais e a importância da conservação desta biodiversidade. Sendo assim, a espécie alvo do nosso estudo, *Clitoria fairchildiana*, vulgarmente conhecida como sombreiro, é uma leguminosa nativa, frequentemente utilizada em programas de arborização urbana e rural. Na literatura, não há relatos sobre a predação de suas sementes por insetos; as notificações raras de insetos que atacam essas plantas são de lepidópteros desfolhadores. Por esta razão, hipotetizamos que as sementes desta espécie possuem um arsenal defensivo inexplorado com potencial inseticida e investigamos a natureza proteica e não proteica de tais defesas e o mecanismo de ação destas moléculas nos insetos modelos *Callosobruchus maculatus* e *Aedes aegypti*. Para isso, identificamos, por técnicas cromatográficas, e caracterizamos, por técnicas de espectrometria de massas e ressonância magnética nuclear, compostos de metabolismo primário e secundário dos cotilédones das sementes de *C. fairchildiana* que se mostraram deletérios ao desenvolvimento e sobrevivência dos insetos modelos. Além disso, investigamos o mecanismo de ação de tais moléculas bioinseticidas, utilizando como estratégias: técnicas de microscopia, análises enzimáticas, cromatografia de afinidade e docking molecular. Identificamos uma proteína semelhante a vicilinas (*Cf*vic), de 12 kDa, altamente tóxica para *C. maculatus* (causando 66 % de redução na massa larval do inseto quando presente em níveis de 0,05% na dieta), que atua como toxina através do mecanismo de ligação a quitina. Essa associação de *Cf*vic a esse polissacarídeo complexo, presente na matriz peritrófica na superfície do intestino médio deste inseto, provavelmente impede a absorção de nutrientes e leva à morte larval. Identificamos também dois rotenoides, 11α-O-β-D-glucopiranosilrotenoide e 6-deoxiclitoriacetal 11-O-n-glucopiranosídio que interferiram no desenvolvimento larval de *Ae. aegypti* ($LC_{50}$ 121.2 PPM), capazes de inibirem em 55% as V-ATPases de larvas tratadas com 80 PPM dos rotenoides. Os rotenoides levaram a um aumento significativo na produção de espécies reativas de oxigênio (ROS) na região do intestino médio posterior das larvas. Isso sugere a que a inibição da V-ATPase leva ao desencadeamento de um processo de estresse oxidativo, que ocasiona a morte larval. As sementes desta espécie, conforme hipotetizávamos, confirmam-se como potenciais fontes de novos bioinseticidas com relevância agronômica e de saúde pública.

**Palavras chave:** *Clitoria fairchildiana,* Cfvic, *Callosobruchus maculatus,* rotenoides, *Aedes aegypti,* vetor, bioinseticidas.

## Abstract

Technological development reveals every day that natural products have a lot of potential for different industrial sectors, such as: in the production of food, drugs, cosmetics, flavorings, insecticides and many others. This also brough to light the importance of studying Brazilian biodiversity, showing the added value of our natural resources and the importance of conserving this biodiversity. Therefore, the target species of our study, *Clitoria fairchildiana*, commonly known as sombrero, is a native legume, often used in urban and rural afforestation programs. In the literature, there are no reports on the predation of its seeds by insects; the rare reports of insects that attack these plants are defoliating lepidopterans. For this reason, we hypothesized that the seeds of this species have an unexplored defensive arsenal with insecticidal potential, and we investigated the protein and non-protein nature of such defenses and the mechanism of action of these molecules in the model insects *Callosobruchus maculatus* and *Aedes aegypti*. For this, we identified, by chromatographic techniques, and characterized, by mass spectrometry and nuclear magnetic resonance techniques, primary and secondary metabolism compounds from the cotyledons of *C. fairchildiana* seeds, that proved to be deleterious to the development and survival of the model insects. Furthermore, we investigated the mechanism of action of such bioinsecticide molecules, using as strategies: microscopy techniques, enzymatic analysis, affinity chromatography and molecular docking. We identified a 12 kDa vicilin-like protein (*Cf*vic) that is highly toxic to *C. maculatus* (causing a 66% reduction in insect larval mass when present at levels of 0.05% in the diet), which acts as a toxin through the chitin binding mechanism. This association of *Cf*vic with this complex polysaccharide present in the peritrophic matrix on the surface of the midgut of this insect, probably prevents the absorption of nutrients and leads to larval death. We also identified two rotenoids, a 11α-O-β-D-glucopyranosylrotenoid and a 6-deoxyclitoriacetal 11-O-n-glucopyranoside, that interfered in the larval development of *Ae. aegypti* ($LC_{50}$ 121.2 PPM), capable of inhibiting in 55% the V-ATPase activity of treated larvae (80 PPM). The rotenoids led to a significant increase in the production of reactive oxygen species (ROS) in the posterior midgut region of the larvae. This suggests that inhibition of V-ATPase leads to the triggering of an oxidative stress process, which causes larval death. The seeds of this species, as we hypothesized, are confirmed as potential sources of new bioinsecticides with agronomic and public health relevance.

**Keywords:** *Clitoria fairchildiana*, Cfvic, *Callosobruchus maculatus*, rotenoids, *Aedes aegypti*, vector, bioinsecticides.

# 1. Introdução

## 1.1 Aspectos gerais

O desenvolvimento tecnológico dos últimos anos revitalizou o interesse na química de produtos naturais em vários campos do conhecimento científico, graças ao desenvolvimento e aprimoramento de técnicas e ferramentas analíticas e de precisão, em especial o interesse voltado a novas descobertas e aplicações multidisciplinares de suas potencialidades como medicamentos e outras drogas (ATANASOV et al., 2021).

Dos aproximados 30.000 compostos naturais conhecidos, 80% são de origem vegetal (SOOD, 2020). Esses compostos bioativos de plantas, chamados fitoquímicos, possuem várias aplicações em diversos setores industriais, como na produção de aditivos alimentares, fármacos, cosméticos, fragrâncias, aromatizantes, inseticidas, biocombustíveis e outros bens de consumo da rotina contemporânea (CHOW et al., 2020).

A adaptação das plantas às diversas condições ambientais resultou na evolução de vias metabólicas secundárias, onde produtos da fotossíntese são canalizados à produção de diversas biomoléculas (TETALI, 2019). Muitas destas são constitutivas, e outras induzidas a partir de estímulos, e estão ligadas aos metabólitos primários por blocos de construção e vias biossintéticas.

## 1.2 *Clitoria fairchildiana*

A planta modelo do estudo é uma espécie não domesticada, da família Fabaceae – Papilionoidae, popularmente conhecida como faveira, sombreiro ou palheteira. É nativa da região amazônica e muito utilizada em programas de arborização urbanos e rurais nas regiões Sudeste e Norte do Brasil (DUCKE, 1949; LORENZI, 1992).

Configura-se como uma espécie de porte arbóreo, de copa volumosa e produtora de flores atrovioláceas que se derivam em frutos deiscentes (DUCKE, 1949; LORENZI, 1992). Produz sementes orbiculares e plano-convexas, exalbuminosas, revestidas por tegumentos castanho-esverdeados; possui cotilédones livres como principais órgãos de reserva e o embrião é invaginado, sendo assim, a anatomia destas sementes segue o padrão da maioria das leguminosas (SILVA & MÔRO, 2008COSTA; DA SILVA; GOMES, 2014) .

Nosso interesse central na espécie *C. fairchildiana* surge na ausência de relatos na literatura sobre pragas predadoras de suas sementes e também no fato de que não foi observado nenhum indício de predação das mesmas em indivíduos desta espécie, regularmente monitorados por pesquisadores de nosso grupo, ao longo de oito anos. As únicas pragas da espécie relatadas, em literatura, foram de insetos e fungos desfolhadores (*Euphalerus clitoriae*, *Urbanus acawoios*,

*Hyperchiria incisa e Erysiphe quercicola*) (FONSECA et al., 2019; MAGISTRALI et al., 2009; TREVISAN et al., 2004; ZANUNCIO et al., 2013).

Sendo assim, a identificação de compostos bioativos relacionados a tal capacidade defensiva destas sementes e a caracterização do seu mecanismo de ação, seria de grande valia para o desenvolvimento de novas formulações de inseticidas naturais, com baixo custo e que não prejudiquem, ou sejam menos prejudiciais ao meio ambiente.

### 1.2.1 Potencial biotecnológico de *Clitoria*

Diversos trabalhos vêm mostrando um grande potencial biotecnológico para espécies do gênero *Clitoria*. Lectinas isoladas a partir de sementes de *C. fairchildiana* foram identificadas, por eletroforese, como duas bandas de cerca de 100 e 116 kDa, capazes de aglutinar eritrócitos de coelho, com atividade anti-inflamatória (LEITE et al., 2012). Também há relato de uma lectina de sementes de *C. ternatea*, capaz de aglutinar eritrócitos humanos e com especificidade de ligação a açúcares do grupo Gal / Gal NAc (NAEEM; HAQUE; KHAN, 2007). Foi relatado, ainda, um inibidor de proteases (tripsina e quimotripsina) em sementes de *C. fairchildiana*, com efeitos antinutricionais sobre a larva de *Anagasta kuehniella*, reduzindo a atividade de proteases intestinais do tipo tripsina em 76% (DANTZGER et al., 2015). Foi relatada também a existência de uma proteína altamente básica em sementes de *C. ternatea*, designada "finotina", a qual mostrou amplo e potente efeito inibitório contra fungos patogênicos de plantas (*Rhizoctonia solani*, *Fusarium solani*, *Colletotrichum lindemuthianum*, *Lasiodiplodia theobromae*, *Pyricularia grisea*, *Bipolaris oryzae* e *Colletotrichum gloeosporioides*), potencial inibitório contra a bactéria *Xanthomonas axonopodis* e também potentes propriedades inibitórias contra os bruquídeos do feijão *Zabrotes subfasciatus* e *Acanthoscelides obtectus* (KELEMU; CARDONA; SEGURA, 2004). Um trabalho de nosso grupo de pesquisa identificou, adicionalmente, um inibidor de tripsina de 13 kDa em sementes de *C. fairchildiana*, o qual foi capaz de reduzir em 87,93% a atividade de enzimas digestivas de larvas de 4º instar de *Aedes aegypti* (DE OLIVEIRA et al., 2015).

Além disso, rotenóides isolados de sementes e raízes de *C. fairchildiana* mostraram atividade antifúngica contra leveduras do gênero *Candida* (SANTOS et al., 2018). E como reforço à hipótese de que sementes de espécies do gênero *Clitoria* possuem um vasto arsenal químico, com potencial inseticida ainda pouco estudado, foram depositadas duas patentes (EP 2677 871 B1 e US 9.271503 B2) de inseticidas produzidos com extrato de *Clitoria ternatea*, cuja composição é descrita como baseada em SPC (Compostos Secundários de Plantas) que afetam o desenvolvimento de insetos-praga de culturas agrícolas, particularmente mariposas e insetos mastigadores ou sugadores de seiva,

os quais interferem com a oviposição e/ou impedem que se alimentem da planta (MENSAH, 2012; 2016).

### 1.3 Arsenais de defesa de sementes contra insetos

Normalmente, em sementes, as defesas são constitutivas, ou seja, estão sempre presentes e previstas na programação genética das espécies. Essas defesas podem ser físicas, tais como dureza, textura e espessura do tegumento, ou químicas, efetivadas pela presença de compostos do metabolismo primário ou secundário das plantas (XAVIER FILHO, 1993).

#### 1.3.1. Proteínas de sementes com ação inseticida

Existem algumas famílias de proteínas tóxicas bem conhecidas, como as lectinas, os inibidores de proteases, inibidores de α-amilases, vicilinas, arcelinas e quitinases (SALES et al., 2000).

Lectinas são glicoproteínas capazes de se ligar de forma específica e reversível a diferentes carboidratos, sem alterar sua estrutura. As lectinas estão amplamente distribuídas na natureza, são proteínas multifuncionais e as mais estudadas são as da família das leguminosas, da tribo Diocleinae (PEUMANS & VAN DAMME, 1998). Essas lectinas de plantas mostraram potencial inseticida para uma grande variedade de insetos, como coleópteros, dípteros e lepidópteros. Aparentemente a resistência à degradação por enzimas digestivas e ou a ligação a receptores intestinais são a causa desta toxicidade, por interferir em funções digestivas, protetoras ou secretoras do intestino destes insetos (YARASI et al., 2011).

Inibidores de proteases têm funções primárias de regulação de proteólise endógena e de armazenamento de aminoácidos e funções acessórias de defesa, através de sua capacidade de inibir a ação catalítica de enzimas proteolíticas de origem exógena, o que leva a uma eficiente estratégia de defesa de plantas contra insetos, através de uma ação antinutricional (VERNEKAR et al., 2001). Tal ação antinutricional decorre da redução do catabolismo de proteínas ingeridas na dieta de insetos, causada pela inibição das enzimas proteolíticas do trato digestivo desses organismos (DE OLIVEIRA et al., 2015).

Inibidores de α-amilases mostram grande potencial na defesa de plantas contra pragas. As enzimas do tipo amilases fazem a clivagem inicial do amido, quebrando-o em oligossacarídeos menores. Os inibidores destas enzimas, portanto, reduzem a capacidade dos organismos que os consomem de hidrolisarem o amido derivado dos alimentos ingeridos (IULEK et al., 2000). Por exemplo, KLUH et al. (2005) relatou um inibidor de α-amilases (αAl-1), que inibiu (analises *in vitro* e *in vivo*) essas enzimas em três ordens de insetos (Coleoptera, Hymenoptera e Diptera), sua atividade inibitória foi demonstrada pela supressão do desenvolvimento das larvas destes insetos.

Vicilinas são proteínas da classe das globulinas 7S, têm função de armazenamento de aminoácidos em sementes e possuem altas massas moleculares (40 a 70 kDa por subunidade). Em leguminosas, são heterogêneas e constituídas de diferentes subunidades. Uma grande variedade de vicilinas de sementes de leguminosas tem mostrado potencial de interferência no desenvolvimento de insetos, como *Callosobruchus maculatus*, pois se ligam fortemente, *in vivo*, à quitina (homopolímero de N-acetil-D-glucosamina,) presente nas matrizes ou membranas peritróficas da superfície do intestino médio deste e de outros insetos (FIRMINO et al., 1996; UCHÔA et al., 2009; VIEIRA BARD et al., 2014). Como por exemplo recentemente Ferreira et al. (2021) mostrou que vicilinas ligantes a quitina do cultivar BRS Xiquexique diminuíram a massa e o comprimento larval de *C. maculatus* em 64,3% e 33,23% respectivamente.

As arcelinas são proteínas de armazenamento de feijões selvagens, que a exemplo das vicilinas, têm função primária de reserva de aminoácidos e secundária como inseticida, principalmente contra coleópteros da família Bruchidae. São encontradas sob diversas isoformas variantes em uma mesma espécie, mas sua expressão é controlada por um único gene (OSBORN et al., 1986). Como exemplo, uma arcelina de sementes de *Lablab purpureus* foi deletéria a *C. maculatus,* alterando a atividade da α-amilase deste inseto (JANARTHANAN & SURESH, 2010).

Quitinases catalisam a hidrólise de quitina, estão presentes de forma constitutiva em sementes e conferem resistência a fitopatógenos que apresentam quitina em suas estruturas corpóreas (ISELI et al., 1996). Por exemplo, SILVA et al. (2018) relatou a toxicidade de quitinases do tegumento da soja sobre o desenvolvimento e sobrevivência de *C. maculatus*, reduzindo a sobrevivência em 77%.

### 1.3.2. Compostos secundários de plantas com ação inseticida

Existe uma enorme variedade de compostos de metabolismo secundário em plantas, os quais podem ser divididos em três grupos químicos distintos: os terpenos, compostos fenólicos e compostos contendo nitrogênio (TAIZ & ZEIGER, 2010).

A maior classe de metabólitos secundários é a dos terpenos. Alguns atuam em processos de desenvolvimento de plantas, como a giberelina (um diterpeno) e os brassinosteróides (triterpenos). No entanto, a maior parte deles está relacionada aos arsenais de defesa das plantas; são compostos tóxicos que detêm os efeitos adversos da herbivoria, imposta por insetos e mamíferos. Como exemplo, há os piretróides, do grupo dos monoterpenos, que fazem parte da composição de inseticidas naturais; eles apresentam insignificante toxicidade a mamíferos e baixa persistência no ambiente. Também aqui estão os óleos essenciais de algumas plantas, como do limoeiro, manjericão e hortelã-pimenta, que constituem-se em compostos de monoterpenos voláteis e sesquiterpenos.

Esses óleos têm propriedades repelentes a insetos já bem conhecidas (MIRZA et al., 2020; SALHA; ABDERRABBA; LABIDI, 2021).

Os compostos fenólicos possuem variadas funções, como por exemplo, proteger contra herbivoria ou atrair polinizadores e dispersores. Eles formam um grupo de mais de 10.000 compostos individuais e possuem um grupo fenol constituído por um grupo funcional carboxila e um anel aromático. Nesse grupo estão os carotenóides e os flavonóides. Alguns isoflavonóides, como as rotenonas, são usadas como inseticidas e os taninos sabidamente afetam a sobrevivência de herbívoros (SANTOS; FURLAN, 2020; WINK et al., 1988).

Os compostos contendo nitrogênio são sintetizados a partir de aminoácidos comuns e também incluem compostos de defesa contra herbivoria. Como exemplo, citam-se os alcalóides e os glicosídeos cianogênicos. Os alcalóides são sintetizados a partir dos aminoácidos lisina, tirosina ou triptofano e atuam, em geral, na defesa contra herbívoros, principalmente mamíferos. Já os glicosídeos cianogênicos não são tóxicos, mas atuam liberando venenos, em alguns casos voláteis, como é o caso do cianeto de hidrogênio que atrapalha a alimentação de insetos herbívoros. Algumas plantas possuem aminoácidos não proteicos que atuam como compostos de defesa. Sua toxicidade pode se dar de várias maneiras, como exemplo bloqueando a síntese ou a absorção de aminoácidos proteicos. Um exemplo é a canavanina, que quando absorvida por herbívoros se liga ao tRNA da arginina e é incorporada nas proteínas do herbívoro no lugar da arginina (OLIVEIRA et al., 1999; SALATINO & SALATINO, 2020).

## 1.4 Insetos-alvo do trabalho

### 1.4.1 *Callosobruchus maculatus*

O besouro *C. maculatus*, conhecido popularmente como “caruncho”, pertence à família Chrysomelidae, ordem Coleoptera, e é a principal praga de grãos armazenados de leguminosas de interesse comercial, principalmente feijões do gênero *Vigna*. O ciclo de vida deste besouro começa com a embriogênese, que dura 5 dias após a oviposição de fêmeas adultas, no tegumento das sementes; o fim deste estágio é marcado pelo início da escavação da larva para dentro da semente, consumindo seus cotilédones. Após essa fase, iniciam-se os 4 instares de vida da larva: o primeiro instar tem duração de 8 a 9 dias; o segundo instar dura de 3 a 4 dias; o terceiro instar também dura de 3 a 4 dias; e o quarto instar dura de 4 a 5 dias. Em seguida, começa a formação da pupa, que leva de 6 a 7 dias para machos e 5 a 6 dias para fêmeas. Por fim, a transformação em adultos leva de 9 a 12 dias para insetos machos e de 10 a 14 dias para fêmeas. Após esse tempo, os insetos adultos eclodem, abandonam as sementes perfuradas e, via de regra, em condições de deterioração que inviabilizam

tanto seu valor comercial para consumo como sua capacidade germinativa (DE SÁ et al., 2014; DEVI & DEVI, 2014).

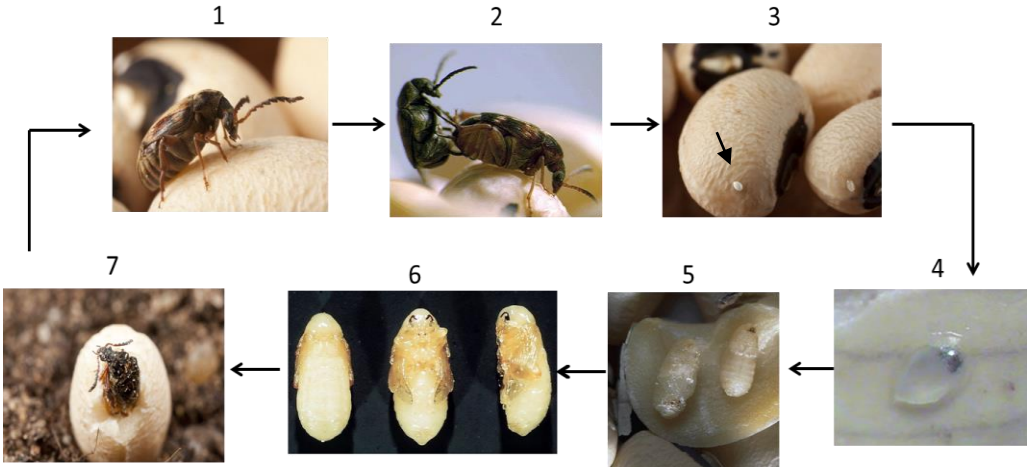


**Figura 1:** Ciclo de vida de *Callosobruchus maculatus*. 1- Fêmea adulta, 2- Acasalamento, 3- Ovos ovipostos na semente (seta preta), 4 - Ovo com larva formada no seu interior, 5 - Larvas dentro das sementes, 6 - Pupas e 7- Emergência do inseto adulto (DE SÁ, 2014).

A infestação por este inseto praga causa prejuízos nutricionais e econômicos no armazenamento de sementes de feijão do gênero *Vigna*, visto que, este gorgulho se alimenta das reservas nutricionais dos cotilédones destas sementes durante o seu desenvolvimento, inviabilizando assim o consumo e germinação das mesmas. Além disso, estas sementes são uma importante fonte proteica na alimentação da população de países em desenvolvimento (REES, 2007; DE SÁ et al., 2014; FERREIRA et al., 2021). Dada a importancia econômica dessa praga, vários estudos têm se dedicado a encontrar sementes resistentes ou compostos vegetais tóxicos para esse inseto. A resistência de genótipos nigerianos de feijão-de-corda foi associada com a presença de formas variantes de vicilinas 7S, as quais possuem habilidade de ligação à quitina, e que são dificilmente digeridas pelas proteases intestinais do inseto, levando-o a uma condição de desnutrição e eventualmente efeitos letais ao desenvolvimento do bruquídeo (FERREIRA et al., 2021; MACEDO et al., 1993; SALES; MACEDO; XAVIER-FILHO, 1992). KUNZ et al. (2017) sugeriram que a internalização de vicilinas é feita por endocitose mediada por um receptor de membrana nos enterócitos do intestino médio de larvas de *C. maculatus*. A avaliação da resistência de certos cultivares de feijão-de-corda ao besouro *C. maculatus* mostrou que alguns deles causaram alteração na oviposição, afetaram a sobrevivência larval, causaram diminuição do peso das larvas e também a redução da atividade de proteases e carboidrases digestivas do inseto (CRUZ et al., 2016). Mais recentemente, MIRANDA et al. (2020) mostrou que a modificação química e *in silico* de

aminoácidos presentes no sítio de ligação a quitina de vicilinas de cultivares resistentes de *V. unguiculata*, alterou a capacidade das mesmas de ligarem-se a este carboidrato e consequentemente interferiu na sua toxicidade ao bruquídeo *C. maculatus*.

