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**JOÃO RICARDO SÁ LEITÃO CAMAROTI**

# FOLHAS DE *Schinus terebinthifolia* (ANACARDIACEAE) COMO FONTE DE AGENTES INSETICIDAS CONTRA *Sitophilus zeamais*, *Plutella xylostella* E *Aedes aegypti*

**Recife**
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Tese apresentada ao Programa de Pós-graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco como parte dos requisitos para obtenção do título de Doutor em Bioquímica e Fisiologia.

**Orientador:** Prof. Dr. Thiago Henrique Napoleão

**Coorientador:** Prof. Dr. Emmanuel Viana Pontual

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## RESUMO

Inseticidas sintéticos são ainda usados no manejo de pragas e vetores, apesar do alto custo, impacto ambiental e seleção de insetos resistentes. Estratégias alternativas para controle de populações de insetos são urgentemente necessárias. A presente tese investigou a atividade inseticida de preparações de folhas de *Schinus terebinthifolia* contra *Sitophilus zeamais*, *Plutella xylostella* e *Aedes aegypti*. Foram avaliados extrato salino (ES), fração enriquecida em derivados cinâmicos (F1) e lectina isolada (SteLL). A composição fitoquímica do extrato foi avaliada por cromatografia de camada delgada e cromatografia líquida de alta eficiência. Foram avaliados os efeitos da ingestão de ES ou na sobrevivência, em parâmetros nutricionais e atividade de enzimas digestivas de *Sitophilus zeamais*. Nos ensaios com *P. xylostella*, foram avaliados o efeito de ES na eclodibilidade dos ovos, a interferência da ingestão de ES e SteLL na sobrevivência de lagartas e na capacidade reprodutiva dos adultos e o efeito deterrente de oviposição de ES. Foi investigada a repercussão da incubação de larvas de *A. aegypti* com ES e F1 no desenvolvimento do intestino médio até o estágio adulto. ES apresentou taninos hidrolisáveis e flavonoides. A ingestão de ES causou mortalidade de *S. zeamais* entre 94 e 97% após 12 dias de incubação. A SteLL não causou mortalidade significativa mas reduziu o ganho de biomassa e a eficiência na conversão do alimento ingerido. O ES inibiu, *in vitro*, a atividade de proteases do intestino de *S zeamais* enquanto SteLL estimulou a atividade de amilase e inibiu proteases. Frente a *P. xylostella*, ES não afetou a eclosão dos ovos, mas causou mortalidade das lagartas com $CL_{50}$ de 14,49% e 11,74% para 96 h e 144 h, respectivamente. O tratamento das lagartas com ES a reduziu a fertilidade dos adultos. ES apresentou efeito deterrente sobre a oviposição. SteLL não apresentou toxicidade para as lagartas de *P. xylostella*. A incubação de larvas de *A. aegypti* com ES e F1 resultou em deformação, hipertrofia e vacuolização das células epitélio intestinal em todos os estágios (larva, pupa e adulto). Houve aumento do número de células em proliferação no intestino de larvas e pupas, indicando problemas nos processos de regeneração. Células marcadas positivamente para caspase-3 foram detectadas em larvas e pupas expostas a ES e F1, indicando a indução de apoptose. Em conclusão, as folhas de *S. terebinthifolia* são fonte de compostos com atividade inseticida para os três insetos avaliados, interferindo na sobrevivência, nutrição, desenvolvimento e reprodução.



**Palavras-chave:** Aroeira-da-praia. Mosquito da dengue. Gorgulho do milho. Traça das brássicas. Inseticida natural.

## ABSTRACT

Synthetic insecticides are still used in pest and vector management, despite the high cost, environmental impact and selection of resistant insects. In this context, alternative strategies for control of insect populations are urgently needed. The present thesis investigated the insecticidal activity of leaf preparations from *Schinus terebinthifolia* against *Sitophilus zeamais*, *Plutella xylostella* and *Aedes aegypti*. The evaluated preparations were saline extract (SE), fraction enriched in cinnamic derivatives (F1) and isolated lectin (SteLL). The phytochemical composition of the extract was evaluated by thin layer chromatography and high performance liquid chromatography. In the evaluation of activity against *S. zeamais*, the effects of SE or SteLL ingestion, nutritional parameters and activity of digestive enzymes. In the experiments with *P. xylostella*, it was evaluated the effect of SE on egg hatchability, the interference of SE ingestion in survival of larvae and on the reproductive capacity of adults and the oviposition-deterrent effect of SE. In addition, toxicity by ingestion of SteLL was evaluated. Finally, it was investigated the repercussion of the incubation of *A. aegypti* larvae with SE and F1 in the development of the midgut until the adult stage. SE showed hydrolysable tannins and flavonoids. Ingestion of SE caused mortality of *S. zeamais* between 94 and 97% after 12 days. The lectin did not present deterrent effect, but reduced the biomass gain and the efficiency in the conversion of the ingested food. The extract inhibited protease activity while SteLL stimulated amylase activity and inhibited proteases. Facing *P. xylostella*, SE did not affect egg hatching, but caused mortality of larvae with $LC_{50}$ of 14.49% and 11.74% for 96 h and 144 h, respectively. Treatment of larvae with SE reduced the fertility of adults. SE presented a deterrent effect on oviposition, with deterrent indexes of 63.42% and 68.02% for 24 and 48 hours, respectively. SteLL showed no toxicity to *P. xylostella*. Incubation of *A. aegypti* larvae with SE and F1 resulted in deformation, hypertrophy and vacuolization of gut epithelial cells at all stages (larva, pupa and adult). There was an increase in the number of proliferating cells in the midgut of larvae and pupae, indicating problems in the regeneration process. Cells positively labeled for caspase-3 were detected in larvae and pupae exposed to SE and F1, indicating the induction of apoptosis. In conclusion, the leaves of *S. terebinthifolia* are source of compounds with insecticidal activity against the three evaluated insects, interfering in the survival, nutrition, development and reproduction.