Outras espécies têm sido estudadas, com objetivo de se descobrirem novas moléculas tóxicas a *C. maculatus*. Estudos mostraram que o tegumento das sementes de *Phaseolus vulgaris* apresenta alguma forma de barreira química ao desenvolvimento pós-embrionário (larval) do besouro. Acredita-se que essa toxicidade seja causada pela redução da atividade das proteases digestivas deste inseto (DE SÁ et al., 2014), mas a natureza química de tal toxicidade não foi identificada. Uma lectina (CrataBL), com potencial para controle do desenvolvimento larval de *C. maculatus*, foi também isolada da casca de *Crataeva tapia*. Os autores mostraram que esta lectina, que agiria como uma proteína multifuncional, foi capaz de reduzir a atividade proteolítica de proteases cisteínicas intestinais do inseto (NUNES et al., 2015). Foi relatada também uma proteína de ligação a quitina na casca de sementes de *Albizia lebbeck*, que se mostrou semelhante a uma protease cisteínica e com atividade tóxica para *C. maculatus*. Entende-se que esta toxicidade possa ser devida à sua ligação a quitina na superfície do intestino médio das larvas, atuando com ação antinutricional (SILVA et al., 2016). JUMBO et al. (2018) mostraram que os óleos essenciais de cravo e canela possuem atividade inseticida comparável a inseticidas sintéticos e afetam significativamente a oviposição de *C. maculatus*.

### 1.4.2 *Aedes aegypti*

Conhecido popularmente como pernilongo ou mosquito da dengue, pertence à ordem Diptera, família Culicidae e sub-família Culicinae (JABEEN et al., 2019). *Aedes aegypti* é uma espécie sinantrópica, ou seja, adaptou-se a viver próximo dos humanos e se beneficiar de condições de permanência e reprodução criadas por ele. Os criadouros utilizados com mais frequência são recipientes com acúmulo de água, como: vasos de planta, garrafas pet, pneus, baldes, calhas entupidas ou desniveladas, ralos pouco utilizados, caixas d'água mal fechadas, bandejas coletoras de ar-condicionado e quaisquer outro local com acúmulo de água parada e "limpa" (presença de alguma matéria orgânica) (VALLE et al., 2021). O ciclo evolutivo desse inseto vetor é do tipo holometábolo: dividido em ovo, larva (4 estádios), pupa e adulto. Isso significa que este apresenta estádios de desenvolvimento muito distintos e por isso o indivíduo adulto não se assemelha em nada com os estádios larvais. A diferença estrutural é tão grande que até mesmo os nichos ecológicos de cada estádio podem ser muito diferentes, ocasionando pressões seletivas bastante distintas. Sendo assim, numa observação rápida pode parecer que os diferentes estádios de vida são diferentes organismos. O mosquito *Ae. aegypti* é um bom exemplo deste fenômeno, visto que as larvas vivem em ambiente

aquático e os adultos são alados podendo viver nos ares e/ou repousando em superfícies terrestres. Além disso, o canal alimentar das larvas é autolisado e completamente substituído durante o processo de pupação, reconstruindo o aparelho digestivo do adulto (CHRISTOPHERS, 1960; LINSER & DINGLASAN, 2014).

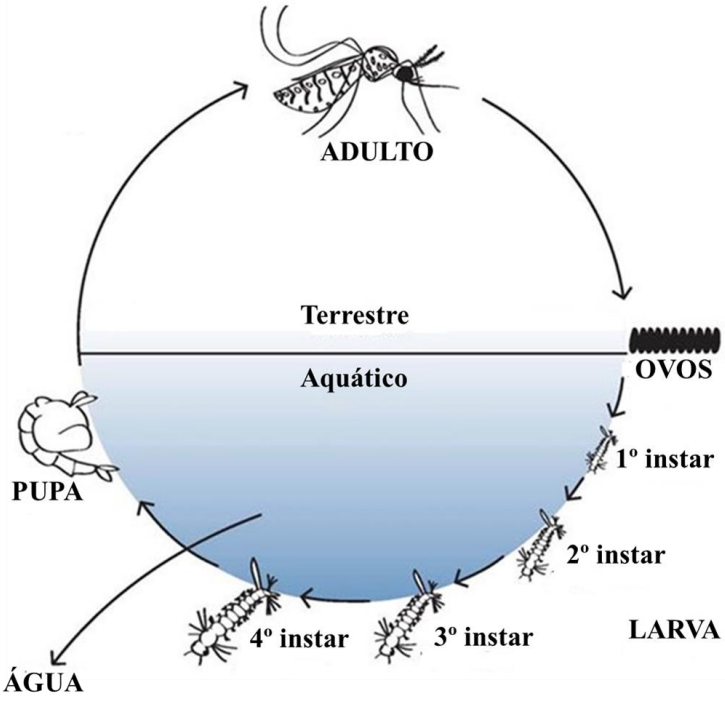


**Figura 2 –** Ciclo evolutivo do *Aedes aegypti,* adaptado de COON et al. (2014).

Este inseto é um vetor de várias arboviroses, como dengue, febre amarela, chikungunya e zika (WHO 2020). Devido ao potencial de distribuição deste vetor, algumas destas arboviroses espalharam-se rapidamente no mundo todo nos últimos anos, causando graves epidemias, o que o tornou um vetor de importância clínica em várias regiões do planeta (PATTERSON; SAMMON; GARG, 2016; SILVA; SANTOS; MARTINS, 2020). A dengue é a mais prevalente e pode afetar 3,9 bilhões de pessoas, em 129 países, sendo responsável por 96 milhões de casos sintomáticos e 40.000 mortes por ano (WHO 2020).

Estratégias de controle da população do mosquito vetor destas arboviroses urgem, especialmente devido à inexistência de vacinas eficazes e medicamentos virais específicos. Por essa razão, a utilização de inseticidas é ainda uma das estratégias de prevenção mais eficazes (WHO, 2021). No entanto, devido ao desenvolvimento de resistência por parte do mosquito *Ae. aegypti*, a substituição destes inseticidas ao longo dos anos foi inevitável e os primeiros organoclorados foram substituídos pelos organofosforados e estes, mais recentemente, pelos piretróides (NAUEN, 2008). Infelizmente, já há registros de populações de *Ae. aegypti* resistentes também a piretróides (ZHENG et al., 2019).

Sendo assim, a busca por compostos naturais com atividade inseticida tem sido uma alternativa para ampliar o leque, possibilitando uma alternância de inseticidas, e com isso dificultar-se o desenvolvimento de resistência no controle deste vetor. Além disso, estes compostos normalmente são biodegradáveis e de baixo custo. DE OLIVEIRA et al. (2016) mostrou que o extrato do sisal aumenta a produção de óxido nítrico em hemócitos de *Ae. aegypti*, induzindo a morte celular. Este resultado sugeriu que este extrato poderia ser utilizado como matéria-prima para novos inseticidas ecológicos e baratos. PROCÓPIO et al. (2015) observou que o extrato das folhas da *Schinus terebinthifolius* (aroeira vermelha) causou danos no intestino médio e fragmentação do DNA, interferindo no desenvolvimento e sobrevivência das larvas de *Ae. aegypti*. E atribuiu o efeito larvicida deste extrato a derivados do ácido cinâmico e flavonóides. Em seguida, estudos mostraram o potencial larvicida de dezessete derivados do ácido cinâmico, contra larvas de quarto instar de *Ae. aegypti*. E sugeriram, através de análises de modelagem molecular, que a atividade larvicida destes compostos pode ser atribuída a utilização de múltiplos alvos, como: a inibição de uma anidrase carbônica (CA), uma histona desacetilase (HDAC2) e dois co-transportadores de cátion-cloreto dependentes de sódio (CCC2 e CCC3) (ARAÚJO et al., 2021)

# 2. Objetivos

## 2.1. Objetivo geral

Identificar e caracterizar compostos de metabolismo primário e secundário de sementes de *Clitoria fairchildiana* que se mostrem deletérios ao desenvolvimento e sobrevivência dos insetos modelo *Callosobruchus maculatus* e *Aedes aegypti*, investigando o mecanismo de ação de tais moléculas.

## 2.2. Objetivos específicos

– Isolar e identificar proteínas com potencial inseticida para o bruquídeo *C. maculatus*;

– Caracterizar a estrutura primária destas proteínas tóxicas ao bruquídeo e inferir suas estruturas tridimensionais;

- Isolar e identificar compostos do metabolismo secundário de sementes de *C. fairchildiana* e avaliar a toxicidade sobre o desenvolvimento de larvas de terceiro instar de *Ae. aegypti*;

- Caracterizar a estrutura dos compostos de metabolismo secundário com atividade inseticida sobre as larvas de *Ae. aegypti*;

- Investigar o mecanismo de ação dos compostos com atividade bioinseticida.

Nota: As estratégias metodológicas adotadas ao longo deste trabalho, bem como os resultados obtidos e a discussão dos mesmos, contextualizados com a literatura relevante, serão apresentados sob a forma de dois artigos científicos (Capítulos 1 e 2, a seguir), já submetidos à apreciação dos corpos editoriais dos periódicos.

# Capítulo I – Proteínas de sementes de *Clitoria fairchildiana* com ação tóxica ao bruquídeo *Callosobruchus maculatus*

(Artigo submetido a revista *Pesticide Biochemistry and Physiology*)

# A vicilin-like protein extracted from *Clitoria fairchildiana* cotyledons was toxic to *Callosobruchus maculatus* (Coleoptera: Chrysomelidae)

Running title: **A chitin-binding vicilin-like protein toxic to cowpea weevil**

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Abbreviations: *Cf*vic – *Clitoria fairchildiana* vicilin; ChBS – Chitin-binding sites; ChBD – Chitin-binding domains; BLAST - Basic Local Alignment Search Tool; MS/MS – Tandem mass spectrometry; CID – Collision-induced dissociation; NAG - N-acetyl D-glucosamine; NCBI - National Center for Biotechnology Information; PDB – Protein Data Bank; RH – Relative humidity.

**Abstract**

*Callosobruchus maculatus* is the main pest cowpea (*Vigna* unguiculata). Given its relevance as an insect pest, studies have focused in finding toxic compounds which could prevent its predatory action towards the seeds. *Clitoria fairchildiana* is a native Amazon species, whose seeds are refractory to insect predation. This characteristic was the basis of our interest in evaluating the toxicity of its seed proteins to *C. maculatus* larvae. Seed proteins were fractioned, according to their solubility, to albumins (F1), globulins (F2), kaphyrins (F3), glutelins (F4), linked kaphyrins (F5) and cross-linked glutelins (F6). The fractionated proteins were quantified, analyzed by tricine-SDS-PAGE and inserted into the diet of this insect pest in order to evaluate their insecticidal potential. The most toxic fraction to *C. maculatus*, the propanol soluble F3, was submitted to molecular exclusion chromatography and all of the peaks obtained, F3P1, F3P2, F3P3, caused a reduction of larval mass, especially F3P1, seen as a major ~12 kDa electrophoretic band. This protein was identified as a vicilin-like protein by mass spectrometry and BLAST analysis. The alignment of the *Cf*vic (*C. fairchildiana* vicilin) peptides with a *V. unguiculata* vicilin sequence, revealed that *Cf*vic has at least five peptides (ALLTLVNPDGR, AILTLVNPDGR, NFLAGGKDNV, ISDINSAMDR, NFLAGEK) which lined up with two chitin binding sites (ChBS). This finding was corroborated by chitin affinity chromatography and molecular docking of chitin-binding domains for N-Acetyl-D-glucosamine and by the reduction of *Cf*vic chitin affinity after chemical modification of its Lys residues. In conclusion, *Cf*vic is a 12 kDa vicilin-like protein, highly toxic to *C. maculatus*, acting as an insect toxin through its ability to bind to chitin structures present in the insect midgut.

Keywords: Chitin-binding, cowpea weevil, butterfly pea tree, insecticidal protein, plant defenses.

## 1. Introduction

The beetle *Callosobruchus maculatus*, popularly known as cowpea weevil, belongs to the order Coleoptera: Chrysomelidae family and is the main pest of the bean species *Vigna unguiculata*.

The infestation by this insect pest causes nutritional and economic damages in the stored seeds of the *Vigna unguiculata* species, since this weevil feeds on the nutritional reserves of the cotyledons during its development, thus preventing their consumption and germination. In addition, these seeds are an important source of protein in the diet of the population of developing countries (Ress, 2007; De Sá et al., 2014; Ferreira et al., 2021). The use of chemical insecticides is still one of the most used control strategies for these insect pests. However, the indiscriminate use of these insecticides led to the development of resistance of this beetle, besides increasing environmental contamination (Satya et al., 2016; Munawar et al., 2020).

Given the economic importance of this pest, several studies have been dedicated to finding resistant seeds or toxic plant compounds for this insect, which would allow an alternation of insecticides, thus hindering the development of resistance by this bruchid and reducing environmental contamination. The seed resistance of Nigerian cowpea genotypes was associated with the presence of variant forms of 7S vicilins, which have the ability to bind chitin, and which are difficult to digest by the insects intestinal proteases, leading to a condition of malnutrition and possibly lethal effects on the development of the bruchid (Macedo et al., 1993; Sales et al., 2001; Uchôa et al., 2009). However, little is known about the molecular process of internalization of these proteins, although vicilins have already been found in hemolymph, midgut, fat body and Malpighian tubules, a systemic effect has not yet been investigated (Uchôa et al., 2006; Souza et al., 2010). It is known that the internalization of vicilins is done by endocytosis mediated by a membrane receptor in the midgut enterocytes of *C. maculatus* larvae. However, bruchid-resistant seed variant vicilins inhibit transcytosis,

resulting in the accumulation of proteins in the midgut cells of these larvae, causing a pro-oxidative scenario, which may explain the deleterious effect on the development of *C. maculatus* larvae (Kunz et al., 2017; 2018). More recently, Miranda et al. (2020) showed that chemical and "in silico" modification of amino acids present in the chitin binding site of vicilins from resistant cultivars of *V. unguiculata* altered their ability to bind this carbohydrate and consequently interfered with their toxicity to bruchid *C. maculatus*. It is also known that seed vicilins from other leguminous species (*Phaseolus vulgaris, Phaseolus lunatus, Canaval. ensiformis* and *Glycine max*), which are not hosts to *C. maculatus*, strongly inhibit the larval development of this insect and bind to a chitin matrix (Yunes et al., 1998).

The model plant of this study is *Clitoria fairchildiana* R.A. Howard, a non-domesticated species from the Fabaceae – Papilionoidae family, popularly known in some regions as butterfly pea tree or as "sombreiro", in Brazil. It is native to the Amazon region and widely used in urban and rural afforestation programs in the Southeast and North regions of Brazil (Ducke, 1949; Lorenzi, 1992). Our central interest in this species stems from the absence of reports in the literature about predatory insects of their seeds, a very peculiar condition confirmed by eight years of monitoring of the species by our own research group. In addition, some studies have shown biotechnological potential for species of the genus *Clitoria*. A protease inhibitor was identified in *C. fairchildiana* seeds, with anti-nutritional effects on *Anagasta kuehniella* larvae, reducing the activity of trypsin-type intestinal proteases by 76% (Dantzger et al., 2015). The existence of a highly basic protein in *C. ternatea* seeds, called finotin, was also reported, which showed a broad and potent inhibitory effect against several plant pathogenic microorganisms and potent inhibitory properties against the bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus* (Kelemu et al., 2004). A 13 kDa trypsin inhibitor isolated from *C. fairchildiana* seeds was seen able to reduce by 87.93% the activity of digestive enzymes of 4$^{th}$-instar *Aedes aegypti* larvae (Oliveira et al., 2015).

The main objective of this work was to conduct a survey of proteins from the cotyledons of the undomesticated legume *C. fairchildiana*, the butterfly pea tree, displaying toxic action over the development and survival of *C. maculatus*.

## 2. Materials and Methods

### 2.1 Biological materials

#### 2.1.1 *Clitoria fairchildiana*

The seeds were collected on the campus of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil. They were then dried at 28° C and stored for further work. The voucher number HUENF 9492 has been allocated for the plant material exsiccate deposited in the University Herbarium.

#### 2.1.2 Insects

Insects were obtained from colonies of *C. maculatus*, kept in the Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). Colony insects were kept on seeds of a commercial variety of *V. unguiculata*, that is susceptible and the preferred diet to these beetles, at 28 °C, 60-80 % r.h., and under a photoperiod of L12:D12.

### 2.2 Methodologies

#### 2.2.1 Protein extraction

Based on the solubilities in solvents such as water, dilute saline, alcohol–water mixtures, and dilute alkali and acid (Shewry and Halford, 2002), seed storage proteins have been classified into four groups namely - albumins, globulins, prolamins, and glutelins. Following protocol by Luo et al. (2014), we prepared six storage protein fractions from the *C. fairchildiana* seeds: the four above mentioned classes plus one subclass of prolamins and one subclass of glutelins. Quiescent cotyledons were ground by using a SL - 30 SOLAB seed knife grinder until obtention of a flour, which was further sieved through a 48 mesh sieve. Proteins

from twenty grams of cotyledonary flour were extracted sequentially with the six solvents listed below, at 20 ºC, and then centrifuged at 10,000 x g for 20 min at 4 ° C; supernatants were collected and set aside after each step. Initially, the flour was extracted using distilled $H_2O$ (1:10 m/v ratio) with stirring for 30 min. We repeated this step with the obtained sediment and the final soluble fraction was denominated F1 (albumins). Proteins from the pellets were extracted sequentially with 200 mL of the following solutions: 0.5 M NaCl for 60 min (resulting soluble fraction, F2, globulins); 60 % (v/v) 2-propanol for 4 h (resulting soluble fraction, F3, kafirins); 0.1 M borate buffer, pH 10.8, for 4 h (resulting soluble fraction, F4, glutelins); 2-propanol with 1 % β-mercaptoethanol for 4 h (resulting soluble fraction, F5, cross-linked kafirins); and 0.1 M borate buffer, pH 10.8, containing 1 % β-mercaptoethanol and 1 % sodium dodecyl sulfate (SDS) for 18 h (resulting soluble fraction, F6, cross-linked glutelins). The final residue was discarded, and all fractions were lyophilized and stored at -20° C. Supplemental Figure S1 shows a scheme of this fractionation procedures.

### 2.2.2 Protein concentration

The protein concentration of the samples was determined by the bicinchoninic acid method (BCA) (Smith et al., 1985), using bovine serum albumin as a reference protein for the standard curve. The readings were carried out using the Thermo Plate - TP Reader spectrophotometer at 540 nm. Alternatively, a NanoDrop 2000 was also used for protein concentration, according to standards set by the equipment and under readings at 280 nm.

### 2.2.3 Visualization of proteins by tricine-SDS-PAGE

All isolated protein fractions were subjected to tricine-SDS-polyacrylamide gel electrophoresis (Schägger and von Jagow, 1987). The gel was run at constant voltage (20 V) for 16 h and loaded samples contained 27 μg of protein / lane. The stacking gel was made of 9.7 % acrylamide and resolving gel of 18 % acrylamide. After the run, gels were stained with

Coomassie R-250 brilliant blue and then bleached with a solution of 40 % methanol and 10 % acetic acid before being photographed and alternatively stained using silver nitrate.

### 2.2.4 Feeding toxicity assays

In order to test the toxic action of *C. fairchildiana* protein fractions to *C. maculatus*, an artificial seed system was employed (Macedo et al., 1993). Peeled commercial *V. unguiculata* seeds were ground until the obtention of a fine flour. The *Vigna* flour was homogeneously mixed with variable concentrations (w/w) of *C. fairchildiana* protein fractions, using a mortar and pestle. Artificial seeds (400 mg; 8 mm in diameter and 5 mm in height) were obtained by pressing the mixture into a cylindrical brass mould with the help of a hand press. Control artificial seeds were made exclusively with susceptible *V. unguiculata* flour, without addition of protein fractions from *C. fairchildiana*. A small group of adult female and male insects (5 from each gender) were kept in the same glass container for three days to allow mating, before collecting females to be transferred to the bioassay glass bottles. Artificial seeds were removed from the mould and exposed to three-days-old, fertilized *C. maculatus* females, during 24 h, kept in glass bottles at 28 °C and 60–80% RH, inside a B.O.D. incubator. Subsequently, the females were removed from the bottles, and only three eggs were left on each seed, with any excess eggs being removed, to avoid food and spatial competition between future hatched larvae. Both control and test seeds were further incubated for a period of 18 days (28 °C and relative humidity 60–80 %), and after this period, infested seeds were opened, and the number and fresh weight of each larva was recorded using a precision balance. We expressed the weight as zero when no larvae were found, or when the larvae were found dead and so small that we could not record any weight from them.

### 2.2.5 Isolation of insect toxic proteins

The protein fraction (F3), which showed higher toxicity to the insect, was initially subjected to a molecular exclusion chromatography in Sephadex G-50, using 10 g of resin

(Sigma), hydrated in distilled water in the proportion of 1:10 (m/v); 40 mg of F3 dissolved in 1200 μL of eluent buffer (100 mM sodium phosphate buffer, 100 mM NaCl, pH 7.6) was applied to the packed into a 55 cm height x 2 cm wide column and chromatography was at a flow rate of 700 μl per min and fractions collected every minute. Protein peaks detected at 280 nm were dialyzed against distilled water for 24 h (with 4 changes of water during this time interval) and lyophilized, before incorporated into insect diet.

### 2.2.6 Identification of proteins by mass spectrometry

Target 12kDa protein band was excised from electrophoretic gel, transferred to 0.5 ml tubes, and cut into smaller pieces. Digestion was performed using trypsin (Promega V511A) according to method described by Shevchenko et al. (1996).

The silver nitrate-stained samples were destained with a solution of ferricyanide 30 mM and sodium thiosulfate 100 mM, at a 1:1 ratio, for 5 min. This washing procedure was repeated until the destaining was complete, followed by 3 washes with 400 μL of water for 5 min each. Samples were dried in a speed-vacuum centrifuge and then rehydrated with 15 μL of an ice-cold trypsin solution (20 ng/μL in 40 mM ammonium bicarbonate, pH 8.0) and left on ice for 45 min. After gel reswelling, 20 μl of 40 mM ammonium bicarbonate was added to the samples followed by incubation for 16 h at 37° C. Following digestion, the peptides were transferred to new 0.5 mL tubes and re-extracted with 30 μL of 1:1 (v/v) 5 % formic acid/50 % acetonitrile and ultrasonicated for 10 min. The re-extracted solution was added to the first extracted solution, concentrated in a speedvac to 10 μL and stored at -20° C for later use.

C18 Zip-Tip micropipette tips activated with 50 % acetonitrile in water and equilibrated with 0.1 % TFA in water, were used to desalt the peptides. The tips were washed ten times with 0.1 % TFA in water. Tip-retained peptides were eluted using 1.5 μL of 50 %/0.1 % (v/v) TFA in water.

Reverse-phase nanochromatography coupled with nanoelectrospray high resolution mass spectrometry was performed for tryptic digest identification. For each sample, 8 μL of desalted tryptic peptide digests were applied to a trap column packed with 3 μm of 120 A Reprosil-Pur C18 AQ matrix (Dr. Maisch GmbH - Germany) and to a separation column packed with the same matrix, directly to a self-pack 10-15 μm Pico Tip empty column (home made using a laser puller P 2000 Sutter Instrument CO.). Chromatography was carried out on an EASY-nLC II Instrument (Thermo Scientific, USA). The samples were loaded onto the trap column at 2000 nL/min, while chromatographic separation occurred at 200 nL/min. Mobile phase A consisted of 0.1 % (v/v) formic acid in water and mobile phase B of 0.1 % (v/v) formic acid in acetonitrile. The gradient conditions were: 2 to 40 % B for 58 min and up to 80 % B for 2 min. This concentration was maintained for 2 min more, before the column was re-equilibrated. The eluted peptides were introduced to an LTQ XL/Orbi/Trap MS (Thermo, USA) for analysis. The voltage source was set at 1.9 kV, capillary temperature at 200 °C and the tube lens voltage at 100 V. The full ion trap, the MSn AGC and FTMS full AGC target values were 30,000, 10,000 and 500,000, respectively. The MS1 spectra were acquired on the Orbitrap analyser (300 to 1,700 *m/z*) at a 60,000 resolution (for *m/z* 445.1200). For each spectrum, the 10 most intense ions were submitted to CID fragmentation followed by MS2 acquisition on the linear trap analyser. The dynamic exclusion option was enabled. The parameter settings were: repeat count = 1; repeat duration = 30 s; exclusion list size = 500; exclusion duration = 45 s and exclusion mass width = 10 ppm.

The peptide mass profiles were analysed using Peaks Studio 8 (Bioinformatics Solutions Inc.). Searches were performed using the Uniprot-viridiplantae database with 7000 entries; search parameters for monoisotopic peptide masses allowed three missed enzymatic cleavage and accepted the carbamidomethylation of the cysteine residues and the oxidation of methionine as fixed and variable modifications, respectively.

### 2.2.7 Structural studies of F3P1

The obtained data were analyzed using the BLAST (Basic Local Alignment Search Tool) tool from NCBI (National Center for Biotechnology Information). Once the protein data indicated a vicilin-like protein (here as *Cf*vic), a multiple alignment between the *Cf*vic peptides and the IT81D1053 *C. maculatus*-resistant vicilin sequence, described by Rocha et al. (2018), was performed using the MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle/). A computational simulation of a three-dimensional model of the *Cf*vic structure was performed by placing the *Cf*vic peptides in their corresponding position at the two cupin domains from the full sequence of the IT81D1053 *Vigna* vicilin sequence, according to the sequence alignment. For modelling, we used the Chimera 1.13.1 rc software and the crystal structure of the 7S globulin-1 (PDB ID: 2EA7) from adzuki bean (*V. angularis*) (Fukuda et al., 2008) as a template, according to Rocha et al (2018). The model was validated in relation to their stereo-chemical properties (Ramachandran plots, Cβ deviation parameters, rotamers, bond lengths, bond angles and Cis Peptides) using the MolProbity tool (http://molprobity.biochem.duke.edu). Molecular images were prepared and refined using the Pymol Molecular Graphics System, version v1.3.

Molecular docking analysis of the chitin binding site (ChBS) from the computer-simulated structure of *Cf*vic with an N-acetyl D-glucosamine (NAG) molecule was performed using the AutoDockTools program version 1.5.6 Sep_17_14I.

### 2.2.8 Confirmation of the chitin-binding ability of *Cf*vic

*Cf*vic was tested for its affinity to chitin using chitin batch affinity chromatography, both in its native form and after acetylation of lysine, since these residues were found in their predicted chitin-binding sites. The acetylation process was done by mixing native *Cf*vic in a saturated sodium acetate solution (2% w/v) (Miranda et al., 2020). The mixture was placed in a cold bath under shaking and acetic anhydride was added in the same weight as the protein, in 5 equal portions for 1 h at 0 ºC. The mixture was stirred for another 1 h at 0 °C and then dialyzed

against distilled water for 24 h (with 3 changes of water during this time interval) and lyophilized.

Three mg of each sample (*Cf*vic, acetylated *Cf*vic and a chitin-binding IT81D-1045 vicilin used as control) dissolved in 500 μL of 0.1 M sodium acetate buffer pH 6.0 were separately added to an Erlenmeyer containing the chitin resin (3 mL; Sigma-Aldrich); the suspensions were gently agitated at 20 $^{o}$C, for 30 minutes. The resin-sample batch for each sample was then poured onto a glass column (14.5 x 3.5 cm) and protein separation was performed by initially washing the column with 0.1 M sodium acetate buffer pH 6.0 (10 mL), for perfusion of non-retained proteins, and then with 0.1 M HCl (10 mL), for elution of retained proteins ones. The process was carried out under a flow pump, regulated to collect 800 μL per minute. Collected fractions were analysed by readings at 280 nm and further dialysed against distilled water for 24 h (with 3 changes of water during this time interval) and lyophilized.

## 3. Results

### 3.1 Fractionation of seed proteins and analysis of their action against C. maculatus

Protein fractions of *C. fairchildiana* were tested against *C. maculatus* by using an artificial seed system (Figure 1). All fractions were toxic to *C. maculatus* larvae (Figure 1), and lethal when incorporated at a 0.3 % level in the insect diet. Fractions F1 and F3 were the most toxic since no insects survived when offered 0.1 % concentration. However, F3 was more toxic than F1. Further tests were then performed with F3 (Figure 2), at the concentrations of 0.05 %, 0.1 % and 0.2 %, using a higher number of seeds per concentration, and a 75 % reduction in larval mass was noticed when these insects were fed with the lowest concentration (Figure 2B); from the 0.1 % concentration on, there was no larvae with detectable weight (Figure 2A). These results demonstrated the high toxicity of this fraction.

Electrophoretic profiles of fractionated *C. fairchildiana* proteins were analysed by Tricine-SDS-PAGE (Figure 3), which showed that protein bands of several molecular masses

(Mrs) were present in all fractions. In fraction F3, now on the major target of this work, the major band (white arrow) was between the molecular mass markers of 6.5 and 14.2 kDa.

### 3.2 Chromatographic separation of F3 proteins and analysis of their action against C. maculatus

F3 proteins were separated using Sephadex G-50 molecular exclusion chromatography, from which we obtained three peaks (F3P1 [tubes 20 to 37]; F3P2 [tubes 65 to 82]; F3P3 [tubes 92 to 160]), as shown in figure 4A. From the initial 60 μg of protein applied to the column, a total recovery of 38 μg was obtained: 9 μg at F3P1, 18 μg at F3P2 and 11 μg at F3P3.

These chromatographic peaks were incorporated into the insect diet, at 0.05% in 400 mg artificial seeds. All three peaks had toxic effects, but F3P1 (1.8 μg of protein) showed the highest toxicity (Figure 4B), leading to the lowest larval weight (Figure 4B white bar and arrow in inserted images), 66.1% lower than that of control larva.

The protein profile of the F3P1 peak showed a major band at ~ 12 kDa, when using tricine-SDS-PAGE gels, revealed with silver nitrate (Figure 5 – black arrow). The same band was also seen in F3P2 and at a lower concentration in F3P3, explaining the insecticidal activity of all three fractions.

### 3.3 Identification of Cfvic primary structure and confirmation of the chitin-binding ability of F3P1 proteins

The F3P1 ~12 kDa band (arrow in Figure 5B) was submitted to mass spectrometry and the obtained peptides were further investigated by fingerprinting, using the BLAST database. We observed that 12 peptides of F3/P1 aligned with vicilin proteins from three legume species (*Vigna unguiculata*, *Vigna angularis* and *Glycine max*), as shown in Table 1, and all these 12 peptides showed 100% homology with the peptides screened by the BLAST, except for the *V. unguiculata* vicilin peptide (AWS21467.1 entry), with 91% homology. Considering these results, we performed a global alignment on MUSCLE of the F3P1 *Cf*vic peptide amino acid sequences with the full sequence of an IT81D-1053 *V. unguiculata* β-vignin (Figure 6). We

observed five peptides (ALLTLVNPDGR, AILTLVNPDGR, NFLAGGKDNV, ISDINSAMDR, NFLAGEK) which lined up with two chitin binding sites (ChBS) described by Rocha et al. (2018). Additionally, this alignment shows conservation of many amino acid residues in the *Cf*vic peptides, when compared to the full sequence of the *V. unguiculata* vicilin, as pointed out by the asterisks.

*In silico* protein chitin-binding assay was performed on chemically modified *Cf*vic, aiming at the acetylation of lysine residues found at the ChBS. Chitin affinity chromatography was used to compare the binding abilities of native *Cf*vic, acetylated *Cf*vic and an IT81D 1045 *V. unguiculata* vicilin. The native *Cf*vic tightly bound to the resin in a way that almost no elution was achieved with 0.1 M HCl (Figure 7 – open square line). This binding was stronger than that observed for both the acetylated *Cf*vic (Figure 7 – asterisk line) and IT81D 1045 vicilin (Figure 7 – black triangle line), as observed by the HCl eluted peaks from these samples.

### 3.4 Molecular docking of the Cfvic chitin binding site (ChBS)

After alignment, a computer simulated three-dimensional analysis of the two domains (Cupin 1 and 2) of the *Cf*vic protein was created by using the PDB ID:2EA7 sequence as template and inserting the *Cf*vic peptides in to the IT81D-1045 vicilin sequence, according to the previous MUSCLE alignment. Two chitin binding sites (ChBS), one in each cupin domain (in pink), were located in the modeled structures (Supplementary materials Figure S2) submitted to the MolProbity analysis (Supplementary materials Figures S3 and S4). After refinement, the percentage of Ramachandran outliers was either 0 or < 2.1%, for cupin domains 1 and 2, respectively (Supplementary material; Tables S1 and S2). We then performed a molecular docking analysis where both chitin binding site (ChBS) were confronted with an N-acetyl-D-glucosamine (NAG) molecule deposited in the PDB (http://www.rcsb.org/ligand/NAG). The experiment reveals a binding energy of $\Delta G = -2.52$ at the cupin 1 domain, where asparagine (Asn), aspartic acid (Asp) and proline (Pro) were the

amino acids interacting with the ligand (Figure 8I). For the cupin 2 domain, binding energy of approximately $\Delta G = -3.1$ was estimated, and asparagine (Asn), aspartic acid (Asp), serine (Ser), glycine (Gly), Valine (Val) and Lysine (Lys) were the amino acids involved in the interaction (Figure 8).

## 4. Discussion

As a result of co-evolutionary relationships, plants have developed diverse and sophisticated defense mechanisms against herbivores. However, during the domestication process of agronomically relevant species, inevitable losses of this genetic potential for defense have occurred, and large annual deficits are faced by farmers and food producing companies due to attacks by pests in the field or during the storage of seeds and milled cereals (Tamiru et al., 2015). The most effective way of controlling these predators is the use of chemical insecticides. However, as a consequence of the excessive use of synthetic chemical insecticides insect resistance arises (Guedes, 2016).

As an alternative strategy, the identification and characterization of molecules with insecticide potential from wild plant species has been exploited (War et al., 2012). *C. fairchildiana* is well inserted in this context because it has not undergone a domestication process and, in fact, there are no reports in the literature on predation of its seeds by any known insect class. In the present work, the seed cotyledons from the butterfly pea tree had a wide variety of proteins, with different solubility properties, and insecticide potential towards *C. maculatus.* The propanol-soluble fraction (F3) was the most effective one and it was, therefore, our main focus; other insecticidal molecules from the F1, F2, F4, F5 and F6 fractions are yet to be investigated. These findings reinforce the importance of conserving the plant biodiversity, since it holds valuable biotechnological potential, for development of novel natural insecticides (Umetsu and Shirai, 2020).

In the F3 fraction, a protein with toxic activity against *C. maculatus*, called *Cf*vic, caused a 66.1 % reduction in the weight of the larvae, when used at a concentration of 0.05 % in the insect diet. This represents a high toxicity potential, since variant vicilins, reported in the literature as the toxic factor of Nigerian lines of *C. maculatus*-resistant cowpea, are only effective at much higher concentrations of 0.5 % to 2.0 % (Macedo et a., 1993; Uchôa et al., 2006; Kunz et al., 2018). More recent studies have reported effective concentrations of seed proteins incorporated in *C. maculatus* diets, for example a cowpea cv. BRS Xiquexique chitin binding vicilin (2%) (Ferreira et al., 2021) and a soybean seed coat chitinase (0.1%) (Silva et al., 2018). Only this last work has found such a low effective concentration, as that we have found here for *Cf*vic. It is important to stress that *C. maculatus*, the target insect of the present work, is much less susceptible to a diverse set of plant toxic proteins than other seed-boring insects, as demonstrated by its performance in transgenic pea seeds expressing a *Phaseolus vulgaris* a-amylase inhibitor (Shade et al., 1994).

*Cf*vic was seen to have a molecular mass of ~ 12 kDa and its primary sequence showed high similarities with vicilin sequences deposited in the databanks. Considering that seed storage vicilins are large proteins and typically classified as globulins (salt-soluble seed proteins), we suggest that the propanol-soluble 12 kDa *Cf*vic may be a member of the so-called vicilin-buried peptides (VBP) family (Zhang et al., 2019), derived from vicilin precursor sequences. Other VBPs have been described in the literature and different types of bioactivities have been reported for these peptides, such as trypsin inhibition, cytotoxicity (Yamada et al., 1999), antimicrobial activity (Marcus et al., 2008) and ribosome inactivation (Li et al., 2005). But some VBPs from *Luffa aegyptiaca* and tomato do not show any of these types of activity (Zhang et al., 2019). A possible explanation for this was given by the latter authors, who suggested that VBPs might have evolved specialized functions instead of serving a conserved, generic function. Zhang et al. (2019) also revealed the occurrence of gene sequences indicative of interstitial VBPs in species

from Amborellales to eudicots, including important grass and legume crop species. These exciting findings may indicate a sophisticated plant strategy to "hide" defensive/bioactive molecules within precursor sequences of storage proteins that would serve primarily as a repository of amino acids for germinating seeds.

Based on a further alignment of *Cf*vic with the full sequence of an IT81D-1053 *V. unguiculata* vicilin β-vignin, we observed five peptides (ALLTLVNPDGR, AILTLVNPDGR, NFLAGGKDNV, ISDINSAMDR, NFLAGEK) which aligned with two chitin binding sites (ChBS) described by Rocha et al. (2018). This suggests that the mechanism of action of *Cf*vic may be through its binding to insect chitin-containing structures, similarly to that reported for vicilins from *V. unguiculata* resistant varieties and from other seeds (Sales et al., 1996; Moura et al., 2007). This *in silico* data on the *Cf*vic ability to bind to chitin was experimentally demonstrated and reinforced by the reduction of such affinity after the chemical modification of Lys residues. Miranda et al. (2020) also showed that chemical acetylation of Lys residues and in silico modifications of the Lys223 were responsible for the decrease of chitin affinity of an IT81D 1045 *V. unguiculata* vicilin.

To check the strength of this binding, molecular docking simulations were performed between the peptides of *Cf*vic (ChBS I - AILTLVNPDGR and ChBS II - NFLAGGKDNV, ISDINSAMDR) and a monomer of N-acetyl-D-glucosamine (NAG), revealing spontaneous binding energies for both ChBS ($\Delta G$ = -2.52 for ChBC cupin 1 domain and $\Delta G$ = -3.1 for ChBS cupin 2). These data altogether suggested that the mechanism of action of *Cf*vic, as already described in the literature for other defense-related vicilins, could be exerted through its association to chitinous structures in insect midguts (Firmino et al., 1996; Macedo et al., 2008). Sales et al. (2001) have shown, for *Callosobruchus maculatus* digestive tracts, after immunostaining with antibodies to chitin, a strong immunolabeling of the apical part of microvilli from the midgut epithelium indicating the presence of chitin or chitinous structures in the larval midgut. The authors also showed immunostaining with anti-vicilin IgG, used as a

probe for chitin-binding ability of vicilins present in the insect diet, revealing strong immunolabeling of the apical part of the microvilli (co-localized with the chitin structures), but without apparent staining of the nuclei or epithelial cells. Labeling of vicilins was not much visible in the gut contents of the lumen, according to the same article.

Through chitin affinity chromatography, the *in silico* data on *Cf*vic ChBS were corroborated and the fact that we were not able to efficiently elute the matrix bound-proteins may be related to the specially high levels of insect toxicity of *Cf*vic when compared with other previously studied legume defense-related vicilins (Firmino et al., 1996; Macedo et al., 2008; Silva et al., 2016). Similar irreversible binding to chitin, under most variable elution conditions, has been reported for a 59 kDa exochitinase (exo-ChiO1) from *Streptomyces olivaceoviridis* (Blaak and Schrempf, 1995), which required the use of denaturing 3 M guanidine chloride to be released from a chitin column. Miranda et al. (2020) also showed that different chemical modifications can alter the chitin affinity of the IT81D 1045 *V. unguiculata* resistant vicilin and that such alterations were reflected in changes to insect toxicity. Further analysis of the complete structure of *Cf*vic must be done to fully disclosure the reason for such high chitin affinity and for the possible relationship to the highly toxic activity against *C. maculatus*.

Plants and their predators have been establishing co-evolutionary relationships for thousands of years, influencing the diversification of both on the planet (Ehrlich and Raven, 1964). As a result of these relationships, the plants developed physical and chemical defense systems against predation. However, the domestication of some cultivars intended for human consumption has influenced the performance of predatory organisms and cultivated species become more vulnerable to them than wild species (Tamiru *et al.,* 2015). In this sense, non-domesticated species may represent a precious repository of defense molecules which must be investigated and recovered. *C. fairchildiana*, target species from this study is a non-domesticated Fabacea whose seeds do not have natural predators from the Insecta Class

described in the literature. The identification of *Cf*vic in these seeds can be of great value both for agronomic programs of crop development and transgenic plants, as well as for the development of new natural insecticides for *V. unguiculata* seeds in storage, allowing an alternation of insecticides and decreasing the speed of resistance development of this insect pest.

## 5. Conclusions

A propanol-soluble protein (*Cf*vic) from *C. fairchildiana* seed cotyledons, with a primary sequence similar to vicilins, had high deleterious effects on the larval development of *C. maculatus*. The protein was able to reduce by 66% the larval weight of this bruchid, when offered at a 0.05% concentration in its diet, preventing the emergence of adults. Sequence data and *in silico* analysis suggested that the mechanism of action of *Cf*vic is related to a strong binding to chitinous structures present in the midgut of this insect.

## 6. Ackowledgements

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ and the Conselho Nacional de Desenvolvimento Científico (CNPq). The first author was supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) MSc research grant.

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Legends

**Figure 1 -** Average fresh weight of *C. maculatus* larvae offered protein fractions F1, F2, F3, F4, F5 and F6 from cotyledons of *C. fairchildiana*, through an artificial seed bioassay. C – Control (Artificial seed made from flour of susceptible *V. unguiculata* seeds). The letters a, b, c and d represent significant statistical differences according to ANOVA (Tukey's Multiple Comparison) ($F= 9.031$; $DF= 10,11$; $P <0.0001$). Experiments consisted of 3 seeds per concentration, each seed bearing 3 eggs; replicates were performed as different experimental runs, leading to a total of 6 seeds and 18 eggs per assay.

**Figure 2** - Average survival (A) and fresh weight (B) of *C. maculatus* larvae fed with the F3 protein fraction obtained from *C. fairchildiana* cotyledons, through an artificial seed bioassay. C – Control (Artificial seed made from flour of susceptible *V. unguiculata* seeds). The letters a, b and c represent significant statistical differences according to ANOVA (Tukey's Multiple Comparison) ($F= 47.91$; $DF= 3,23$; $P <0.0001$). Experiments consisted of 5 seeds per concentration, each seed bearing 3 eggs; replicates were performed as different experimental runs, leading to a total of 30 eggs per assay.

**Figure 3** - Electrophoretic visualization of the protein profile of *C. fairchildiana* cotyledon protein fractions on tricine-SDS-PAGE gel. MM represents molecular mass markers from 6.5 to

26.6 kDa. Gels stained with Coomassie Brilliant Blue. Black arrow points to F3 (fraction of choice), with a major band (white arrow).

**Figure 4** - Fractionation of F3 proteins by molecular exclusion chromatography on a Sephadex G-50 column (Panel A) and dietary effects of chromatographic peaks (F3P1, F3P2, F3P3), offered at a 0.05% concentration, on larval weight of *C. maculatus* (Panel B; photographs of larvae are shown in the inserted box). C – Control (Artificial seed made from flour of susceptible *V. unguiculata* seeds). * - Statistically significant differences were calculated according to ANOVA (Tukey's multiple comparison) ($P < 0.0001$). Each experiment consisted of 5 seeds per concentration and duplicate runs, giving a total of 10 seeds and 30 eggs per assay (3 eggs per seed). Black arrows point to F3P1 (fraction of choice from now on).

**Figure 5** - Electrophoretic visualization of the protein profile of the F3 fraction peaks of *C. fairchildiana* cotyledons on tricine-SDS-PAGE gel; MM - molecular mass markers from 26.6 to 6.5 KDa. Gel stained with silver nitrate.

**Figure 6** - Multiple alignment of F3/P1 peptides with the full sequence of the *C. maculatus*-resistant *V. unguiculata* IT81D1053 cultivar, described by Rocha et al. (2018).

**Figure 7** – Chitin affinity chromatography profiles of native *Cf*vic (open squares), acetylated *Cf*vic (asterisk) and IT81D 1045 *Vigna unguiculata* vicilin (black triangle). Arrow represents the addition of the 0.1 M HCl elution buffer. Equilibration buffer was 0.1 M sodium acetate, pH 6.0; 800 µL fractions were collected per minute.

**Figure 8** - Molecular docking of both *Cf*vic chitin binding sites (ChBS) with an N-acetyl d-glucosamine (NAG) molecule, using the AutoDockTools program version 1.5.6 Sep_17_14. I - *Cf*vic in cupin 1 domain (in blue) + NAG (white molecule); II - *Cf*vic in cupin 2 domain (in orange) + NAG (white molecule). ChBS amino acids are shown by its three-letter codes.

**Table Title**

**Table 1:** Analysis of F3P1 peptides fingerprinting, using the BLAST database.

**Supplemental Materials**

**Figure 1 (Supp.):** Scheme of the fractionation of the six classes of seed proteins from cotyledons of *C. fairchildiana*, according to Luo et al. (2014).

**Figure 2 (Supp.):** Computer simulation of the three-dimensional structure of *Cf*vic protein, using as template the *V. angularis* vicilin crystal structure (PDB ID: 2EA7). **A** - Cupin 1 domain and **B** - Cupin 2 domain. In beige, template; in blue, *Cf*vic peptides; in pink, chitin binding sites present in *Cf*vic peptides.

**Figure 3 (Supp.):** Ramachandran plot for cupin-1.

**Figure 4 (Supp.):** Ramachandran plot for cupin-2.

**Table 1**: Summary statistics of the MolProbity analysis of the three-dimensional molecular model of the Cupin 1 domain of *Cf*vic protein. The analysis was performed by submitting the simulated three-dimensional molecular model to the MolProbity web server (http://molprobity.biochem.duke.edu/).

**Table 2:** Summary statistics of the MolProbity analysis of the three-dimensional molecular model of the Cupin 2 domain of Cfvic protein. The analysis was performed by submitting the three-dimensional molecular model to the MolProbity web server (http://molprobity.biochem.duke.edu/).