**Keywords:** Brazilian pepper tree. Dengue mosquito. Maize weevil. Diamondback moth. Natural insecticide.

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### Phytoinsecticides for controlling pests and disease vectors



### Artigo 1



### Artigo 2

**SUMÁRIO**

# 1 INTRODUÇÃO

*Sitophilus zeamais*, popularmente conhecido como gorgulho-do-milho, é uma das principais pragas agrícolas do milho, atacando também arroz, trigo, cevada, aveia, bem como produtos derivados (GOÑI et al., 2017). Tem sido descrita a ocorrência de populações de *S. zeamais* resistentes a inseticidas como piretroides e organofosfatos (FRAGOSO et al., 2007; FREITAS et al. 2016). *Plutella xylostella*, também chamada de traça-das-brássicas, é considerada a principal praga de brássicas no mundo (ZALUCKI et al., 2012; FURLONG et al., 2013; SHEN et al., 2017). A voracidade alimentar das lagartas de *P. xylostella*, desde a eclosão, a curta duração do ciclo de vida e a elevada capacidade reprodutiva em todas as épocas do ano são fatores importantes para elevado impacto dessa praga (ZHOU et al., 2011). *P. xylostella* tem uma grande capacidade de desenvolver resistência, sendo descritas populações resistentes a 95 princípios ativos de inseticidas utilizados atualmente (ZHANG et al., 2016). Já o *Aedes aegypti* é um mosquito cosmopolita, com elevada ocorrência nas áreas urbanas da Ásia, África e Américas Central e do Sul. As fêmeas desta espécie praticam hematofagia pois necessitam das proteínas presentes no sangue de vertebrados para que ocorra a maturação dos ovos (SILVA et al., 2008). É durante o repasto sanguíneo que o mosquito-fêmea age como vetor dos causadores de febre amarela, dengue, febre do vírus Zika (todos do gênero *Flavivirus*) e chikugunya (*Alphavirus*), doenças de alta relevância epidemiológica, principalmente nas regiões tropicais do planeta (LENHART et al., 2007; GUO et al., 2016).

Estratégias para a proteção de culturas e produtos agrícolas contra o ataque de insetos praga, bem como o controle de insetos vetores de doenças estão entre as principais preocupações da indústria de alimentos, da agricultura, e de organizações de saúde em todo o mundo (FAO, 2009; BAHATI, 2018a). O uso de inseticidas sintéticos ainda prevalece como uma das principais formas de manejo de insetos. No entanto, o uso de muitos deles tem um alto custo, polui o meio ambiente, afeta organismos não-alvo e promove a seleção de populações de insetos resistentes (CORRÊA; SALGADO, 2011; SILVA et al., 2016; BELLINATO et al., 2016; BAHRATI; SAHA., 2018).

As plantas desenvolveram diversas formas de defesa para se protegerem contra insetos herbívoros e predadores. A defesa química inclui compostos de diferentes classes, tais como proteínas de defesa (ex. lectinas e inibidores de proteases) ou metabólitos secundários (por exemplo, antocianinas, fenóis, quinonas, alcaloides, flavonoides, saponinas, taninos, terpenos

e rotenoides) (HARBORNE, 1993; HANLEY, et al., 2007). Essas moléculas podem ser exploradas como alternativas aos inseticidas sintéticos atualmente utilizados.

*Schinus terebinthifolia* Raddi é uma planta da família Anacardiaceae, conhecida popularmente como aroeira-da-praia e bastante conhecida por suas propriedades medicinais (FEDEL-MIYASATO et al., 2014; ROSAS et al., 2015). As folhas de *S. terebinthifolia* contêm uma lectina denonimada SteLL, que apresenta atividade antimicrobiana contra bactérias e fungos patogênicos ao homem (GOMES et al., 2013). Extrato salino das folhas dessa planta apresentou atividade larvicida contra *A. aegypti*, causando danos no intestino das larvas e interferindo no seu desenvolvimento (PROCÓPIO et al., 2015). Os autores também mostraram evidências de que a atividade larvicida estava ligada à presença de derivados do ácido cinâmico e flavonoides. Esse extrato contém a lectina SteLL, mas a mesma não foi ativa contra as larvas do mosquito.

A presente tese teve como objetivos ampliar a avaliação do potencial inseticida do extrato salino de folhas de *S. terebinthifolia* e da lectina SteLL, ao investigá-los contra *S. zeamais* e *P. xylostella*, bem como aprofundar o estudo dos efeitos deletérios do extrato e de fração enriquecida em derivados cinâmicos obtida a partir dele sobre o desenvolvimento do intestino médio de *A. aegypti*.

# 2 OBJETIVOS

## 2.1 GERAL

Avaliar o potencial inseticida de extrato, fração enriquecida em derivados cinâmicos ou lectina (SteLL) de folhas de *Schinus terebinthifolia* contra *Sitophilus zeamais*, *Plutella xylostella* e *Aedes aegypti*.

## 2.2 ESPECÍFICOS

- ✓ Revisar o estado-da-arte em fitoinseticidas para controle de pragas agrícolas e vetores de doenças.
- ✓ Determinar o perfil fitoquímico do extrato salino (ES) de folhas de *S. terebinthifolia*.
- ✓ Isolar SteLL a partir do extrato salino, seguindo protocolo previamente estabelecido.
- ✓ Avaliar a toxicidade por ingestão de ES e SteLL para adultos de *S. zeamais*, através da determinação de taxa de mortalidade e parâmetros nutricionais.
- ✓ Investigar os efeitos do ES e SteLL na atividade de enzimas digestivas (protease e α-amilase) do intestino de adultos de *S. zeamais*.
- ✓ Avaliar a toxicidade por ingestão de ES e SteLL para lagartas de *P. xylostella*.
- ✓ Avaliar a sobrevivência de pupas originadas de lagartas (L1) de *P. xylostella* tratadas com ES.
- ✓ Investigar o efeito de ES sobre a eclosão de ovos de *P. xylostella*.
- ✓ Analisar o efeito de ES sobre o comportamento de oviposição e a capacidade reprodutiva de *P. xylostella*.
- ✓ Investigar a ação da ingestão ES e fração enriquecida em derivados cinâmicos (F1) sobre a organização do epitélio intestinal de larvas de *A. aegypti*.
- ✓ Analisar alterações na organização do epitélio intestinal de pupas e adultos de *A. aegypti* originados de larvas tratadas com ES e F1.
- ✓ Determinar o número de células em proliferação e em apoptose no epitélio intestinal de indivíduos de *A. aegypti* em diferentes estágios (larva, pupa e adulto) tratados com ES e F1 no terceiro estágio larval (L3).