Table 1

| Nº | Ratio m/z | F3/P1 peptides | Intensity Sample | Alignment | Query Cover | % Ident | Species | E value | Accession |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 580.795 | QIQNLENYR | 1.73E5 | Vicilin | 100% | 100% | *Vigna unguiculata* | 0.006 | AWS21467.1 |
| 2 | 561.799 | QNKELATYR | 1.34E | Hypothetical protein | 100% | 88.89% | *Vigna angularis* | 0.37 | KOM40650.1 |
| 3 | 472.767 | SNQLQNLK | 6.76E5 | Hypothetical protein | 87% | 85.71% | *Vigna angularis* | 39 | KOM35484.1 |
| 4 | 730.325 | GQNNPFYFDSDR | 1.64E5 | Vicilin | 100% | 100% | *Vigna unguiculata* | 4e-06 | CAP19902.1 |
| 5 | 752.338 | GENNPFYFSSDR | 2.75E5 | β-conglycinin | 100% | 83.33% | *Vigna unguiculata* | 6e-04 | XP_027906391.1 |
| 6 | 1067.05 | IPAGTTFFLVNPDDNENLR | 1.74E5 | Vicilin | 100% | 100% | *Vigna unguiculata* | 6e-13 | AWS21467.1 |
| 7 | 701.339 | NILEASFDSDFK | 9.95E4 | Vicilin | 100% | 100% | *Vigna unguiculata* | e3-05 | CAP19902.1 |
| 8 | 584.844 | ALLTLVNPDGR | 1.1E6 | Vicilin | 100% | 90.91% | *Vigna unguiculata* | 0.017 | CAP19902.1 |
| 9 | 584.844 | AILTLVNPDGR | 1.1E6 | Vicilin | 100% | 100% | *Vigna unguiculata* | 8e-04 | CAP19902.1 |
| 10 | 437.78 | ATVLLMVK | 1.1E6 | Uncharacterized protein | 75% | 100% | *Glycine max* | 56 | XP_006579169.1 |
| 11 | 573.294 | GGGGGGGLGSGGSLR | 1.87E5 | Hypothetical protein | 93% | 92.31% | *Vigna unguiculata* | 0.29 | QCD99976.1 |
| 12 | 616.81 | SGGGGGGVAGAATASR | 1.23E6 | Hypothetical protein | 93% | 75% | *Vigna angularis* | 2.8 | BAU02679.1 |
| 13 | 699.853 | DLDLFISSVDMK | 1.31E5 | β-conglycinin | 100% | 100% | *Vigna angularis* | 1e-05 | XP_017433627.1 |
| 14 | 523.784 | LSSPATLASSL | 2.77E6 | Hypothetical protein | 90% | 88.89% | *Vigna angularis* | 18 | KOM38999.1 |
| 15 | 403.716 | NFLAGEK | 2.29E5 | β-conglycinin | 100% | 100% | *Vigna unguiculata* | 7.7 | XP_027937577.1 |
| 16 | 405.229 | LASYISR | 1.75E6 | Hypothetical protein | 85% | 100% | *Phaseolus vulgaris* | 89 | XP_007158718.1 |
| 17 | 405.229 | IASYLSR | 1.75E6 | Hypothetical protein | 100% | 85.41% | *Vigna unguiculata* | 63 | QCD98090.1 |
| 18 | 405.229 | LASYLSR | 1.75E6 | Hypothetical protein | 100% | 100% | *Phaseolus vulgaris* | 11 | XP_007134114.1 |
| 19 | 484.331 | AIVILVVNK | 0 | Vicilin | 100% | 100% | *Vigna unguiculata* | 0.091 | CAP19902.1 |
| 20 | 569.262 | ISDINSAMDR | 2.32E4 | Hypothetical protein | 90% | 77.78% | *Phaseolus vulgaris* | 15 | XP_007131795.1 |
| 21 | 435.779 | VVSLSIPR | 5.58E7 | Uncharacterized protein | 87% | 100% | *Glycine max* | 14 | XP_003532703.2 |
| 22 | 637.325 | NQEVEEERIK | 9.47E5 | Hypothetical protein | 90% | 87.50% | *Vigna angularis* | 3.7 | KOM45384.1 |
| 23 | 696.358 | ALSSQNEPFNLR | 1.34E5 | Vicilin | 91% | 100% | *Vigna unguiculata* | 3e-04 | AWS21467.1 |
| 24 | 396.225 | ALESLMK | 1.89E5 | Uncharacterized protein | 85% | 100% | *Vigna angularis* | 44 | XP_017410294.1 |
| 25 | 396.225 | ALESIMK | 1.89E5 | Hypothetical protein | 85% | 100% | *Glycine max* | 31 | KAG4906660.1 |
| 26 | 396.225 | AIESLMK | 1.89E5 | Hypothetical protein | 85% | 100% | *Glycine max* | 31 | KAG4999608.1 |
| 27 | 598.794 | NFLAGGKDNV | 2.65E4 | β-conglycinin | 100% | 90% | *Glycine max* | 0.16 | AAB01374.1 |
| 28 | 571.27 | YDKELWCK | 2.3E5 | Uncharacterized protein | 87% | 85.71% | *Glycine max* | 39 | XP_006599667.1 |

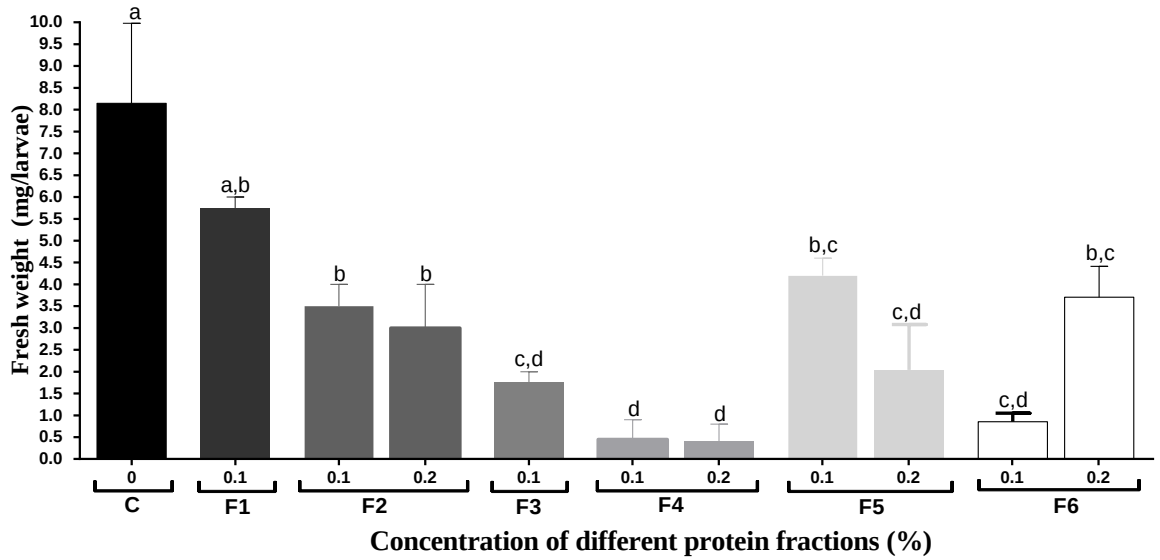



Figure 1

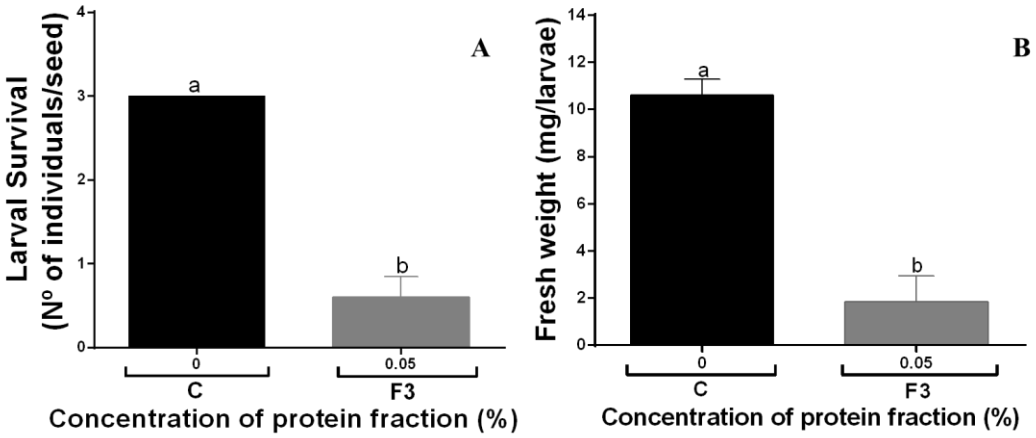
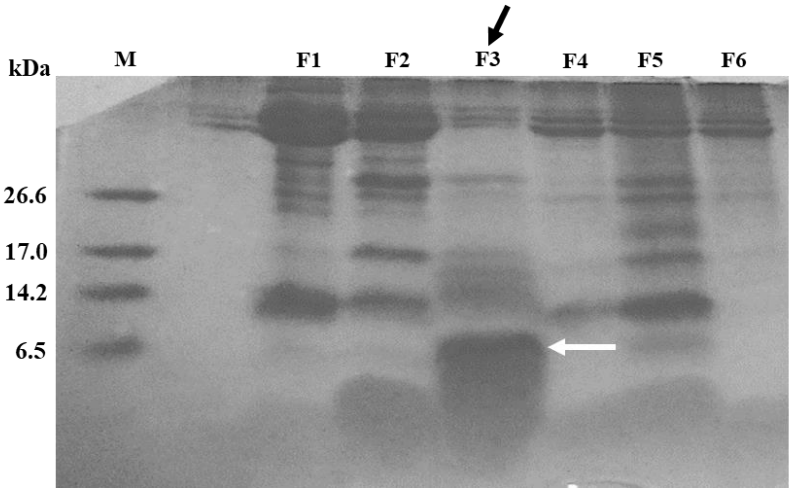



Figure 2

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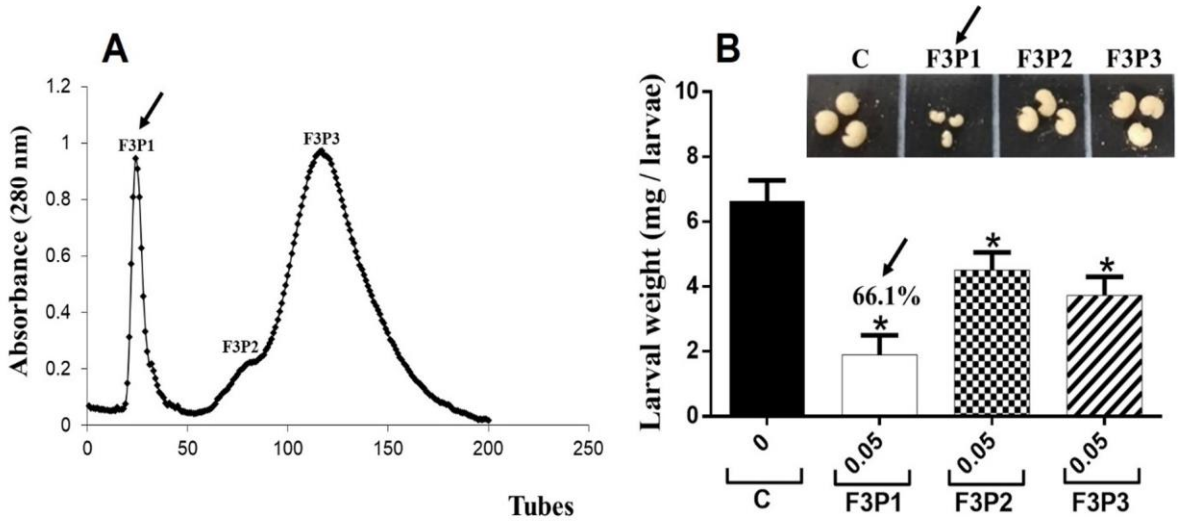



Figure 4

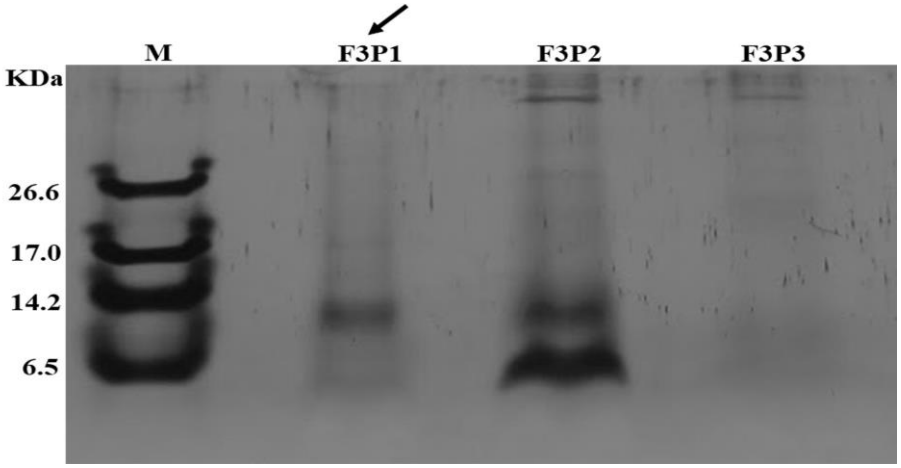
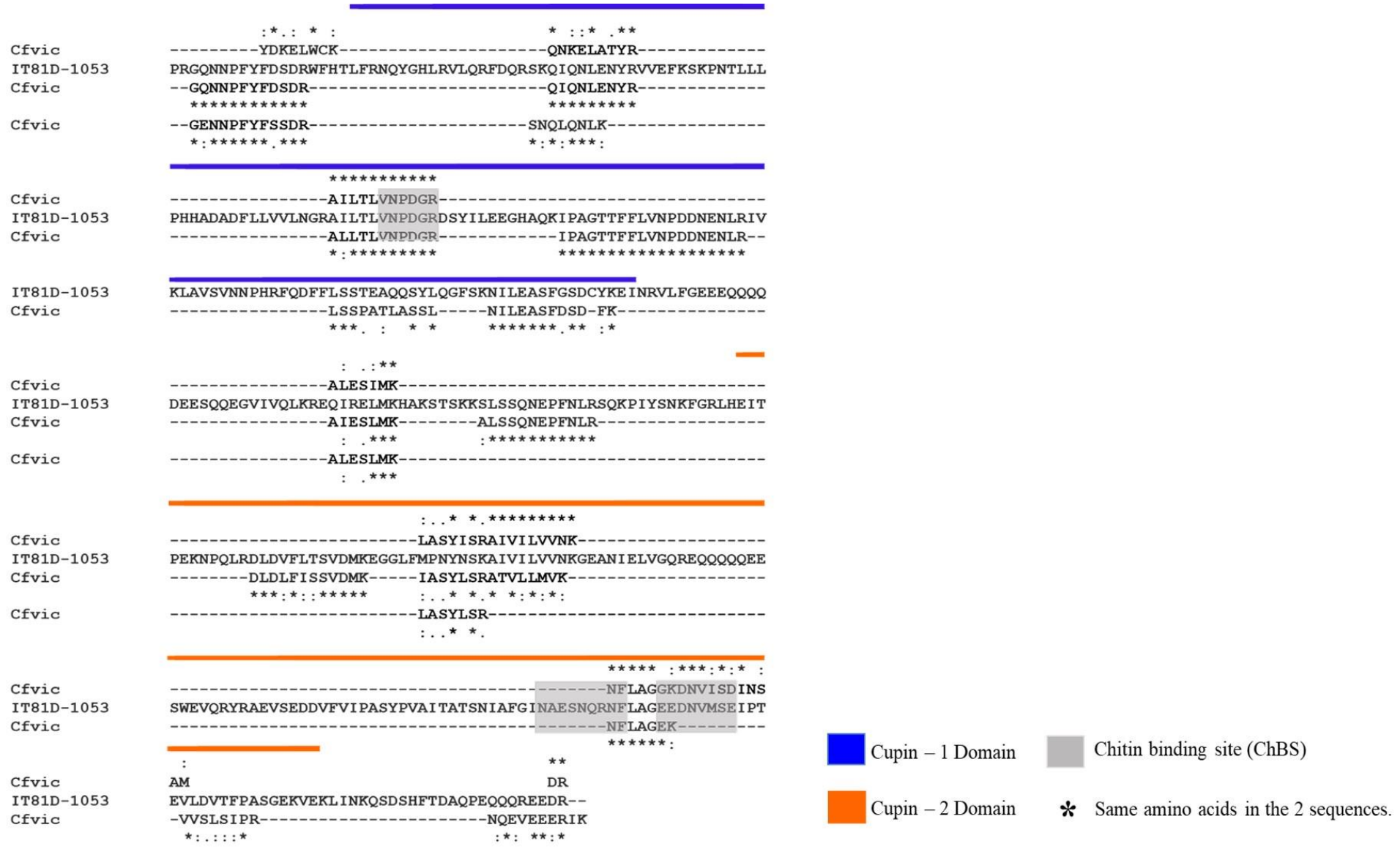



Figure 5


Cfvic
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Cupin – 1 Domain
Chitin binding site (ChBS)
Cupin – 2 Domain
Same amino acids in the 2 sequences.

Figure 6

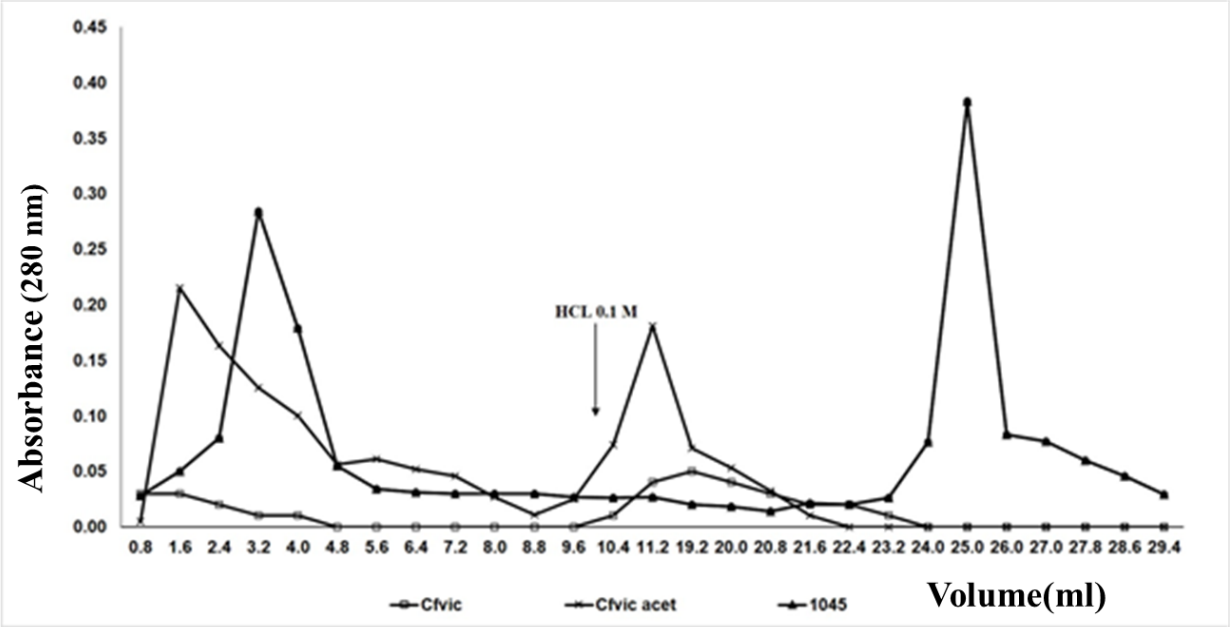



Figure 7

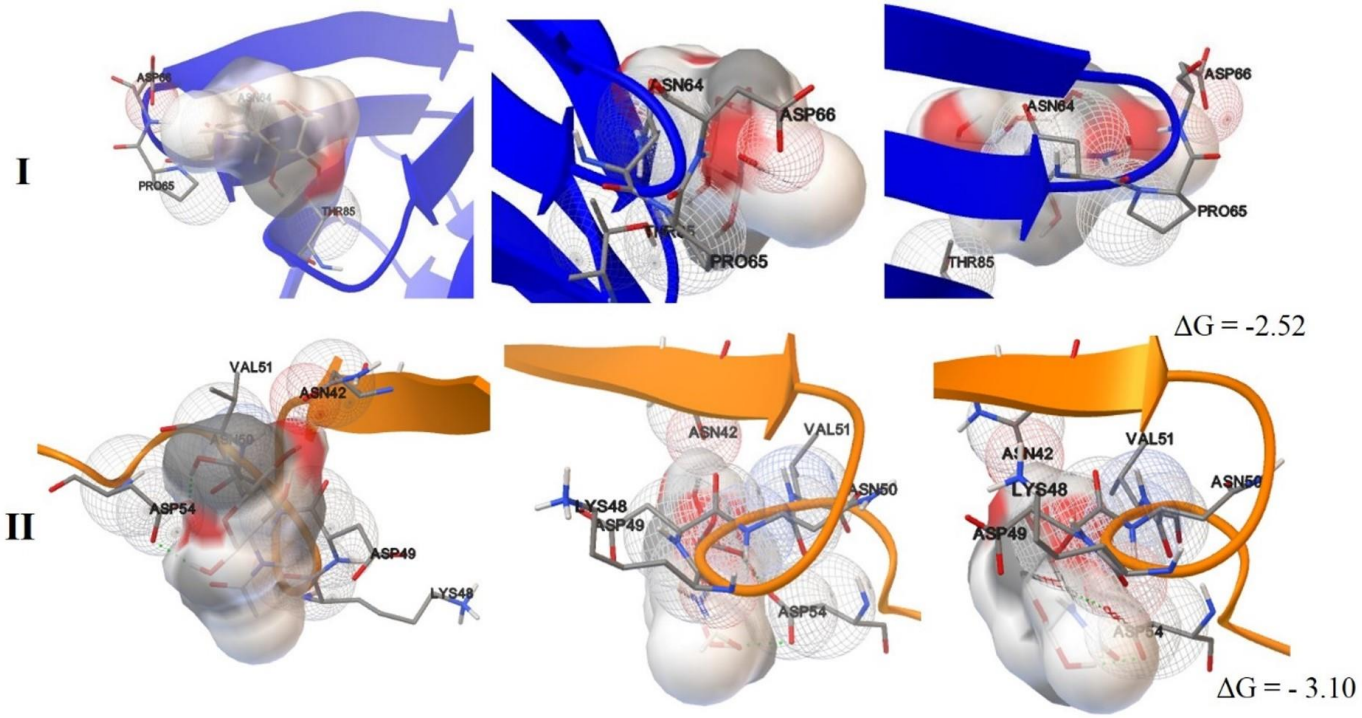



Figure 8

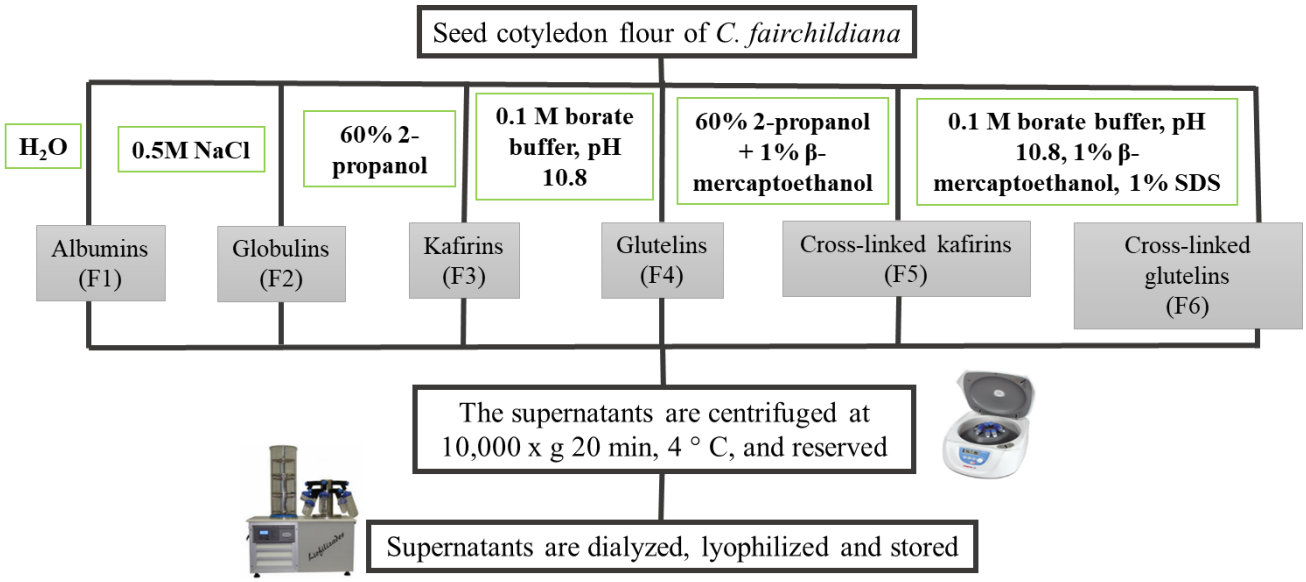



Figure 1 (Supplementary)