# 3 FUNDAMENTAÇÃO TEÓRICA

## 3.1 *Sitophilus zeamais*

*Sitophilus zeamais* Motsc. (Coleoptera, Curculionidae), comumente conhecido como gorgulho-do-milho, é uma das prinicipais pragas do milho em todo o mundo e também pode atacar outras culturas, tais como arroz, trigo, sorgo, cevada, feijão, aveia e algodão. É capaz ainda de atacar frutas como uvas, maçãs e pêssegos, geralmente na fase de maturação, bem como danificar alimentos processados/industrializados, como macarrão, biscoitos, chocolate e frutas secas (GALLO et al., 2002; BOTTON et al., 2005, GOÑI et al., 2017).

O ciclo de vida de *S. zeamais* compreende as fases de ovo, larva, pupa e adulto. As larvas apresentam coloração amarelo-clara e cabeça marrom-escura, enquanto as pupas são brancas. Os adultos (Figura 1) possuem de 2 a 3,5 mm de comprimento, são de cor castanho-escura e apresentam manchas avermelhadas nos élitros. Possuem ainda a cabeça projetada para frente e um rostro curvado, o qual é curto e grosso nos machos (Figura 2A) e longo e afilado nas fêmeas (Figura 2B). Os adultos são também capazes de voar, o que facilita a infestação no campo e nos locais de armazenamento (BOTTON et al., 2005; LORINI et al., 2010).

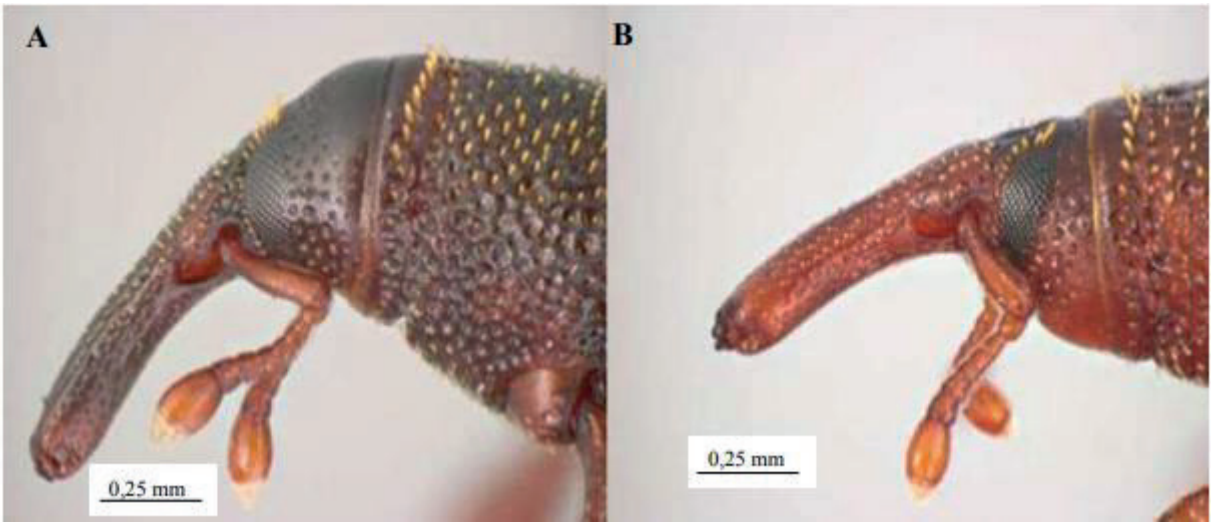
**Figura 1-** Adulto de *Sitophilus zeamais.*

Fonte: http://www.eudaimoniapestcontrol.com/common-pests-in-new-york/22-common-pests-in-storred-products/106-maize-weevil-wikipedia

Após o acasalamento, as fêmeas perfuram os grãos, depositam seus ovos e fecham esse orifício a partir de uma secreção gelatinosa produzida pelo aparelho ovipositor. Geralmente, apenas um ovo é depositado em cada grão. Após a eclosão, a larva passa por 4 estágios, durante os quais alimenta-se do tecido de reserva do grão, até se tranformar em pupa. Após completo o desenvolvimento do inseto adulto, este perfura o grão e sai, reiniciando o ciclo. O inseto adulto também alimenta-se dos grãos. Caso ocorra a postura de

mais de um ovo por grão, a larva mais forte irá se sobrepor às demais, ocorrendo sempre apenas a emergência de um adulto por grão (BOTTON et al., 2005; LORINI et al., 2010; ANTUNES; DIONELLO, 2010).

**Figura 2 –** Diferença entre o rostro de fêmeas (A) e machos (B) de *Sitophilus*.




Fonte: http://www.ufmt.br/pgeagri/arquivos/266571d22954a157df5a846483ca24fb.pdf

O gorgulho do milho apresenta um ciclo de vida relativamente longo, sendo a longevidade média dos adultos de 140 dias. O período médio de pré-oviposição (fase entre a emergência do grão e o início da postura dos ovos) é de 6 dias e as fêmeas então ovipositam em média de 3 ovos por dia durante cerca de 104 dias, totalizando uma média de 282 ovos durante toda sua vida. O tempo médio entre a oviposição e a emergência de insetos adultos é de 34 dias (GALLO et al., 2002; BOTTON et al., 2005; LORINI et al., 2010).

*S. zeamais* é considerado uma praga primária, por ser capaz de causar danos em grãos saudáveis e intactos ainda no campo. Porém, é também capaz de promover infestação cruzada, pois pode atacar tanto grãos em cultura quanto armazenados (BOTTON et al., 2005; LORINI et al., 2010). Os grãos danificados pelo *S. zeamais* têm menor peso, valor nutricional, capacidade de germinação e valor de mercado. Juntamente com outros insetos-praga, *S. zeamais* é responsável por perdas em torno de 24,5% da produção de milho (YUYA et al., 2009; TEFERA et al., 2011) e que podem chegar a 90% no caso de grãos desprotegidos (NWOSU et al. , 2015a,b).

Métodos químicos, físicos e biológicos podem ser utilizados para o controle do gorgulho-do-milho. O uso de inseticidas sintéticos, tais como fosfina e brometo de metila, por meio de técnicas de fumigação e pulverização convencionais, é a estratégia mais utilizada na

proteção de culturas e produtos armazenados contra o ataque por *S. zeamais* (PINTO-JÚNIOR et al., 2004; FARONI; SILVA, 2008). Entretanto, o uso desses compostos está associado com diversos problemas, como a toxicidade para organismos não-alvo, alta toxicidade para manipuladores, contaminação residual e emergência de populações resistentes devido à aplicação intensiva e indiscriminada (NAPOLEÃO et al., 2015).

A resistência de populações de *S. zeamais* a piretroides é descrita desde a década de 1990 (RIBEIRO et al., 2003; FRAGOSO et al., 2003, 2005, 2007) e foi relatada resistência a organotiofosfatos como malathion e fenitrotion em populações no Brasil (GUEDES et al., 1994, 1995). Estudos prevêem um aumento no surgimento de populações resistentes, uma vez que o uso excessivo de inseticidas sintéticos para combater esta praga ainda é predominante (ZHANG et al., 2015; FREITAS et al., 2016). Dessa forma, tem sido avaliadas formas alternativas de controle, tais como o controle biológico utilizando *Lariophagus distinguendus,* um parasitóide himinóptero (ADARKWAH et al., 2012). Um exemplo de controle químico de *S. zeamais* utilizando produto natural é o caso dos isotiocianatos, liberados a partir de glucosinolatos em resíduos de Brassicaceae, como fumigantes. O isotiocianato de alila apresentou efeito fumigante ($LC_{50}$: 5,69 μg/mL), causando colapso do citoesqueleto e disfunções mitocondriais (ZHANG et al., 2017).

### 3.2 *Plutella xylostella*

A traça-das-brássicas, *Plutella xylostella* L. (Lepidoptera: Plutellidae) é um microlepidóptero de origem europeia, provavelmente, mas atualmente distribuído em toda a América, Europa, Sudeste da Ásia, Austrália e Nova Zelândia (CAPINERA, 2015). É considerada como a principal praga da couve, repolho e outras brássicas no Brasil e no mundo (BOIÇA-JUNIOR et al., 2005, SHEN et al., 2017).

Durante o seu ciclo de vida, *P. xylostella* sofre metamorfose completa, passando pelas fases de ovo, lagarta (fase larval composta por 4 estágios), pupa ou crisálida e adulto. O período de desenvolvimento de ovo até adulto depende da temperatura ambiental: por exemplo, a 15 ºC o ciclo dura 34 dias, enquanto a 35 ºC ocorre em 12 dias (CASTELO BRANCO et al., 1997). Os ovos são ovais e achatados, amarelados ou de cor verde pálida, e medem, em média, 0,44 mm de comprimento por 0,26 mm de largura (Figura 3A). São depositados individualmente ou em pequenos grupos de 2 a 8 ovos na face abaxial da folha, próximo a nervura central ou, ocasionalmente, em outras partes da planta. As fêmeas

depositam, em média, 150 a 360 ovos e o tempo até a eclosão é de 2 a 4 dias (GALLO et al., 2002; THULER et al., 2009; CAPINERA, 2015).

**Figura 3-** *Plutella xylosyella*. Ovos (A), lagartas (B), pupas (C) e adultos (D).


Fontes: (A) http://keyun-biocontrol.en.made-in-china.com/product/pqVmIDMjEsYX/China-Live-Insect-Plutella-Xylostella-Eggs.html. (B) http://www.pestnet.org/fact_sheets/cabbage_diamond_back_moth_020.htm. (C), http://bugguide.net/node/view/800747, (D) https://bay.ifas.ufl.edu/newsletters/2015/05/15/the-diamondback-moth-a-major-pest-of-cole-crops

Após a eclosão, as lagartas de primeiro estágio minam as folhas (cavam galerias), alimentando-se do parênquima por 2 ou 3 dias. Em seguida, abandonam essas minas e passam a alimentar-se da epiderme, perfurando as folhas e inutilizando-as para a comercialização (IMENES et al., 2002). As lagartas (Figura 3B) são verde-claras, com a cabeça de cor parda e pelos escuros sobre o corpo, podendo medir de 7 a 10 mm de comprimento no último estágio, o qual atingem em cerca de 9 a 10 dias após eclosão (CARNEIRO, 1983; GALLO, et al., 2002). A duração dos estágios larvais depende da temperatura e da cultura hospedeira. Por exemplo, períodos menores de desenvolvimento são relatados em climas mais quentes e foi observado que houve uma variação na duração do período larval em culturas de couve-flor bola (8,7 dias) e repolho midouri (10,7 dias) (VIANA et al., 2008).