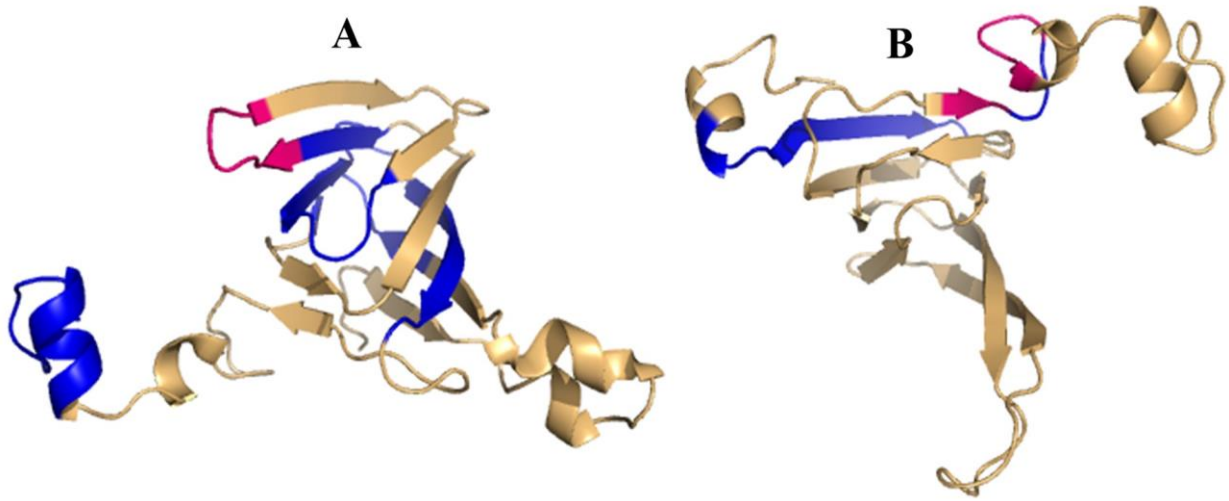
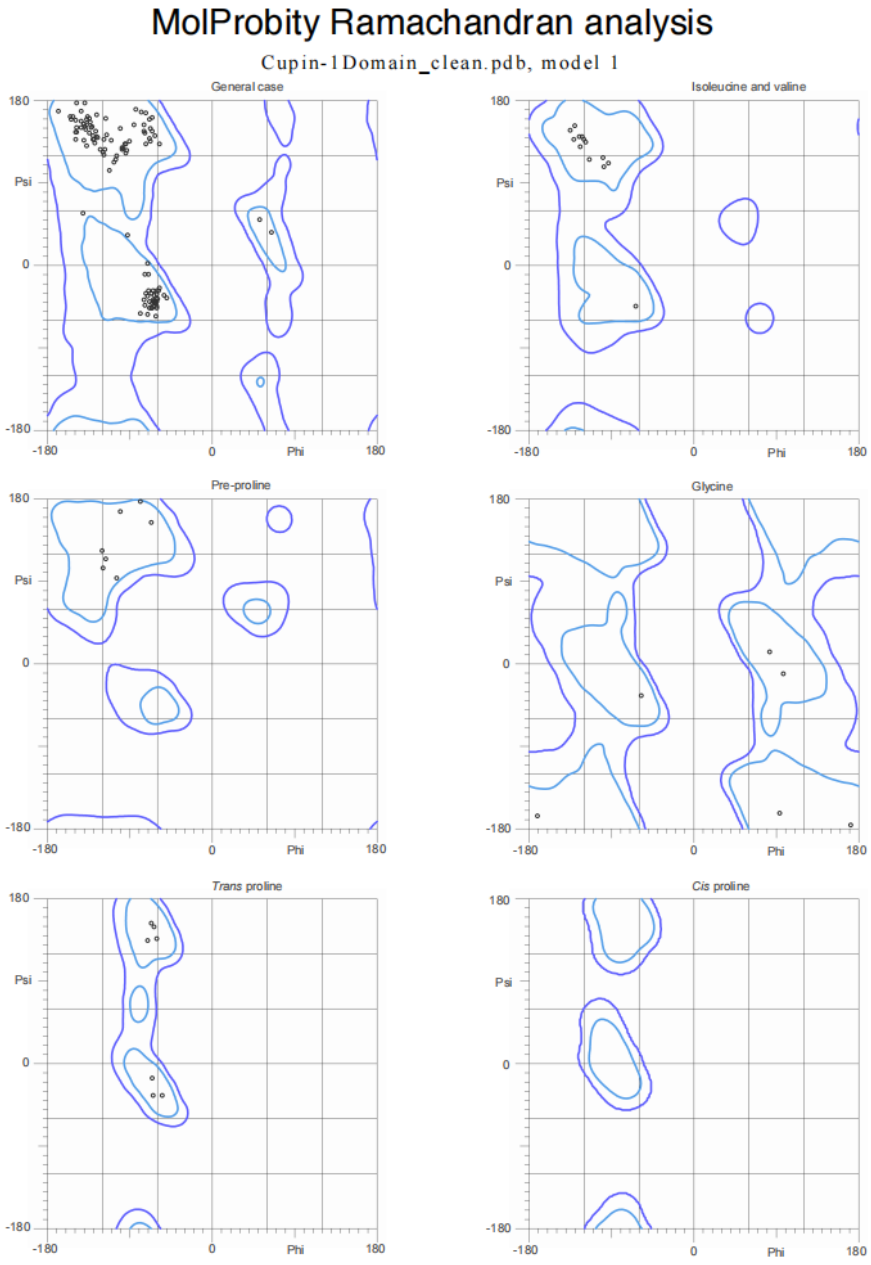



Figure 2 (Supplementary)



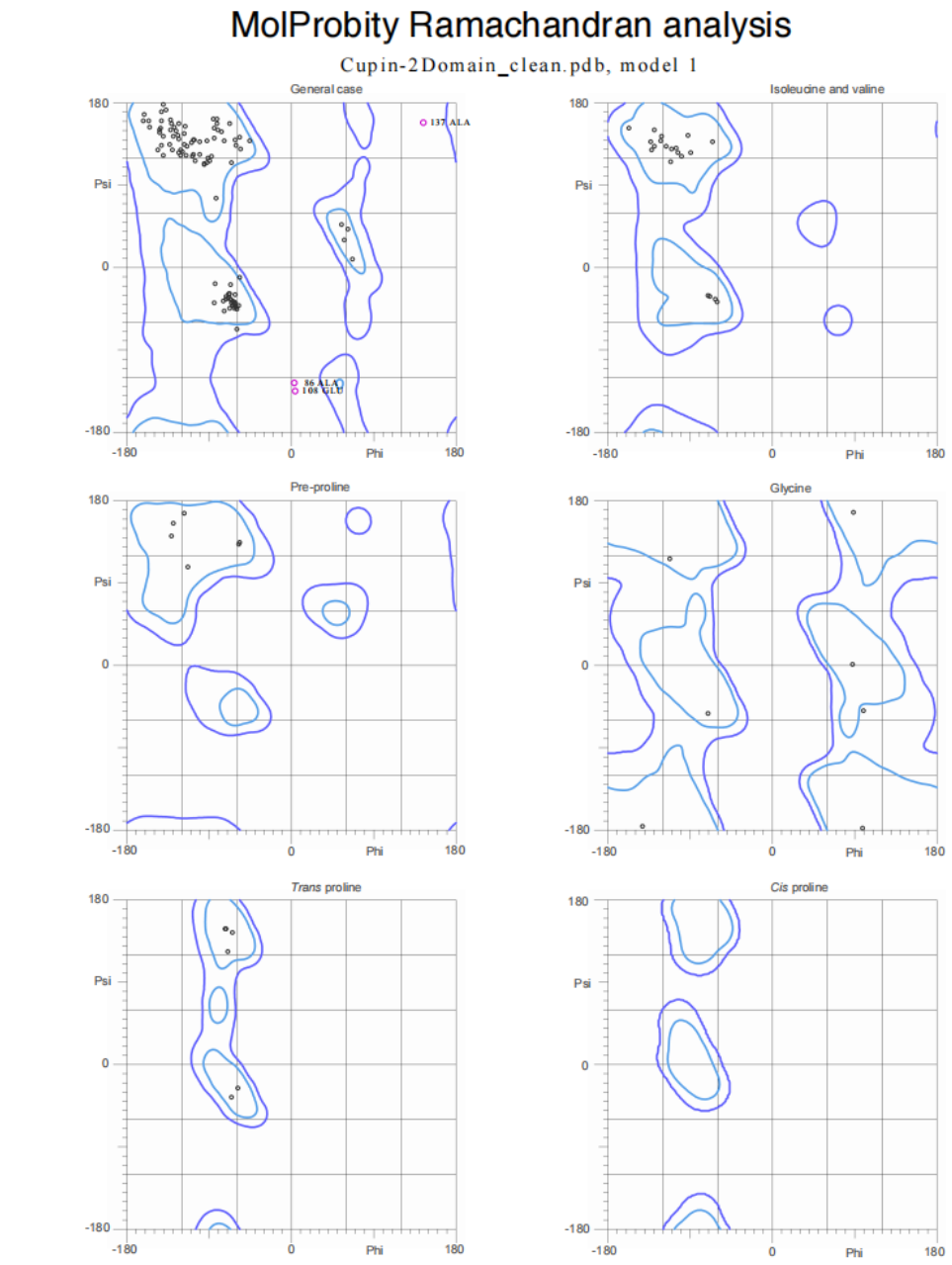

98.6% (143/145) of all residues were in favored (98%) regions.
100.0% (145/145) of all residues were in allowed (>99.8%) regions.

There were no outliers.

http://kinemage.biochem.duke.edu

Lovell, Davis, et al. Proteins 50:437 (2003)

Figure 3 (Supplementary)




96.5% (136/141) of all residues were in favored (98%) regions.
97.9% (138/141) of all residues were in allowed (>99.8%) regions.

There were 3 outliers (phi, psi):
86 ALA (3.7, -127.5)
108 GLU (4.3, -136.8)
137 ALA (145.5, 159.2)

http://kinemage.biochem.duke.edu

Lovell, Davis, et al. Proteins 50:437 (2003)

Figure 4 (Supplementary)

Table S1 - Summary statistics of the MolProbity analysis of the three-dimensional molecular model of the Cupin 1 domain of *Cf*vic protein. The analysis was performed by submitting the simulated three-dimensional molecular model to the MolProbity web server (http://molprobity.biochem.duke.edu/).

| | | | | |
|---|---|---|---|---|
| Protein Geometry | Poor rotamers | 6 | 4.51% | Goal: <0.3% |
| | Favored rotamers | 116 | 87.22% | Goal: >98% |
| | Ramachandran outliers | 0 | 0.00% | Goal: <0.05% |
| | Ramachandran favored | 143 | 98.62% | Goal: >98% |
| | Cβ deviations >0.25Å | 0 | 0.00% | Goal: 0 |
| | Bad bonds: | 0/ 1226 | 0.00% | Goal: 0% |
| | Bad angles: | 27 / 1664 | 1.62% | Goal: <0.1% |
| Peptide Omegas | Cis Prolines: | 0 / 7 | 0.00% | Expected: ≤1 per chain, or ≤5% |

† - In the two column results, the left column gives the raw counts, right column gives the percentages.

Table S2 - Summary statistics of the MolProbity analysis of the three-dimensional molecular model of the Cupin 2 domain of Cfvic protein. The analysis was performed by submitting the three-dimensional molecular model to the MolProbity web server (http://molprobity.biochem.duke.edu/).

| | | | | |
|---|---|---|---|---|
| Protein Geometry | Poor rotamers | 3 | 2.44% | Goal: <0.3% |
| | Favored rotamers | 108 | 87.80% | Goal: >98% |
| | Ramachandran outliers | 3 | 2.13% | Goal: <0.05% |
| | Ramachandran favored | 136 | 96.45% | Goal: >98% |
| | Cβ deviations >0.25Å | 0 | 0.00% | Goal: 0 |
| | Bad bonds: | 0 / 1129 | 0.00% | Goal: 0% |
| | Bad angles: | 20 / 1526 | 1.31% | Goal: <0.1% |
| Peptide Omegas | Cis Prolines: | 0 / 6 | 0.00% | Expected: ≤1 per chain, or ≤5% |

† - In the two column results, the left column gives the raw counts, right column gives the percentages.

# Capítulo 2 – Compostos do metabolismo secundário de sementes de *Clitoria fairchildiana* tóxicos ao mosquito *Aedes aegypti*

(Artigo submetido a revista *Pesticide Biochemistry and Physiology*)

# Rotenoids from *Clitoria fairchildiana* (Fabaceae) seeds affect the cellular metabolism of larvae of *Aedes aegypti* L. (Culicidae)

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**Abstract**

Plant bioactive compounds have shown great potential as bioinsecticidal agents and therefore have become an alternative for the development of new insecticide formulations. Non-domesticated species may represent a treasure chest of defensive molecules which must be investigated and rescued. *Clitoria fairchildiana* is a non-domesticated Fabaceae, native from the Amazonian Forest whose seeds are exquisitely refractory to insect predation. Secondary metabolites from these seeds have been fractionated by different organic solvents and the $CH_2Cl_2$ fraction (CFD - Dichloromethano *Clitoria fairchildiana*), as the most toxic to 3rd instar *Aedes aegypti* larvae ($LC_{50}$ 180 PPM), was subjected to silica gel chromatography, eluted with a gradient of $CH_2Cl_2$: MeOH and sub fractioned in nine fractions (CFD1 - CFD9). All obtained fractions were tested in their toxicity to the insect larvae. Two rotenoids, a 11α-O-β-D-glucopyranosylrotenoid and a 6-deoxyclitoriacetal 11-O-n-glucopyranoside, were identified in the mixture of CFD 7.4 and CFD 7.5, and they were toxic ($LC_{50}$ 121.2 PPM) to 3rd instar *Aedes aegypti* larvae, leading to exoskeleton changes, cuticular detachment and perforations in larval thorax and abdomen. These *C. fairchildiana* rotenoids have interfered with the acidification process of cell vesicles in larvae midgut and caused inhibition of 55% of V-ATPases activity of larvae treated with 80 PPM of the compounds, when compared to control larvae. The rotenoids have also led to a significant increase in the production of reactive oxygen species (ROS) in treated larvae, especially in the hindgut region of larvae intestines, indicating a triggering of an oxidative stress process to these insects.

**Keywords:** Glucopyranosylrotenoid; Butterfly pea tree; Dengue mosquitoes; Oxidative stress; V $H^+$-ATPase activity.

## 1. Introduction

*Aedes aegypti* is a vector of several arboviruses, such as dengue, yellow fever, chikungunya and zika (Webb, 2016). Due to the wide distribution of this vector, some of these arboviruses have spread rapidly around the world in recent years, causing serious epidemics, which has made it a vector of clinical importance in various regions of the planet (Patterson et al., 2016; Silva et al., 2020). Dengue is the most prevalent among these diseases and can affect 3.9 billion people in 129 countries, being associated to 96 million symptomatic cases and 40,000 deaths per year (WHO, 2021).

Strategies to control the mosquito populations have become necessary due to the lack of effective vaccines and specific viral drugs to deal with the transmitted arboviruses. The use of insecticides is one of the most frequent employed prevention strategies (WHO, 2021). However, due to the development of resistance by the *Ae. aegypti* insect, it has been necessary to replace these insecticides formulations frequently over the years. Initially organochlorines were replaced by organophosphates and these were then replaced by pyrethroids (Nauen, 2008). However, there are already reports of resistance to pyrethroids by some *Ae. aegypti* populations (Zheng et al., 2019). Another drawback for this strategy is the insecticides pollution of earth and aquatic biomes (Halstead et al., 2015), what represents a growing concern for life quality on the planet.

Thus, the search for natural compounds with insecticidal activity aims not only to expand the range, enabling an alternation of insecticides, reducing the development of resistance in *Ae. aegypti*, but also the use of molecules that may be less harmful to natural ecosystems. These compounds are usually biodegradable and inexpensive. Oliveira et al. (2016) showed that sisal extract increases nitric oxide production in *Ae. aegypti* hemocytes, inducing cell death. This result suggested that this extract could be used as a raw material for new ecological and cheap insecticides. Procópio et al. (2015) observed that the extract from leaves of *Schinus terebinthifolius* caused damage to the midgut and DNA fragmentation, interfering with the development and survival of *Ae. aegypti* larvae. The authors attributed the larvicidal effect of this extract to derivatives of cinnamic acid and flavonoids. Larvicidal potential of seventeen cinnamic acid derivatives against fourth instar larvae of *Ae. aegypti* has been shown and through molecular modeling analysis the larvicidal activity of these compounds was linked to a multi-target mechanism of action involving inhibition of a carbonic anhydrase (CA), a histone deacetylase (HDAC2), and two sodium-dependent cation-chloride co-transporters (CCC2 e CCC3) (Araújo et al., 2020).

The evolutionary cycle of this vector insect is of the holometabolous type, divided into egg, larva (4 stages), pupa and adult (Christophers, 1960).

In this study, we evaluated the larvicidal activity of secondary metabolites from cotyledons of *Clitoria fairchildiana* seeds towards third instar larvae of *Ae. aegypti*. This species is an undomesticated legume, native to the Amazon region, commonly named as the butterfly pea tree, whose seeds are highly refractory to predation by insects since there are no reports in the literature on predation of its seeds by any known insect class. We identified two rotenoids, highly toxic to the insect, which were seen to cause relevant diminishment of acid compartments in insect larvae midguts, inhibition of V-ATPase activity in midgut cells and a significant increase in the levels of reactive oxygen species (ROS), suggesting a mechanism of action through a burst of oxidative stress in the digestive system of these larvae.

## 2. Materials and methods

### 2.1. Biological Materials

#### 2.1.1. *Clitoria fairchildiana*

Seeds of *C. fairchildiana* trees were collected on the campus of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil. They were then dried at 28°C and stored for further work. The voucher number HUENF 9492 has been allocated for the plant material exsiccate deposited in the University Herbarium.

#### 2.1.2. Insects

*Aedes aegypti* larvae of the Rockefeller strain were obtained from the insectary kept at the Laboratory of Biotechnology (LBT) of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF). Mosquitoes were raised at room temperature between 20º and 30º C, in cages, and fed on sterile 10% sucrose solution. A glass container filled with sterile distilled water was kept inside each cage to maintain humidity. Larvae were fed on a sterile finely ground commercial fish food. Pupae were rinsed and transferred to sterile distilled water and maintained in separate cages until adult emergence. Production of eggs was induced after providing blood meal (mouse or lamb) to the insects. Females laid eggs on moisture filter paper 3 and 4 days after blood meal. After 2 days, desiccated eggs were transferred to plastic containers filled with deoxygenated distilled water for larvae eclosion.

### 2.2. Extraction and isolation of secondary compounds of *C. fairchildiana* seeds

The procedures were performed as described by Passos et al. (2019). The quiescent seeds were dried in an oven for 72 h at 37ºC, dehulled and the cotyledons were ground with a SL-30

SOLAB seed crusher until the flour was obtained, which was then sieved in a 48-mesh sieve. The flour obtained (412.6 g) was extracted with 1.1 L of methanol at room temperature for 36 h. The obtained extract was submitted to a rotaevaporator for evaporation and condensation and then resuspended in a solution of MeOH:$H_2O$ (1:3 v/v) and partitioned with $CH_2Cl_2$, EtOAc and n-butanol, sequentially. The $CH_2Cl_2$ fraction (CFD) was subjected to silica gel chromatography (SGC), eluted with a gradient of $CH_2Cl_2$: MeOH and nine subfractions were obtained (CFD1 - CFD9). Out of these, the CFD7 fraction (1.21 g) was rechromatographed through SGC, eluted with a gradient of $CH_2Cl_2$: MeOH and produced six subfractions (CFD7.1 - CFD7.6). Rotenoids were isolated and identified in the mixture of CFD 7.4 and CFD 7.5 fractions.

### 2.3. Chemical characterization of compounds from secondary metabolism of *C. fairchildiana* seeds

NMR analysis was performed on a Bruker Ascend 500 (500 MHz for 1H and 125 MHz for 13C) and we used TMS as an internal standard. Chemical changes ($\delta$) in ppm and coupling constants (J) in Hz were analysed following Passos et al. (2019). We performed the UPLC analysis on a Shimadzu LC-20A instrument (Kyoto, Japan). The separation was made on a Shimadzu XR-ODS C18 column (75 mm x 2.1 mm; Phenomenex, Torrance, CA, USA). The mobile phases used were: A - ultra pure water with 0.1% formic acid and B - acetonitrile with 0.1% formic acid. The gradient elution was: 0–1 min 30 % B, 1–15 min 30–52 % B, 15–17 min 52–100 % B, 17–20 min 100 % B, 20–23 min from 100 – 30 % B, 23–25 min 30 % B. The injection volume was of 20 μL of the initial CFD 7.4 and CFD 7.5 fractions mixture. The UV/ DAD was monitored at 280 nm. The HRESI-MS mass spectra was obtained on a microOTOF-Q II BrukerDaltonics mass spectrometer, using the positive ion analysis mode (Mathias and Oliveira, 2019).

### 2.4. Larvicide activity assay

The larvicide assay of the extracts and fractionated compounds was carried out in accordance with the standards established by WHO (2005). From a stock solution, where the extracts and compounds were diluted in ultrapure water, different dilutions were prepared for the tests. We used 10 third instar larvae for 20 ml of each solution and the tests were performed in triplicate. The negative control was done using ultrapure water. The larvae were incubated for 24 h at 28º C. After this incubation period, mortality was analyzed by the movement of larvae, and these were also observed under a stereomicroscope. Statistical analysis of data and $LC_{50}$ were obtained using the Graph Pad Prism 6 program.

### 2.5. Morphological effects of *C. fairchildiana* rotenoids on *Ae. aegypti* larvae

Third instar *Ae. aegypti* larvae were divided into groups of 15 larvae for each treatment. Two test groups and a negative control group were used. Firstly, all groups were treated equally with rotenoids (80 PPM and 160 PPM) and the negative control larvae group with ultrapure water for 24 h. Then the larvae were fixed in an aqueous solution containing 2.5 % glutaraldehyde, 4 % formaldehyde and 0.1 M phosphate buffer for 24 h. Subsequently, the samples were subjected to three washes with 0.1 M phosphate buffer for 30 min and post-fixed in 1 % osmium tetroxide and 0.1 M phosphate buffer for 1 h. After washing again in the same buffer, the larvae were dehydrated in a crescent series of acetone (20 %, 50 %, 70 %, 90 % and 3 times 100 % super-dry), with a duration of 1 h, at each step. After dehydration, the larvae were subjected to a critical point to replace all acetone with liquid $CO_2$ under high pressure conditions (Bal-Tec Critical Point Dryer - CPD 030). Images were obtained with a scanning electron microscope (Zeiss – EVO 40) at a voltage acceleration of 15 kV.

### 2.6. Investigation of *C. fairchildiana* rotenoids action on *Ae. aegypti* metabolism

#### 2.6.1. Acidic compartments analysis

Third instar *Ae. aegypti* larvae were divided into groups of 6 larvae for each treatment. Two test groups and a negative control group were used. All groups were initially treated equally, with a solution containing 10 mM DTT (Edwards and Jacobs-Lorena, 2000), 0.03 % agar and 50 µg/mL gentamicin, overnight. Afterwards, the larvae were washed with ultrapure water. Then, the test larvae were treated with CFD-RI and CFD-RII rotenoids (0.08 mg/mL and 0.16 mg/mL, respectively) and the negative control larvae with ultrapure water, for 24 h. After that time, 10 mM quinacrine dihydrochloride (Sigma Aldrich-Q3251) was added for 2 h. Finally, the larvae were washed and transferred to microplate wells filled with water. The fluorescence labeling was observed by microscopy (Axioplan-Carl Zeiss) and to quantify it a Zen 2.3 software (Blue Edition) was employed. All larvae observed had their proventriculus active, indicating that they were alive at the time of analysis.

#### 2.6.2. Isolation of membrane vesicles

Isolation of total membranes was performed by differential centrifugation as described by Ribeiro et al. (2012), with some adaptations. A number of 400 larvae were used for control and another 400 larvae were tested with rotenoids (treated with 80 PPM of the solution containing CFD-RI and CFD-RII). After treatment for 24 h, these larvae were homogenized in a glass

homogenizer with a Teflon pistil ("potter") in an extraction buffer pH 7.0 (250 mM sucrose, 10 % glycerol, 1 mM EDTA, 0.1 mM Tris, 0.4 % PVPP, 0.08 % BSA, 2 mM DTT, 1 mM PMSF, 1 mM benzamidine and 0.01 % protease inhibitor cocktail). After maceration of the larvae in the buffer, the homogenate was subjected to the first centrifugation (HITACHI Himac centrifuge), 1,000 g at 4ºC for 20 min, to eliminate nuclei and impurities. The pellet was discarded, and the supernatant was subjected to another centrifugation (10,000 g, 4ºC, 20 min). The precipitate corresponding to the mitochondria was resuspended in 1 ml of resuspension medium (20 mM MOPS pH 7.2, 10 % glycerol, 12.5% sucrose, 1 mM PMSF, 1 mM benzamidine, 2 mM DTT and 0.01 % protease inhibitor cocktail). The supernatant was subjected to another centrifugation (100,000 g, 4ºC, 1 h). The precipitate corresponds to the microsomal fraction, which contains the plasma membranes of vesicles, which was resuspended in 1 mL of resuspension medium. The samples were kept in cryogenic Eppendorf tubes, stored at -70ºC for further analysis.

### 2.6.3. V H+-ATPase activity assay

V-ATPase activity was determined as described by Ribeiro et al. (2012), with adaptations. Microsomal fraction (0.30 mg/mL of isolated vesicles) from the larvae was added to 1 ml reaction containing HEPES-Tris buffer pH 7.2 (50 mM), KCl (100 mM), $MgSO_4$ (5 mM), ATP (1 mM). The inhibitor used was concanamycin (0.1 mM). The hydrolysis reaction was stopped by adding 200 mL of 20 % TCA (trichloroacetic acid) at the times of 0, 15, 30, 45 and 60 min from the start. Subsequently, 0.5 mL of dye solution was added to each tube, from the stock solution (5 % ammonium molybdate solution and 2.06 % sulfuric acid) with 5 % ascorbic acid in a 100:1 ratio. After 10 min, the absorbance reading of the samples was taken in a spectrophotometer at 750 nm. The ATPase activity was determined by the measurement of inorganic phosphate (Pi) released during the hydrolysis of ATP, through the difference between the total activities of the enzymes when no inhibitor is added and the activity of the enzymes in the presence of the inhibitor.

### 2.6.4. Oxidative stress analysis

Third instar *Ae. aegypti* larvae were divided into groups of 6 larvae for each analysis. Two test groups and a negative control group were used. Firstly, all groups were treated equally with a solution containing 10 mM DTT (Edwards and Jacobs-Lorena, 2000), 0.03 % agar and 50 µg/mL gentamicin, overnight. Afterwards, the larvae were washed with ultrapure water. Then the test larvae were treated with CFD-RI and CFD-RII rotenoids (0.08 mg/mL and 0.16 mg/mL, respectively) and the negative control larvae with ultrapure water, for 24 h. After that time, 10 mM ROS marker 2',7' dichlorofluorescein diacetate (Sigma Aldrich - D6883) was added for 2 h.

Finally, the larvae were washed and transferred to microplate wells filled with water. The fluorescence labeling was observed by microscopy (Axioplan-Carl Zeiss) and to quantify it, the Zen 2.3 software (Blue Edition) was employed.

## 3. Results and discussion

### 3.1 Secondary metabolites of *C. fairchildiana* toxic to 3rd instar larvae of *Ae. aegypti*

The total MetOH extract of *C. fairchildiana* cotyledons caused the death of all *Ae. aegypti* larvae at concentrations of 0.1 %, 1 % and 2 % (data not shown). From this initial MetOH extract, the partition of dichloromethane ($CH_2Cl_2$) was the one, among the four obtained, that showed toxicity to the larvae. Survival analysis (Fig. 1A) showed significant reduction for 73 % of control larvae at the concentration of 0.1 mg/mL, for 43 % at 0.2 mg/mL, and for 0 % survival at concentrations of 0.4 mg/mL onwards. The observed $LC_{50}$ (Fig. 1B) was 0.18 mg/mL or 180 PPM. When considering the anatomical aspects of these larvae (Fig. 2), it was possible to observe a whitish excretion in the mouth of the larvae raised at a 0.2 mg/mL of $CH_2Cl_2$. There was also an indication of intoxication and the appearance of dark spots along the body of the larvae at concentrations of 0.4, 0.8 and 1 mg/mL, suggesting some possible melanization responsive process, since melanization has been associated to diverse insect immunity abilities, as reviewed by Nakhleh et al. (2017).

Secondary metabolites isolated extracted from the partition of dichloromethane ($CH_2Cl_2$) of *C. fairchildiana* seeds were analysed by a set of two-dimensional nuclear magnetic resonance spectroscopy techniques (Table S1 and Figs. S1 a S5 -Suppl. Material). They were identified as two rotenoids (Fig. 3) and named CFD-RI and CFD-RII. They are isomers, with CFD-RI being present in greater amounts (75.9%) than CFD-RII (24.1%). Both had been previously described in the literature: CFD-RI isolated from *C. fairchildiana* seeds and identified as 11α-O-β-D-glucopyranosylrotenoid (MATHIAS et al., 1998) and CFD-RII, from *C. fairchildiana* roots and identified as 6-deoxyclitoriacetal 11-O-n-glucopyranoside ((SILVA; BERNARDO; PARENTE, 1998). However, no biological activity had been attributed to either of them. The molecular mass of the isolated rotenoids were determined and confirmed by high resolution mass spectrometry (HRESIMS; Fig. S6 – Suppl. Material). Tandem mass spectrometry (MS/MS) of the major CFD-RI compound of m/z 341.1052 is shown in Fig. S7 (Suppl. Material). A fragmentation mechanism of CFD-RI is proposed in Fig. S8 (Suppl. Material), based on both mass spectra profiles shown in Figs. S6 and S7. Tandem mass spectrometry (MS/MS) of the minor component CFD-RII compound of m/z 357.3300 is shown in Fig. S9 (Suppl. Material). A fragmentation mechanism of

CFD-RII is proposed in Fig. S10 (Suppl. Material), based on both mass spectra profiles shown in Figs. 8 and 9 (Suppl. Material).

Rotenoids are natural compounds considered chemical relatives of rotenone. Due to its properties, rotenone is considered ideal for use as an agricultural insecticide, as it is susceptible to photo decomposition by ultraviolet light (sunlight), has a toxicity with a half-life of 1 to 3 days, does not threaten as a pollutant of groundwater as it rapidly deteriorates in the soil and, furthermore, its absorption via the gastrointestinal tract is minimal and readily degraded by the liver (LAZO; GUILLOT; MILLER, 2014). The larvicidal activity of the isolated CFD-RI and CFD-RII rotenoids (Fig. 4A) showed that from the concentration of 0.08 mg/mL there was a significant reduction in larval survival to 67%; with 0.16 mg/mL, the survival dropped down to 33%; with 0.32 mg/ml, to 7%; and 0.64 mg/ml, to 0%. The $LC_{50}$ was calculated as 0.121 mg/ml or 121.2 PPM (Fig. 4B).

Morphological changes in the exoskeleton as a whole and more noticeable in the cuticle of larvae treated with the isolated rotenoids were observed by scanning electron microscopy (Fig. 5). As indicated by the arrowheads, the treatment provoked an apparent cuticular detachment in the thorax and in the abdomen of the treated larvae (80 PPM and 160 PPM) and the arrows show cuticle perforation in the thorax and abdomen regions.

Rotenone is a known inhibitor of complex I of the electron transport chain (ETC) since pioneer works from Palmer and coworkers (1968). Due to the rotenoid nature of *C. fairchildiana* isolated compounds, we investigated possible alterations in acidification processes of cell vesicles, using a specific-fluorescent marker. The acridine derivative quinacrine which is a weak base that binds ATP (Bodin and Burnstock, 2001). The results (Fig. 6A) showed that quinacrine effectively marked the acidified vesicles of the untreated larvae (green staining highlighted by the arrowheads). It is possible to observe the marking of acidified vesicles in both the anterior and the posterior midguts. After treatments with rotenoids (0.08 and 0.16 mg/ml), the diminishment of acid compartments through fluorescence quenching was noticeable. The quantification of this quenching (Fig. 6B - I and II) in the anterior midgut showed that it was 3.2 (with 0.08 mg) and 4 times (with 0.16 mg) lower compared to the control larvae and, in posterior midgut, 1.7 (with 0.08 mg) and 2.6 times (with 0.16 mg) than the control. These results indicated that rotenoids affected the acidification of midgut cells of larvae and, consequently, the cell metabolism. According to Zhuang et al. (1999), V-ATPases are responsible for the acidification of these vesicles. Therefore, we measured the activity of these enzymes and observed an inhibition of 55% when compared to the rate of V-ATPases activity in control larvae. It was also possible to observe (Fig. 7) increasing reductions, in the enzyme rate, of 1.8, 1.9 and 2.2 times as the time of reaction went from 30 to 45

and to 60 min, respectively. Linser et al. (2009) reports that V-ATPases act by alkalinizing the midgut of these larvae and that the pH varies according to their location. We further investigate a possible induction of oxidative stress in the intestine of these larvae by the isolated CFD-RI and CFD-RII rotenoids and observed that, in the posterior midgut (PM) (Figs. 8A-I and 8B-I), it was possible to observe a significant increase in the production of reactive oxygen species (ROS) in treated larvae with the rotenoids (at 80 and 160 PPM) of about 2.2 and 2.6 times more when compared to the control larvae. and in the hindgut (HG) (Figs. 8A-II and 8B-II), even larger increases in ROS levels were noticed (5.7 and 7.4 times with each tested concentration, when compared to the control). These results suggest that these rotenoids cause oxidative stress in the intestine of these larvae and may be related to the inhibition of V-ATPases, since they are located in the apical membrane of the posterior midgut, which has longer microvilli, increasing the contact and nutrient absorption surface areas (PATRICK et al., 2006; ZHUANG; LINSER; HARVEY, 1999). We also believe that the morphological changes observed in Figure 5 can be related to the high production of reactive oxygen species (ROS) in the exoskeleton of these larvae, just as Adamski (2007) shows changes in the cuticle of *Spodoptera exigua* larvae, by metabolic disturbances caused by ROS. Zhang et al. (2019) also show oxidative damage caused by the presence of ROS in the cell cytoplasm, leading to a large outflow of $Ca^{2+}$ in the mitochondria and inducing cell necrosis that damages the structure of the peritrophic membrane, leading to barrier dysfunction in the midgut. Furthermore, oxidative stress may be causing mitochondrial dysfunction and thus inducing cell death (ZHANG et al., 2019).

Therefore, the inhibition of V-ATPase probably triggers a process of oxidative stress, which leads to changes in the exoskeleton and their death. In addition, these are water soluble which facilitates their use as larvicides and are chemical cousins of rotenone, which is considered an ideal insecticide for use in agriculture.

## 4. Acknowledgements

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ and the Conselho Nacional de Desenvolvimento Científico (CNPq). The first author was supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) MSc research grant.

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**Legends**

**Figure 1:** Survival (A) and % of mortality in parts per million (PPM) (B) of *Aedes aegypti* 3$^{rd}$ instar larvae raised on aquatic media containing increasing concentrations of dichloromethane partition ($CH_2Cl_2$) of the initial methanolic *C. fairchildiana* seed extract. C - control negative with water. $LC_{50}$ was calculated using GraphPad Prism 6. The letters a, b, c and d represent significant statistical differences according to ANOVA (Tukey's multiple comparison) (F= 85.67; DF= 6,14; P

467 < 0.0001). The experiment consisted of 10 larvae per concentration and was performed in
468 triplicate, giving a total of 30 larvae per trial.

469 **Figure 2:** General morphological features of 3rd instar *Aedes aegypti* larvae exposed to different
470 concentrations (mg/mL) of the dichloromethane partition observed under a stereomicroscope. C -
471 control group. Scale bar = 1 mm.

472 **Figure 3:** Structures of rotenoids isolated from *C. fairchildiana* seeds, elucidated by one- and two-
473 dimensional Nuclear Magnetic Resonance ($^{1}H$ e $^{13}C$ NMR).

474 **Figure 4:** Survival (A) and % of mortality in parts per million (PPM) (B) of *Aedes aegypti* 3rd
475 instar larvae raised in the presence of the isolated compounds from the dichloromethane ($CH_2Cl_2$)
476 partition. C - control negative with water. $LC_{50}$ was calculated using GraphPad Prism 6. The letters
477 a, b, c and d represent significant statistical differences according to ANOVA (Tukey's multiple
478 comparison) (F= 419.1; DF= 6,14; P < 0.0001). The experiment consisted of 10 larvae per
479 concentration and was performed in triplicate, giving a total of 30 larvae per trial.

480 **Figure 5 –** Scanning electron microscopy (SEM) of 3rd instar not treated larvae (A, D, G and J),
481 treated with 80 PPM of isolated rotenoids (B, E, H and K), and with 160 PPM of isolated rotenoids
482 (C, F, I and L) of *Aedes aegypti*. (HD – Head; THO – Thorax; ABD – Abdomen).

483 **Figure 6** - Change in acidification of vesicles in *Aedes aegypti* control and test larvae's intestines.
484 **A -** Quinacrine fluorescence labeling. The scale bar corresponds to 100µm. [Superior insert in
485 panel A – Larval body drawing (Linser et al., 2009), as guidance]; **B -** Quenching fluorescence
486 quantification in: I - GC/AMG of untreated larvae and in treated larvae with rotenoids (80 PPM
487 and 160 PPM); II - PMG of untreated larvae and in treated larvae with rotenoids (80 PPM and 160
488 PPM). GC - gastric cecum; AMG - anterior midgut; PMG - posterior midgut.

489 **Figure 7 -** V-ATPase concanamycin-sensitive hydrolytic activity (U = µmolPi.mg-1.min-1) was
490 determined in plasma membrane-enriched vesicles from *Aedes aegypti* control and test larvae,
491 raised on 80 PPM of isolated rotenoids.

492 **Figure 8 –** Oxidative stress in the gut of the larvae. **A -** Fluorescence labeling of ROS. [Superior
493 insert in panel A – Larval gut drawing with its divisions and corresponding pHs (Linser et al.,
494 2009), as guidance]. The scale bar corresponds to 100µm. **B –** Fluorescence quantification by the
495 Zen 2.3 software in: I - PMG of untreated larvae and in treated larvae with rotenoids (80 PPM and
496 160 PPM); II - HG of untreated larvae and in treated larvae with rotenoids (80 PPM and 160
497 PPM). PMG - posterior midgut; HG – hindgut; ROS - oxygen-reactive species.

**Legends (Supplementary Materials)**

**Table S1 -** $^{13}C$ - (100 MHz) and $^{1}H$ - (400 MHz) NMR data of the rotenoids CDF - RI and CDF - RII, δ in PPM and multiplicities and J in Hz, including results obtained by heteronuclear HSQC correlated with shift 2D ($1J_{HC}$) and HMBC ($nJ_{HC}$ n = 2 and 3).

**Figure S1** - $^{1}H$ NMR spectrum of the rotenoids in the mixture of CFD 7.4 and CFD 7.5 fractions (500 MHz, $CDCl_3$).

**Figure S2 -** $^{13}C$ - DEPTQ NMR spectrum of the rotenoids in the mixture of CFD 7.4 and CFD 7.5 fractions (125 MHz, $CDCl_3$).

**Figure S3** - $^{1}H$-$^{1}H$ COSY spectrum of the rotenoids in the mixture of CFD 7.4 and CFD 7.5 fractions.

**Figure S4 -** HSQC spectrum of the rotenoids in the mixture of CFD 7.4 and CFD 7.5 fractions.

**Figure S5 -** HMBC spectrum of the rotenoids in the mixture of CFD 7.4 and CFD 7.5 fractions.

**Figure S6 -** HRESIMS mass spectra of the isolated rotenoids (CFD-RI e CFD-RII).

**Figure S7 -** MS/MS spectrum from peak 1 of *m/z* 341.1052 (CFD-RI).

**Figure S8 -** Proposed fragmentation mechanisms of the peak 1 (CFD-RI) to justify principal peaks revealed by HRESIMS, including results of MS/MS.

**Figure S9 -** MS/MS spectrum from peak 2 of *m/z* 357.3300 (CFD-RII).

**Figure S10 -** Proposed fragmentation mechanisms of the peak 2 (CFD-RII) to justify principal peaks revealed by HRESIMS, including results of MS/MS.

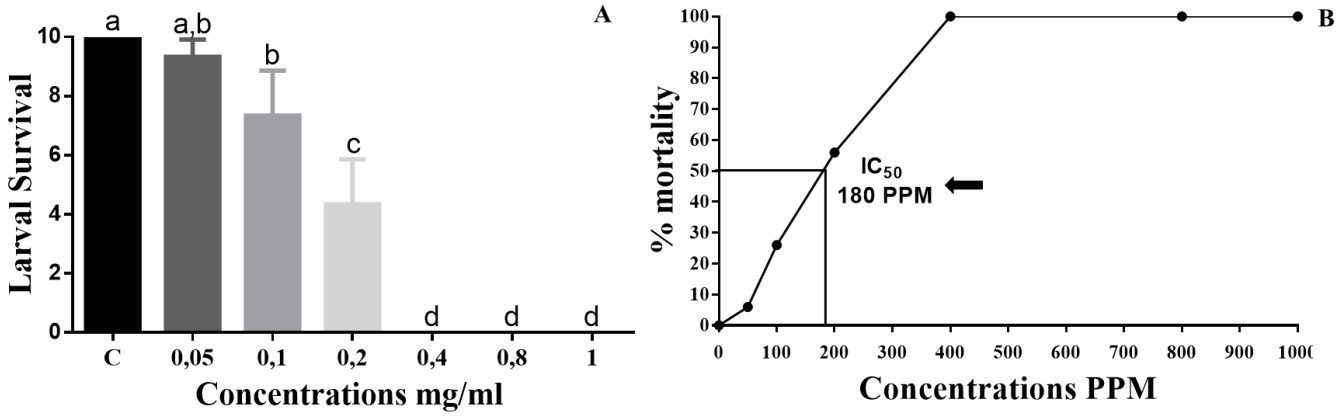



Figure 1

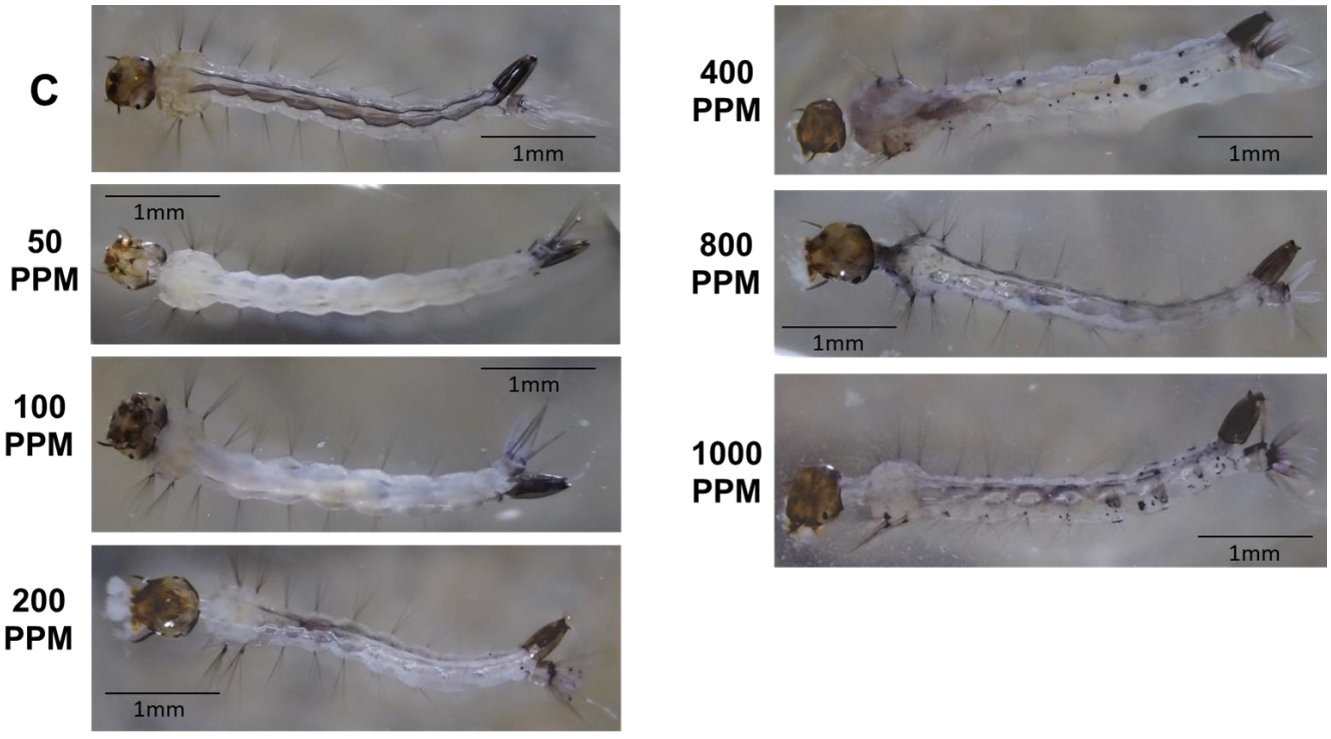
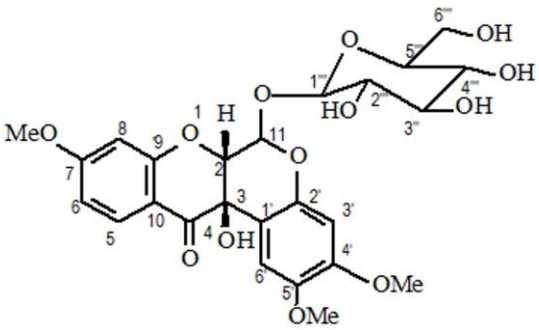
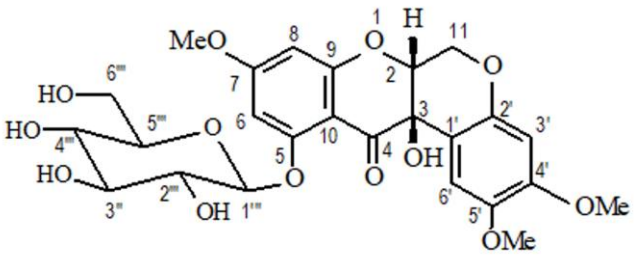