No último estágio larval, as lagartas tecem um casulo de rede aberta e coloração branca na superfície das folhas, iniciando o processo de pupação. O período de pupação varia de 4 a 15 dias, dependendo da temperatura. A pupa (Figura 3C) é amarelada e mede de 7 a 9 mm de comprimento. Os adultos (Figura 3D), em sua maioria, emergem durante as primeiras 8 h do período de fotofase, correspondendo a mariposas pequenas, esbeltas e acinzentadas com antenas pronunciadas e cerca de 6 mm de comprimento, apresentando uma mancha alongada de cor creme ou marrom-claro na região dorsal. Os adultos apresentam hábitos

noturnos e o acasalamento ocorre no crepúsculo do mesmo dia em que surgem. As fêmeas iniciam a oviposição logo após o acasalamento, atingindo seu pico entre 19:00h e 20:00h. O período de oviposição dura, em média, 4 dias (TAKELAR;SHELTON, 1993; GALLO et al., 2002; CAPINERA, 2015).

Em culturas desprotegidas, infestações por *P. xylostella* podem causar danos que levam à perda de 100% da produção. O ciclo de vida curto e o alto potencial reprodutivo durante todo o ano contribuem para o elevado impacto dos surtos desta praga (TAKELAR; SHELTON, 1993; CASTELO-BRANCO; GATEHOUSE, 2001; ULMER et al., 2002; TORRES et al., 2006). Uma grande diversidade de inseticidas sintéticos é utilizada para proteger as brássicas contra *P. xylostella,* principalmente nos trópicos e subtrópicos, onde os danos atingem grandes proporções (RIBEIRO et al., 2017). O custo total para prevenção e controle da *P. xylostella*, em todo mundo, pode chegar a 5 bilhões de dólares por ano (FURLONG et al., 2013).

*P. xylostella* tem a capacidade de desenvolver rapidamente resistência a inseticidas (YOU et al., 2013, ZHANG et al., 2016). São descritas populações de *P. xylostella* resistentes a 95 princípios ativos de inseticidas químicos em todo o mundo (APRD, 2017). No Brasil, tem sido descrita a resistência de *P. xylostella* a piretroides, avermectinas, indoxacarb, benzoilureia e diamidas (SANTOS et al., 2011; OLIVEIRA et al., 2011; RIBEIRO et al., 2017).

Himenópteros pertencentes aos gêneros *Diadegma* e *Diadromus*, *Microplitis* e *Cotesia*, e *Oomyzus* são conhecidos inimigos naturais de *P. xylostella.* Produtos derivados de bactérias (Por exemplo, Bti) e mico-insecticidas, por exemplo os obtidos a partir de *Zoophthora radicans* e *Beauveria bassiana*, estão sendo cada vez mais aplicados ou investigados para controle biológico. Vírus, nemátodos e microsporídeos também possuem potencial como biopesticidas para *P. xylostella* (SARFRAZ et al., 2005; GUZMÁN-FRANCO et al., 2008; ETEBARI et al., 2011; BERTOLACCINI et al., 2011).

### 3.3 *Aedes aegypti*

*Aedes aegypti* L. (Ordem Diptera, Família Culicidae) é um mosquito originário do continente africano e atualmente considerado cosmopolita, com elevada ocorrência nas áreas urbanas da Ásia, África e Américas Central e do Sul (CONSOLI; OLIVEIRA, 1994; SIMOY

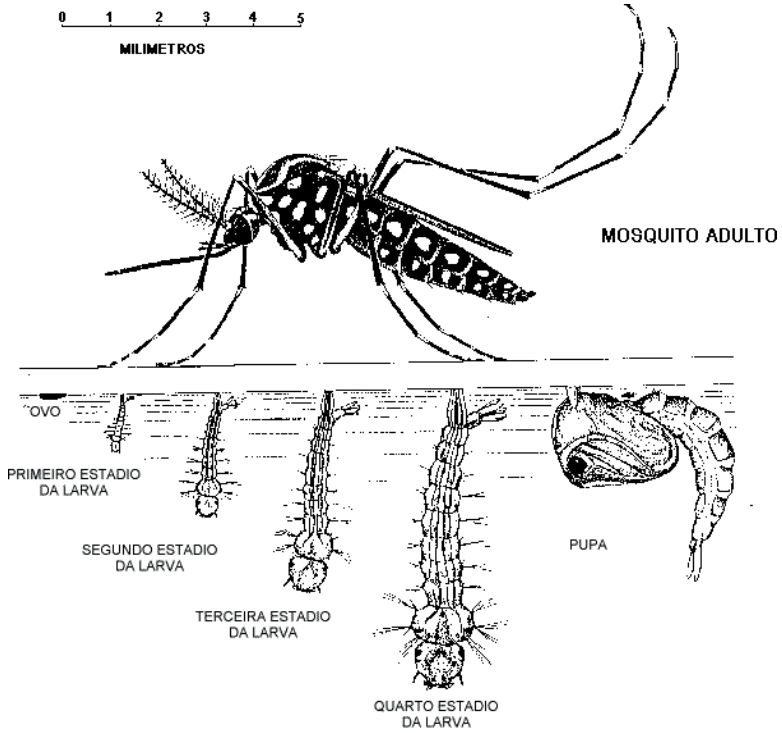
et al., 2015; BEZERRA et al., 2016). O mosquito *A. aegypti* mede menos de 1 cm, é de cor preta com manchas brancas no tórax e abdome e listras brancas nas pernas (Figura 4).

**Figura 4-** O mosquito *Aedes aegypti.*

Fonte: http://www.brasil.gov.br/saude/2016/02/notificacao-nos-casos-de-zika-virus-passa-a-ser-obrigratoria/aa47-aedes.jpg/view

O ciclo de vida desse mosquito compreende quatro fases: ovo, larva, pupa e adulto, sendo as três primeiras fases imaturas e aquáticas e a última fase madura/reprodutiva e aérea (Figura 5). O mosquito sofre metamorfose completa durante seu desenvolvimento, passando por alterações tanto na estrutura externa quanto nos órgãos internos (GULLAN; CRANSTON, 2007; SILVA et al., 2008; SIMOY et al., 2015).