Figure 2




**CFD-RI**

**11α-O-β-D-Glucopyranosylrotenoid**




**CFD-RII**

**6-deoxyclitoriacetal 11-O-n-glucopyranoside**

Figure 3

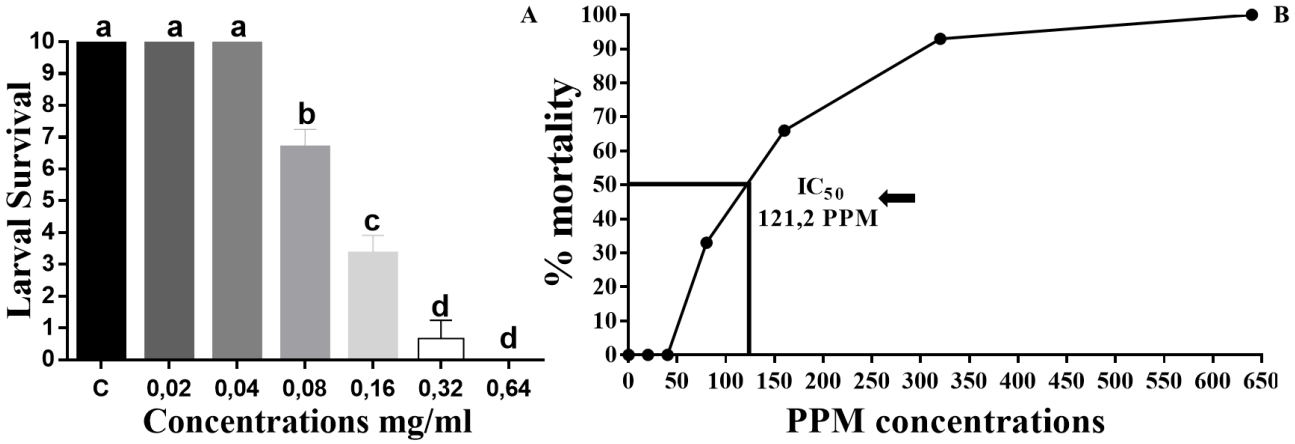



Figure 4

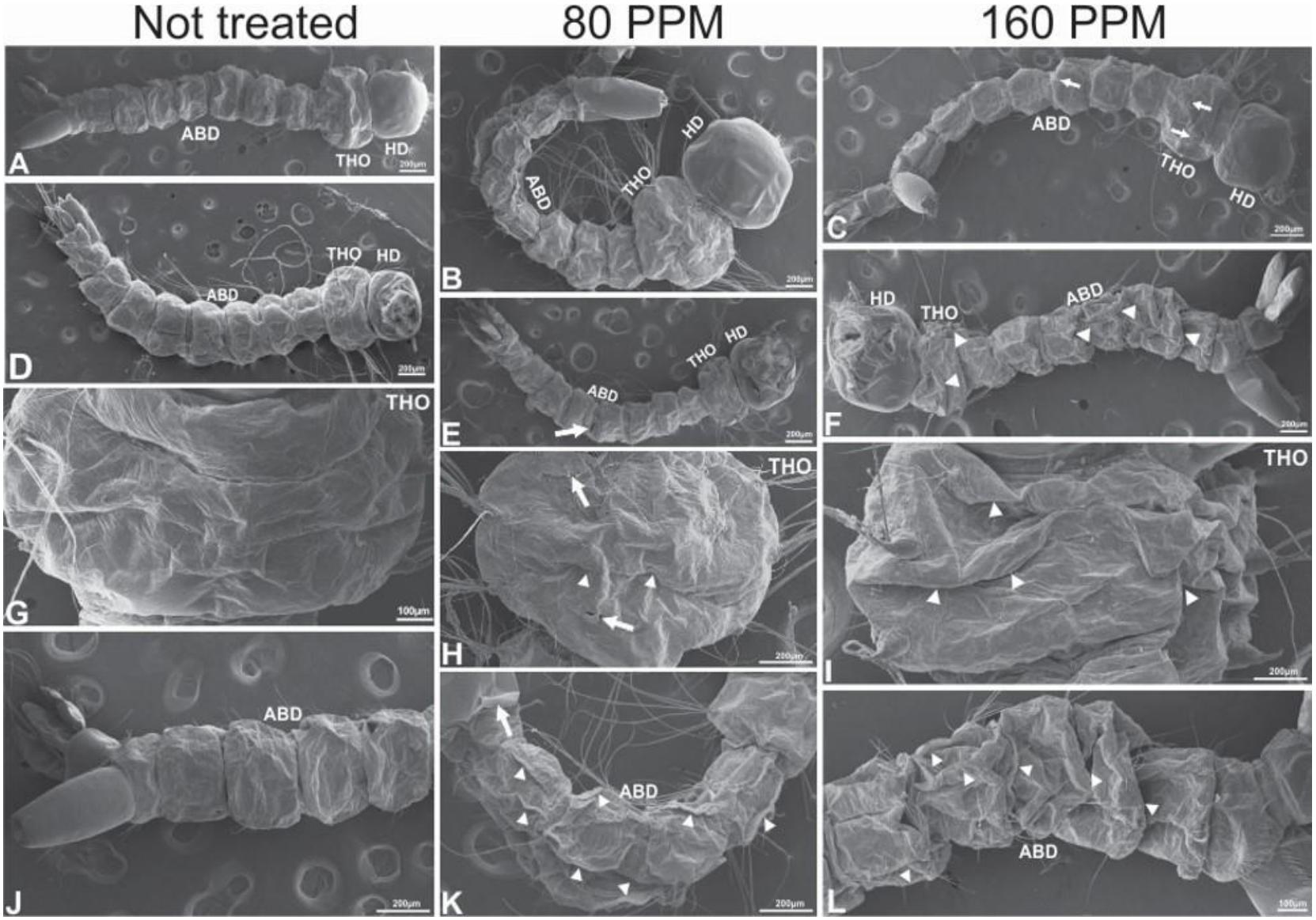

Not treated
80 PPM
160 PPM
A
ABD
THO
HD
B
ABD
THO
HD
C
ABD
THO
HD
D
ABD
THO
HD
E
ABD
THO
HD
F
HD
THO
ABD
G
THO
100µm
H
THO
200µm
I
THO
200µm
J
ABD
200µm
K
ABD
200µm
L
ABD
100µm

Figure 5

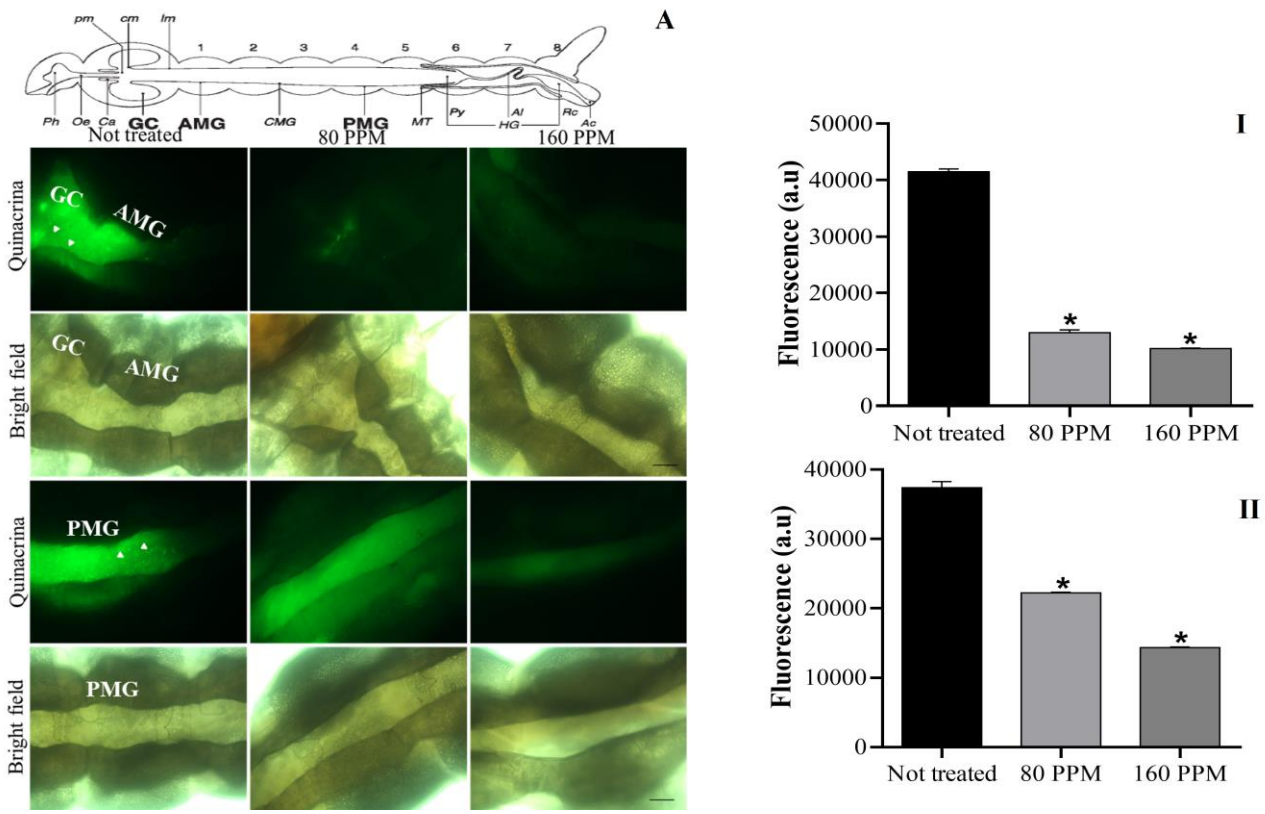



Figure 6

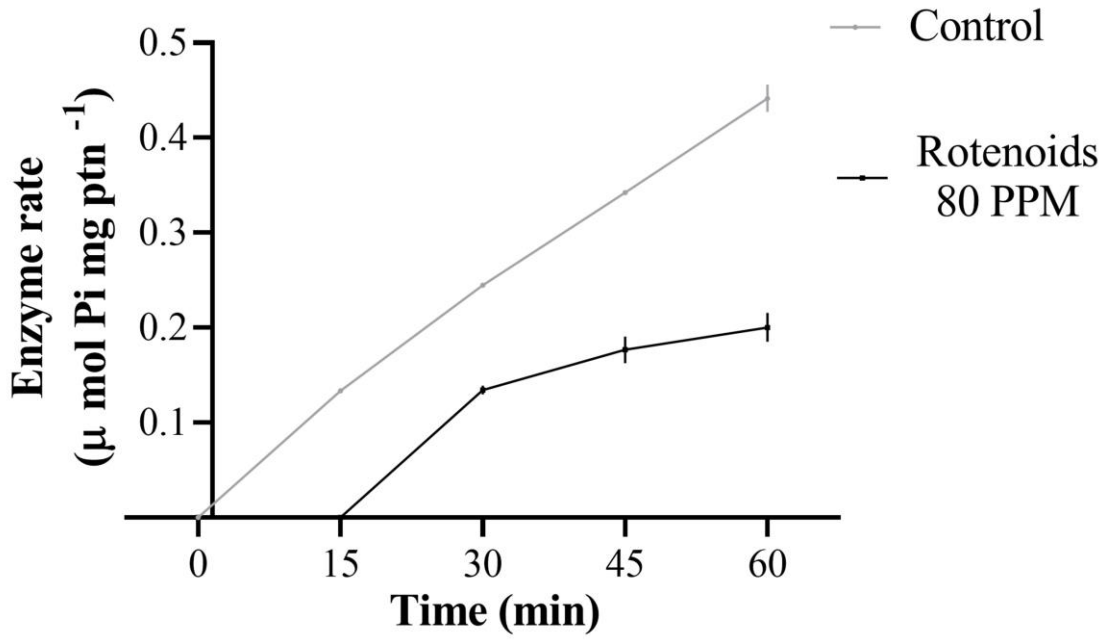



Figure 7

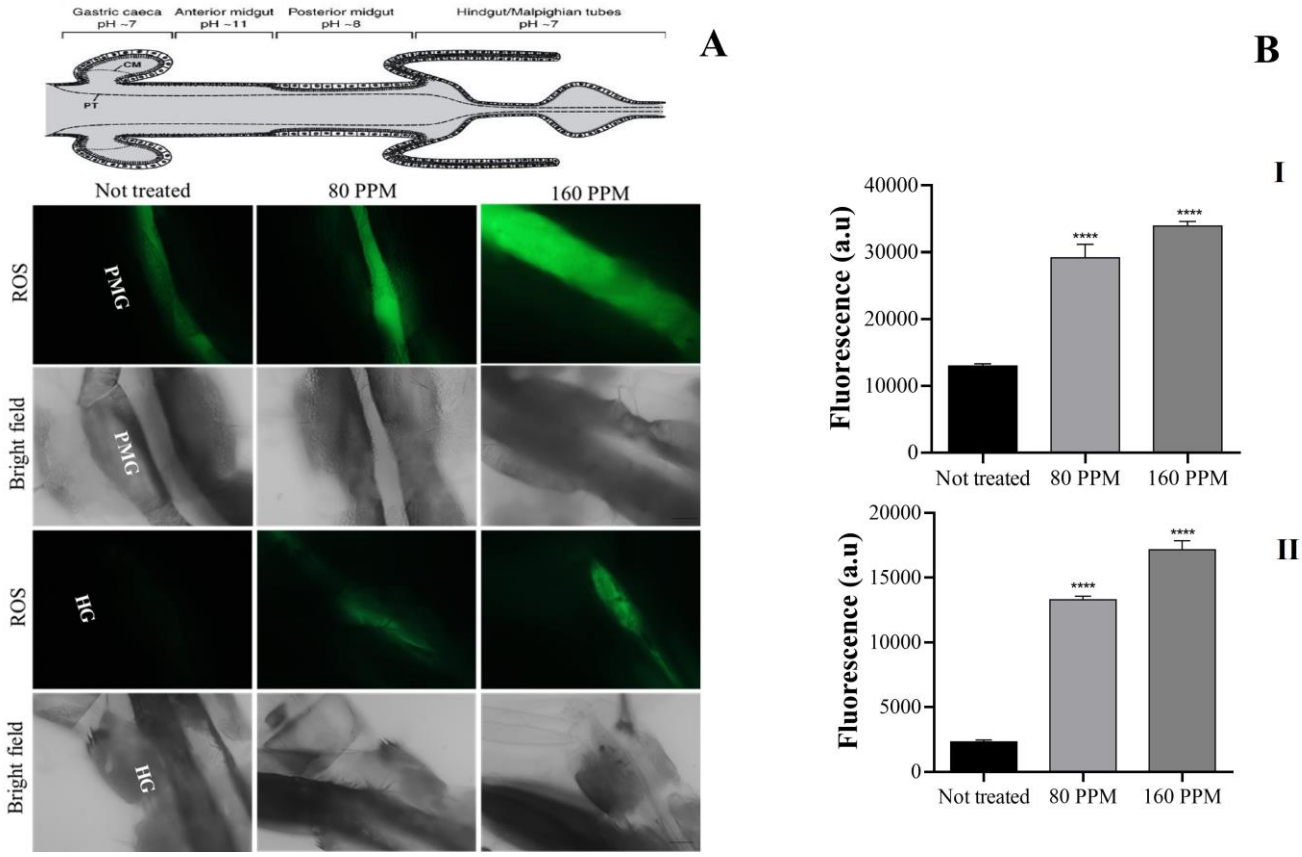



Figure 8

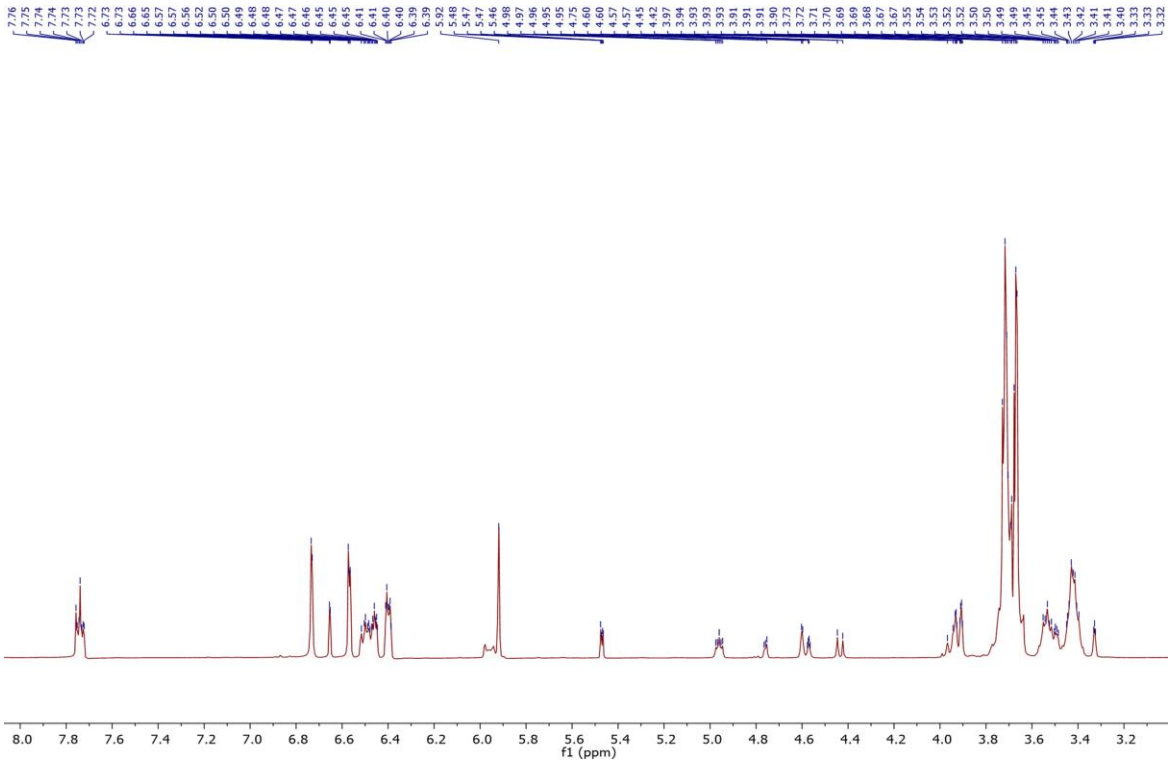



Figure S1 (Supplementary)

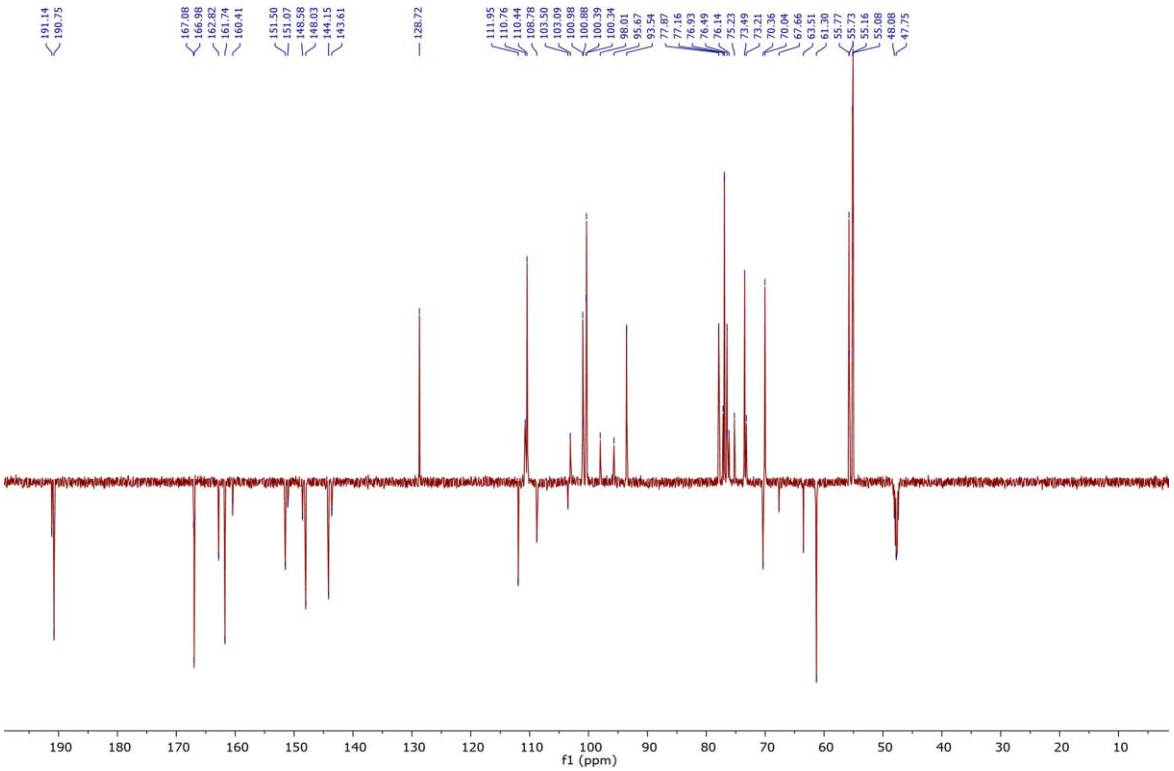



Figure 2S (Supplementary)

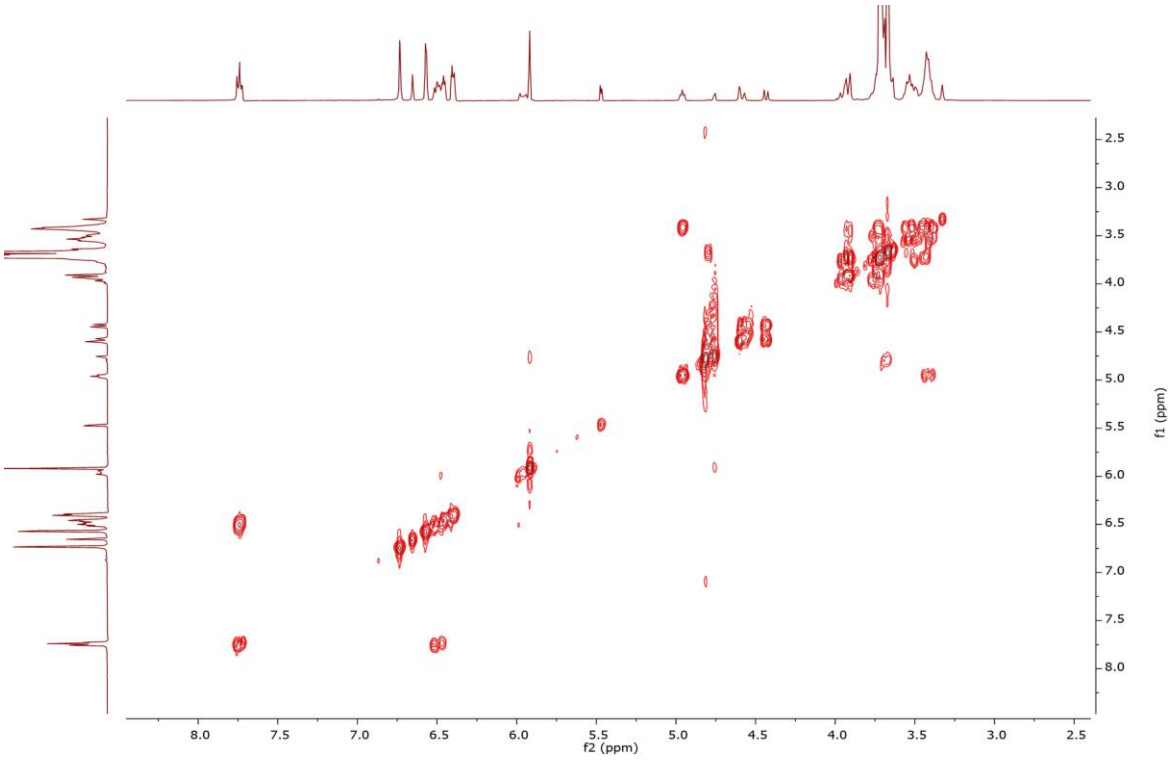



Figure 3S (Supplementary)