**Figura 5-** O ciclo biológico do *Aedes aegypti.*




Fonte: http://www.dengue.org.br/mosquito_aedes.html

Em seu habitat silvestre original, a fêmea adulta deposita os ovos em buracos de árvores, axilas de folhas, cascas de frutos e outras coleções de água. No ambiente urbano, se adapta às situações impostas pela ocupação humana, reproduzindo-se em criadouros artificiais tanto abandonados a céu aberto e preenchidos pelas águas das chuvas, como aqueles utilizados para armazenar água para uso doméstico. Uma vez que muitos destes criadouros são pequenos e descartáveis, oferecem apenas um habitat temporário, pois podem sofrer dessecação, perturbações ou serem destruídos. Contudo, os ovos de *A. aegypti* apresentam alta resistência à desidratação, podendo permanecer viáveis, fora da água, por um período de até 1 ano, o que representa um sério obstáculo para sua erradicação (CONSOLI; OLIVEIRA, 1994; BESERRA et al., 2010; REITER, 2007; CHAVES et al., 2014). Esta condição permite que os ovos sejam transportados a grandes distâncias, em recipientes secos, tornando-se um importante meio de dispersão do inseto (MINISTÉRIO DA SAÚDE, 2011).

Em condições ambientais ideais de umidade e temperatura, a eclosão das larvas no primeiro estágio (L1) ocorre em um período de 2 a 5 dias, uma vez que os ovos sejam estimulados pelo contato com a água. Seguem-se três mudas sucessivas levando aos segundo, terceiro e quarto estágios larvais (L2, L3 e L4) (FORATTINI, 1962; SIMOY *et al*., 2015). As larvas são compostas por cabeça, tórax e abdômen (Figura 6) e, embora aquáticas, necessitam do oxigênio do ar, emergindo para a superfície da água para respirar através de um sifão ou tubo de sucção de ar. A fase larvária compreende o período de maior alimentação e crescimento. A alimentação é essencialmente composta por matéria orgânica, tais como algas, bactérias e esporos de fungos presentes em seus habitats (FORATTINI, 1962; CONSOLI; OLIVEIRA, 1994).

Após o quarto estágio, a larva passa à fase pupal, durante a qual não se alimenta e não libera resíduos metabólicos. As pupas têm aspecto de vírgula (Figura 6) e quase sempre se encontram próximas à superfície da água, o que facilita a emergência dos insetos adultos. O corpo da pupa é dividido em cefalotórax (cabeça e tórax unidos) e abdômen e possui um par de tubos respiratórios, que atravessam a água e permitem a respiração. Nesse estágio já é possível diferenciar o sexo, sendo as fêmeas maiores que os machos, característica que se estende aos adultos (MINISTÉRIO DA SAÚDE, 2011; SIMOY et al., 2015).

O tempo de pupação pode variar entre um dia até algumas semanas, dependendo da temperatura da água (SIMOY et al., 2015; GRESH et al., 2015). A duração do amadurecimento larval depende de condições tais como: temperatura, disponibilidade de alimento e densidade larvária no criadouro. Em condições ótimas (água não poluída, pH neutro, umidade relativa em torno de 75%, temperatura entre 26 ºC e 27 ºC e luminosidade

controladas, simulando os períodos dia e noite), o período entre a eclosão da larva e a pupação não excede 5 dias (ANJOLETTE; MACORIS, 2016). Quando em baixa temperatura (abaixo de 21 °C) e escassez de alimentos, este intervalo pode se prolongar por semanas (SIMOY et al., 2015; GRESH et al., 2015)

**Figura 6 –** Larvas (A) e pupas (B) de *Aedes aegypti*


Fonte: http://www.fiocruz.br/ioc/cgi/cgilua.exe/sys/start.htm?infoid=342&sid=32

Logo após emergir, o inseto adulto procura pousar sobre as paredes do recipiente, assim permanecendo durante várias horas, o que permite o endurecimento do exoesqueleto, das asas e, no caso dos machos, a rotação da genitália em 180º. Os machos se diferenciam das fêmeas por serem menores e possuírem antenas plumosas e palpos mais longos (MINISTÉRIO DA SAÚDE, 2011). O mosquito adulto vive em média 45 dias, mantém características urbanas e alimenta-se de seivas das plantas. As fêmeas desta espécie também praticam hematofagia pois necessitam das proteínas presentes no sangue de vertebrados para que ocorra a maturação dos ovos. O repasto sanguíneo costuma acontecer nas primeiras horas da manhã e nas últimas da tarde (SILVA et al., 2008). É durante a alimentação sanguínea que o mosquito-fêmea age como vetor de agentes patógenos (IOC, 2017).

*A. aegypti* atua como vetor dos causadores de febre amarela, dengue, febre do vírus Zika (todos do gênero *Flavivirus*) e chikugunya (um *Alphavirus*), doenças de alta relevância epidemiológica, principalmente nas regiões tropicais do planeta (LENHART et al., 2007; GUO et al., 2016). A dengue é atualmente considerada a mais importante doença transmitida por mosquitos ao redor do mundo, sendo também a doença viral de propagação vetorial mais

rápida. Está presente em cerca de 128 países, com 40% da população mundial vivendo em áreas de risco. A incidência da dengue aumentou 30 vezes nos últimos 50 anos e é estimado que aproximadamente 390 milhões de novos casos são reportados anualmente e 2,5% das pessoas infectadas morrem. Em 2018 foram estimados 446.150 casos de dengue, com incidência de 45,9/100.000 pessoas (WHO, 2018a,b).

A chikungunya é uma doença emergente que tem se espalhado por regiões tropicais e subtropicais. Tem sido reportada em mais de 60 paises na Ásia, África, Europa e nas Américas. Em 2016, houve um total de 349.936 casos suspeitos e 146.914 casos confirmados em laboratório em todo mundo. O Brasil foi o país que relatou a maioria dos casos (265.000 casos suspeitos). A doença apresenta como sintomas febre alta e dor nas articulações que podem persistir por longos períodos (semanas, meses ou anos) (WHO, 2017).