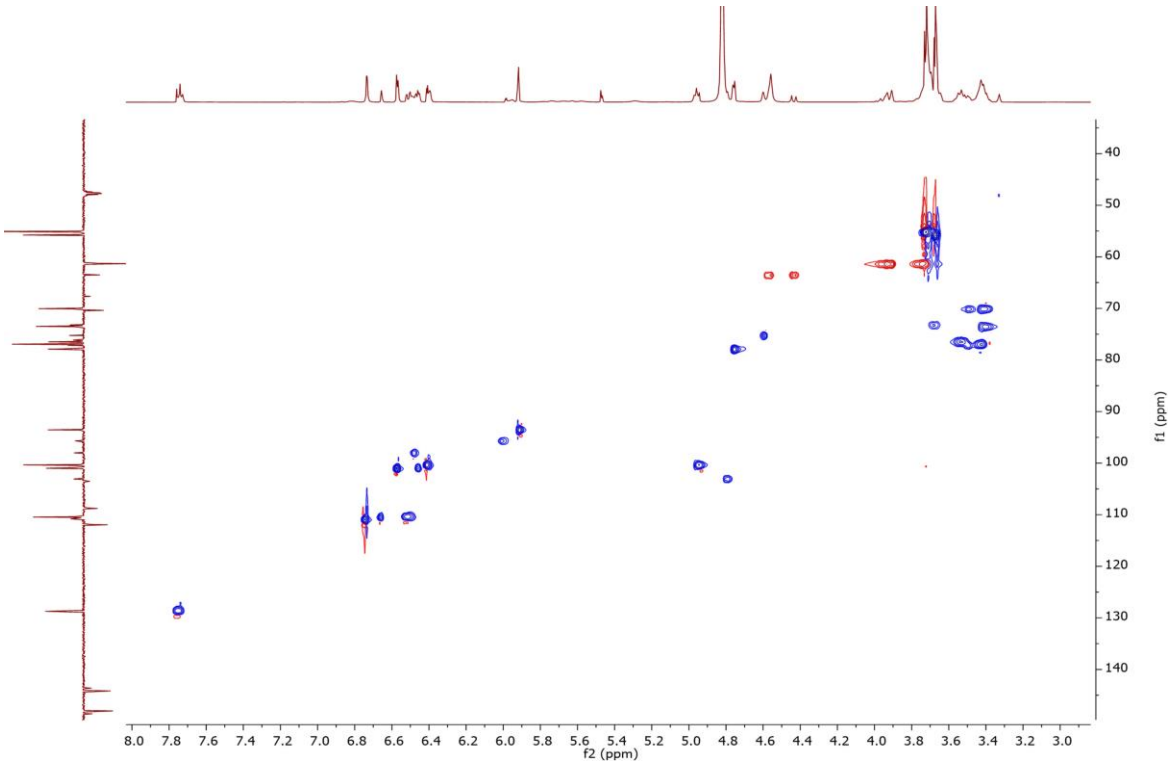



Figure 4S (Supplementary)

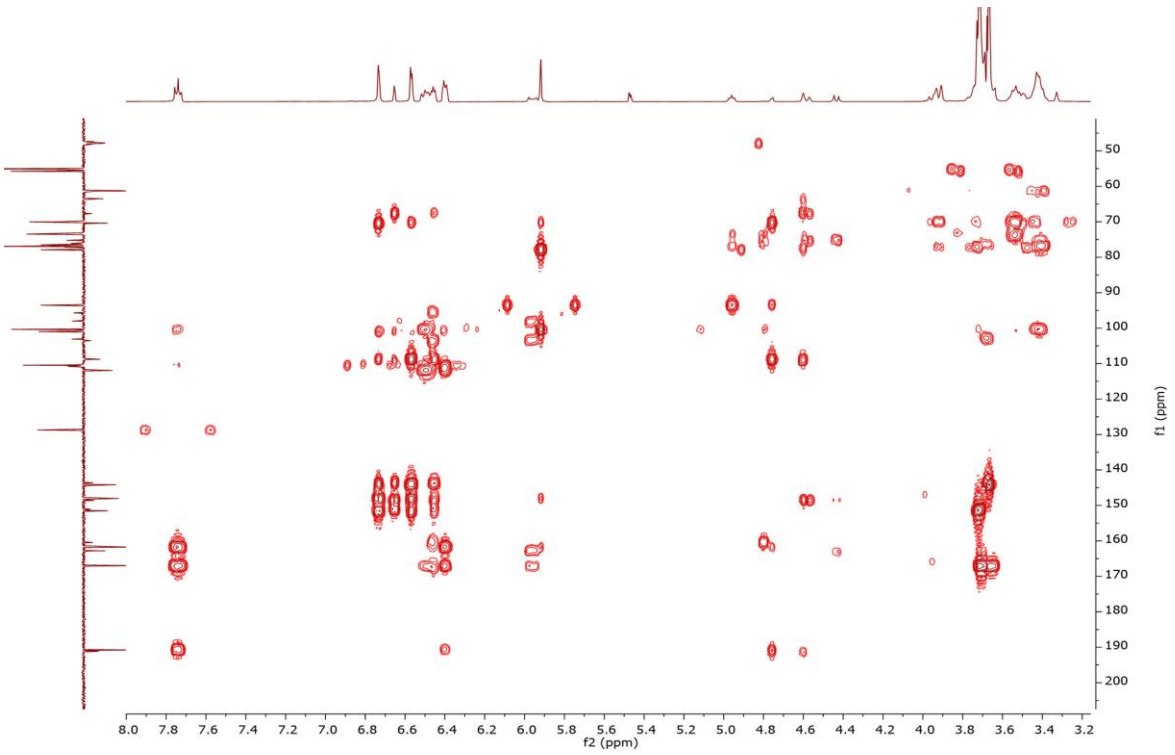



Figure 5S (Supplementary)

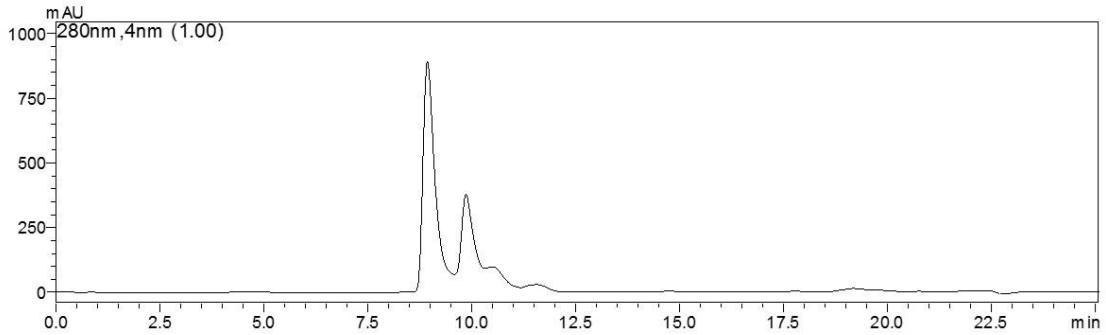
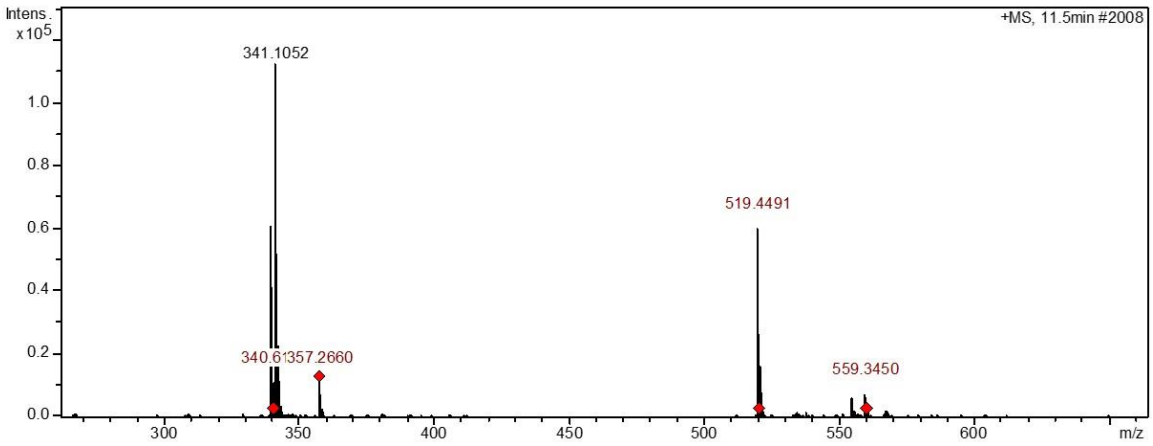



Figure 6S (Supplementary)

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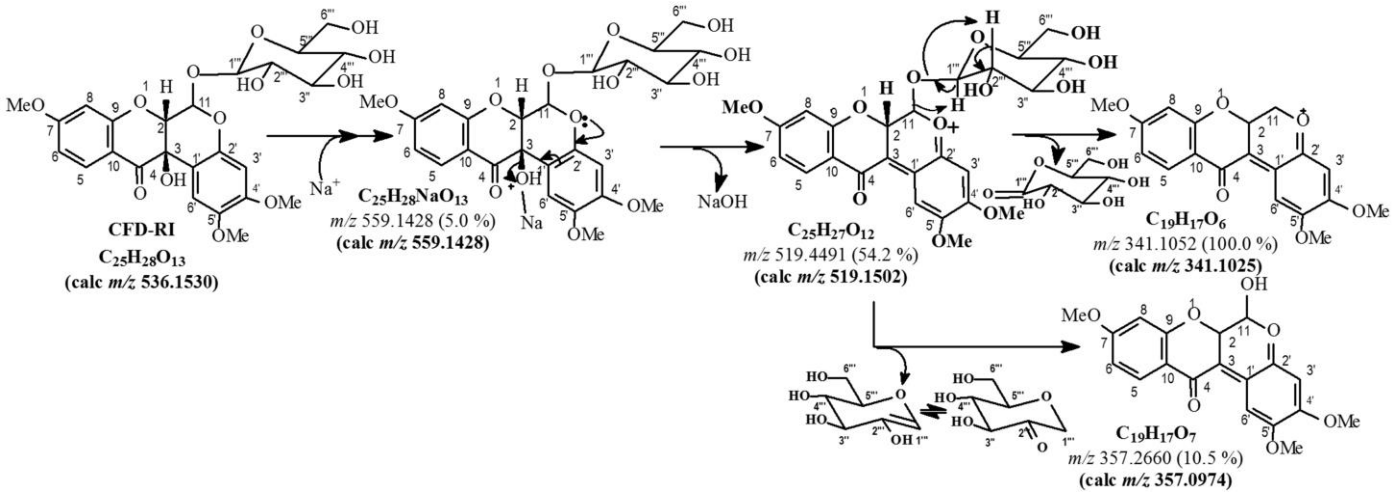


Figure 8S (Supplementary)

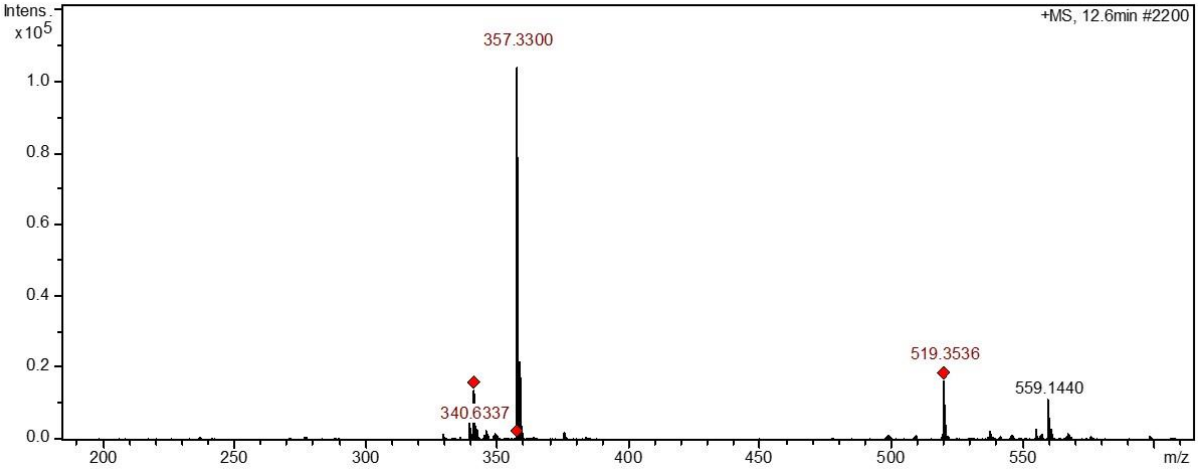


Figure 9S (Supplementary)

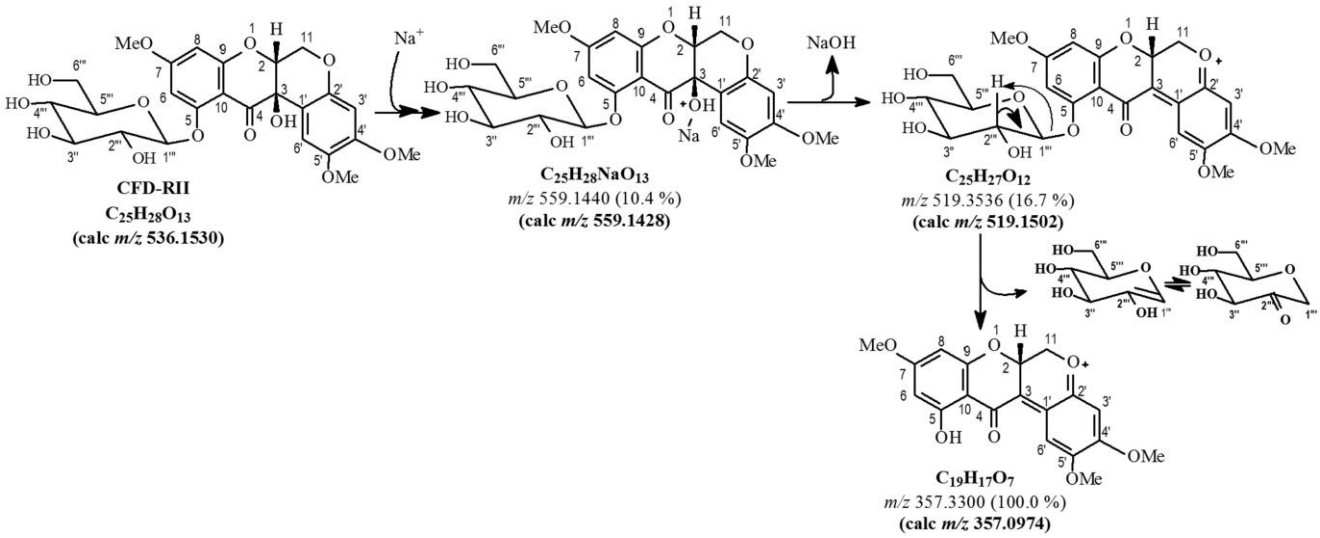


Figure 10S (Supplementary)

**Table S1.** $^{13}C$ - (100 MHz) and $^{1}H$ - (400 MHz) NMR data of the rotenoids CDF - RI and CDF - RII, δ in PPM and multiplicities and J in Hz, including results obtained by heteronuclear HSQC correlated with shift 2D ($1J_{HC}$) and HMBC ($nJ_{HC}$ n = 2 and 3).

| | **CFD-RI, 75.9 %** | | | | **CFD-RII, 24.1 %** | | | |
|---|---|---|---|---|---|---|---|---|
| | HSQC | | HMBC | | HSQC | | HMBC | |
| | $\delta_C$ | $\delta_H$ | $^2J_{CH}$ | $^3J_{CH}$ | $\delta_C$ | $\delta_H$ | $^2J_{CH}$ | $^3J_{CH}$ |
| **C** | | | | | | | | |
| 3 | 70.89 | - | H-2 | H-6' | 67.05 | - | H-2 | H-6'; H-16a |
| 4 | 190.67 | - | | H-2; H-5 | 191.15 | - | | H-2 |
| 5 | - | - | - | - | 162.82 | - | H-6 | |
| 7 | 167.02 | - | H-6; H-8 | H-5; MeO-7 | 167.16 | - | H-6; H-8 | MeO-7 |
| 9 | 161.78 | - | H-8 | H-2; H-5 | 160.76 | - | H-8 | |
| 10 | 112.06 | - | | H-6; H-8 | 103.61 | - | | H-2; H-6; H-8 |
| 1' | 108.98 | - | H-6' | H-2; H-3' | 108.88 | - | | H-2 |
| 2' | 148.02 | - | H-3' | H-6' | 148.60 | - | H-3' | H-6'; H-11b |
| 4' | 151.60 | - | H-3' | H-6'; MeO-4' | 151.19 | - | H-3' | H-6'; MeO-4' |
| 5' | 144.20 | - | H-6' | H-3'; MeO-5' | 143.66 | - | H-6' | H-3'; MeO-5' |
| **CH** | | - | | | | | | |
| 2 | 78.00 | 4.72 (s) | H-11 | | 75.22 | 4.58 (dl, 1.5) | H-11b | |
| 5 | 128.59 | 7.78 (d, 8.9) | | | - | - | - | - |
| 6 | 110.36 | 6.63 (dd, 8.9, 2.3) | H-5 | H-8 | 95.66 | 6.14 (d, 2.3) | | |
| 8 | 100.29 | 6.48 (d, 2.3) | | H-6 | 98.00 | 6.55 (d, 2.3) | | |
| 11 | 93.52 | 5.90 (d, 0.9) | H-2 | H-1" | - | - | - | - |
| 3' | 100.30 | 6.62 (s) | | | 100.87 | 6.51 (s) | | |
| 6' | 111.17 | 6.76 (s) | | | 110.65 | 6.68(s) | | |
| $CH_2$ | | | | | | | | |
| 11 | - | - | - | - | 63.52 | 4.56 (dd, 11.9, 2.9)<br>4.453 (d, 11.9) | | |
| MeO | | | | | | | | |
| 7 | 54.97 | 3.81 (s0 | | | 55.05 | 3.78 (s) | | |
| 4' | 55.16 | 3.80 (s) | | | 55.04 | 3.78 (s) | | |
| 5' | 55.80 | 3.70 (s) | | | 53.80 | 3.69 (s) | | |
| Carbohydrate unit | | | | | | | | |
| CH-1" | 100.99 | 4.94 (d, 7.8) | H-2" | H-5"; **H-11** | 103.27 | | | |
| CH-2" | 73.47 | 3.32 | H-1"; H-3" | H-4" | 73.19 | | | |
| CH-3" | 76.91 | 3.48 (t, 8.6) | H-2" | | 76.47 | | | |
| CH-4" | 70.02 | 3..37 (t, 8.6) | H3" | 2H-6" | 70.04 | | | |
| CH-5" | 76.91 | 3..72 (m) | 2H-6" | | 76.47 | | | |
| $CH_2$-6" | 61.29 | 3.89 (dd, 11.9, 1.7)<br>3.74 (d, 11.9) | H-5" | | 61.29 | | | |

## 3. Considerações Finais

Conforme nossas observações pessoais e os registros da literatura nos levavam a crer, as sementes de *Clitoria fairchildiana* provaram-se como um valioso repositório químico para o desenvolvimento de novas formulações de bioinseticidas com relevância agronômica e à saúde pública. Os compostos vegetais aqui encontrados, de ambas naturezas proteica (*Cf*vic) e não proteicas (rotenoides), mostraram-se letais ao desenvolvimento de ambos insetos modelos, quando utilizados em baixas concentrações.

*Cf*vic (Vicilina de *Clitoria farichildiana*), uma proteína de 12 kDa, inicialmente fracionada como uma kafirina e depois caracterizada como uma proteína semelhante a vicilina (um possível peptídeo derivado de vicilina VBPs) foi capaz de reduzir o peso larval de *C. maculatus* em 66%, quando inserida em uma concentração de 0,05% na sua dieta, inviabilizando a emergência de adultos. Dados experimentais e *in silico* sugerem que o mecanismo de ação de *Cf*vic está relacionado a uma alta força de ligação a quitina, visto que foram encontrados pelo menos 5 peptídeos (ALLTLVNPDGR, AILTLVNPDGR, NFLAGGKDNV, ISDINSAMDR, NFLAGEK) alinhados em dois sítios de ligação a quitina (ChBS) já descritos anteriormente na literatura e ambos ChBS mostraram alta energia de ligação a um monômero de N-acetil-D-glucosamina, demonstrando tratar-se de uma reação espontânea ($\Delta G<0$). Esses resultados foram confirmados por testes em cromatografia de afinidade a quitina e reforçados pela diminuição da afinidade após modificação química de *Cf*vic por acetilação dos resíduos de lisina (Lys). Sendo assim, provavelmente, esta alta força de ligação de *Cf*vic a estruturas quitinosas presentes no intestino médio deste inseto leva a morte larval por desnutrição.

Já os rotenoides (11α-O-β-D-glucopiranosilrotenoide e 6-deoxiclitoriacetal 11-O-n-glucopiranosídio) isolados, mostraram-se capazes de inibir em 55% a atividade das V-ATPases das larvas de terceiro instar de *Aedes aegypti* quando utilizados na concentração de 80 PPM e o cálculo estimado da $LC_{50}$ da mistura dos dois rotenóides na solução de cultura das larvas foi de 121.2 PPM. Estes causaram também alterações no exoesqueleto destas larvas, e a partir da concentração de 80 PPM foi possível observar um descolamento cuticular e perfurações no tórax e no abdômen das mesmas. Observamos ainda, um estresse oxidativo no intestino médio posterior e ainda mais acentuado no seguimento final do intestino destas larvas. Sendo assim, provavelmente a inibição da V-ATPase desencadeia um processo de estresse oxidativo, que leva a alterações no exoesqueleto e a morte das mesmas. Além disso, estes são solúveis em água o que facilita

sua utilização como larvicidas e são primos químicos da rotenona, que é considerada como um inseticida ideal para o uso na agricultura.

Este trabalho também reforça a importância do estudo da biodiversidade brasileira, mostrando à população em geral, o valor agregado de nossas riquezas naturais e a importância da conservação desta biodiversidade.

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