O primeiro surto de febre do vírus Zika ocorreu em 2007 na Micronésia. Em seguida, foram registrados surtos na Polinésia Francesa em 2013 e 2014. Em 2015, o vírus Zika se espalhou nas Américas e, desde então, foi relatado em mais de 40 países deste continente (OMS, 2018c). A sintomatologia da febre do vírus Zika é semelhante àquela da dengue e inclui febre baixa, erupções cutâneas, artralgia e hiperemia conjuntival. A infecção pelo vírus Zika em mulheres grávidas ganhou notoriedade recentemente devido à sua forte associação com a síndrome de Guillain-Barré e com a microcefalia em recém-nascidos (JOHANSSON et al., 2016; MIRANDA et al., 2016, WHO, 2018c).

A atual situação epidemiológica dessas três doenças em todo o mundo tem levado a estudos que visam o desenvolvimento de diferentes estratégias de controle por diversos setores públicos. A primeira vacina contra a dengue, Dengvaxia (CYD-TDV), desenvolvida pela Sanofi Pasteur, foi registrada no México em dezembro de 2015. CYD-TDV é uma vacina tetravalente recombinante, viva atenuada e profilática e baseia-se numa programação de 3 doses aplicadas ao longo de 12 meses. É indicada para indivíduos de 9 a 45 anos de idade que vivem em áreas endêmicas e está autorizada no México, Brasil, Filipinas e El Salvador. Contudo, algumas restrições ao uso da vacina tem sido apontadas tais como maior eficácia em indivíduos já soropositivos para o vírus DEN e o risco de desenvolvimento de doença severa em indivíduos soronegativos (AGUIAR et al., 2016, 2017).

Considerando que: (1) o uso de vacinas contra a dengue ainda está sendo estabelecido, (2) a vacina disponível não está no calendário de vacinação e não tem, ainda, uma grande abrangência; (3) ainda não existem vacinas para febre do vírus Zika e chikugunya; o controle da população do *A. aegypti* ainda continua sendo uma das principais estratégias para reduzir a disseminação da doença. Recomenda-se que a prevenção das doenças causadas por esse vetor

seja feita a partir de estratégias para a redução da densidade vetorial e pode ser direcionada para ovos, larvas ou insetos adultos. O controle das populações de vetores deve minimizar a transmissão da doença sem prejudicar outros organismos e o meio ambiente (AGUIAR et al., 2017, WHO, 2017, BAHATI et al., 2018, WHO, 2018c).

O controle mecânico é geralmente empregado para combater insetos vetores e é baseado na eliminação ou proteção adequada de potenciais criadouros. O controle biológico baseia-se na regulação do tamanho das populações de mosquitos usando predadores (por exemplo *Megacyclops formosanus, Gambusia* sp.), parasitoides (*Strelkovimermis spiculatus, Romanomermis culicivorax*), patógenos (*Bacillus thuringiensis israelenses, Metharizium anisopliae*) ou competidores (MULLA et al., 1982; ROSE et al., 2001; RODRIGUEZ et al., 2005; KALIMUTHU et al., 2017). O controle químico corresponde ao uso de inseticidas para reduzir a população dos mosquitos através de aplicação direta ou indireta em concentrações apropriadas (BOYCE et al., 2013; GRASSWITZ e FIMBRES, 2013; ANDORNO e LOPEZ, 2014).

O uso de inseticidas químicos ainda prevalece como uma das principais formas de controle dos mosquitos devido à sua eficiência imediata e baixo custo inicial (ROUBOS et al., 2014). Os organoclorados, organofosforados, carbamatos e piretroides, dentre outros, são compostos amplamente utilizados para controle dos insetos adultos. Os organofosforados são utilizados também como larvicidas (RANSON et al., 2010). As legislações mais recentes em vários países colocaram maiores restrições no uso de inseticidas, principalmente aqueles pouco seletivos (HILLOCKS, 2012). Isso estimulou a adoção de inseticidas mais ecológicos e o desenvolvimento de estratégias integradas de controle de mosquitos. Os produtos alternativos recomendados pela Organização Mundial da Saúde para o controle de mosquitos incluem biolarvicidas Bti (*Bacillus thuringiensis* serovar. *israelensis*) e Bs (*Bacillus sphaericus*) e o inseticida natural espinosade (WHO, 2016).

### 3.4 Fitoinseticidas

O uso indiscriminado de inseticidas sintéticos polui o ambiente, pode ser perigoso para humanos, animais, plantas e outros organismos não-alvo, e leva à seleção de indivíduos resistentes (BENHALIMA et al., 2004; KEMABONTA; ODEBIYI, 2005; CORRÊA e SALGADO, 2011; KIM; LEE, 2014). Nesse contexto, tem crescido a busca por inseticidas naturais, principalmente os de origem vegetal.

Plantas e insetos convivem por mais de 350 milhões de anos. Como estratégia de evolução, as plantas desenvolveram formas de defesa para se protegerem contra insetos herbívoros e predadores. Isso resultou em um complexo sistema de defesa, sendo as plantas capazes de reconhecer moléculas estranhas e sinais de células danificadas por insetos, ativando diferentes tipos de resposta (HOWER; JANDER, 2008; VERHAGE et al., 2010; HARE, 2011).

Diversos compostos defensivos têm sido largamente avaliados como inseticidas. Os fitoinseticidas podem corresponder ao próprio material vegetal (geralmente em pó), produtos derivados (cinzas de madeira) ou compostos obtidos por extração em soluções aquosas ou solventes orgânicos (MENEZES, 2005). Os princípios ativos presentes em fitoinseticidas podem ser metabólitos primários, como proteínas defensivas (ex. lectinas e inibidores de proteases) ou metabólitos secundários (por exemplo, antocianinas, fenóis, quinonas, alcaloides, flavonoides, saponinas, taninos, terpenos e rotenoides) (HARBORNE, 1993; HANLEY, et al., 2007).

Estes compostos podem causar mortalidade de insetos em todos os estágios, promover alterações morfológicas, interferir no comportamento (através de efeitos irritantes, repelentes, atraentes, deterrentes), atrasar o desenvolvimento e reduzir a fertilidade, entre outros efeitos, sendo de grande relevância no controle integrado de insetos (CAVALCANTE et al., 2006; COSTA et al., 2012; DELETRE et al., 2013; NAVARRO et al., 2013; PONTUAL et al., 2014; PROCÓPIO et al., 2015; MOHANKUMAR et al., 2016).

Polatoglu et al. (2016) verificaram que o óleo essencial extraído de *Salvia veneris* apresentou atividade fumigante e toxicidade por contato contra espécies de *Sitophilus,* além de provocar a inibição da atividade da acetilcolinesterase. O óleo essencial de *Artemisia vestita* apresentou forte efeito fumigante ($CL_{50}$: 13,42 mg/L de ar) e toxicidade por contato ($DL_{50}$: 50,62 mg/inseto adulto) contra *S. zeamais* (CHU et al., 2010). Napoleão *et al*. (2013) concluíram que o extrato de folhas de *Myracrodruon urundeuva* induziu a mortalidade de *S. zeamais* quando incorporado em dieta artificial ($CL_{50}$: 72,4 mg/g) além de causar perda de biomassa devido a diminuição da capacidade de conversão de alimentos ingeridos. Extrato aquoso de folhas de *Tradescantia spathacea* diminuiu a eficiência de ganho relativo de biomassa e taxa de ganho relativo de biomassa em *S. zeamais* (PROCÓPIO et al., 2015).

Extrato em diclorometano preparado a partir do caule de *Bauhinia scandens* var. *horsfieldii* apresentou toxicidade por contato contra larvas de *P. xylostella* ($DL_{50}$*:* 2,76 e 2,15 µg/larva em 24 e 48 horas, respectivamente). Heptacosano e hexacosano eram os principais principios ativos desse extrato (POONSRI et al., 2015). Wei et al. (2015) avaliaram o efeito

do óleo essencial de *Chenopodium ambrosioides* contra larvas de *P. xylostella* e relataram efeito fumigante, toxicidade por contato, deterrência alimentar, inibição da atividade de enzimas detoxificantes de inseticidas, tais como carboxilesterase e glutationa S-transferase, e inibição da pupação.

Extrato metanólico da raiz de *Rubia cordifolia* apresentou ação ovicida (70,40%) a 500 mg/L e atividade larvicida com $CL_{50}$ de 102,23 mg/L contra *A. aegypti* (MUNUSAMY et al., 2016). El-Sheikh et al. (2016) avaliaram a atividade inseticida do extrato de *Tribulus terrestris*, obtido utilizando éter de petróleo como solvente, contra *A. aegypti* e relataram atividade larvicida com $CL_{50}$ de 64,6 ppm, além de observarem um efeito tóxico para pupas (57,1% e 100% de mortalidade a 100 e 400 ppm, respectivamente) e redução da emergência de adultos resultantes de larvas tratadas. Atividade repelente de 100% a uma dose de 1,5 $mg/cm^2$ também foi observada. Um inibidor de protease isolado de extrato de flores de *Moringa oleifera* apresentou atividade larvicida para larvas recém eclodidas de *A. aegypti* e é um agente antibacteriano para a microbiota do intestino de larvas no estágio L4 (PONTUAL et al., 2014). Inibidor de protease isolado de sementes de *Cassia leiandra* reduziu em 50% a atividade de proteases intestinais de *A. aegypti* e apresentou um efeito larvicida com $CL_{50}$ de 0,0228 M (DIAS et al., 2017). O óleo essencial da sementes de *Syagrus coronata* foi capaz de promover a morte de larvas de *A. aegypti* ($CL_{50}$: 21,07 ppm) e exerceu efeito deterrente de oviposição. Os resultados indicaram que a atividade larvicida se deve à ação dos ácidos decanoico e dodecanico, enquanto que o efeito deterrente da oviposição provavelmente está ligado à presença de ácido octanoico no óleo essencial (SANTOS et al., 2017).

O uso de inseticidas de origem natural, como aqueles derivados de plantas mostra-se como uma importante alternativa para os problemas causados pelos inseticidas sintéticos, por apresentarem geralmente uma toxicidade mais seletiva, serem mais facilmente biodegradáveis e permanecerem por menos tempo no meio ambiente.

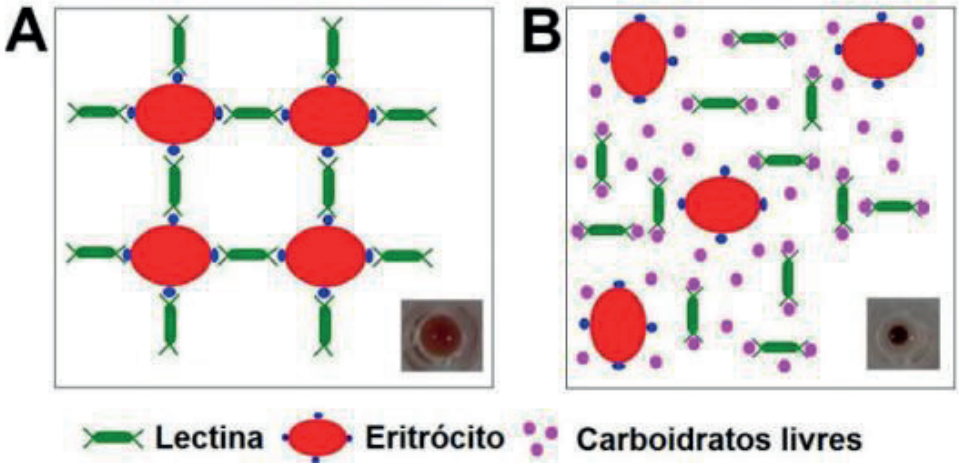
### 3.4.1 Lectinas

Lectinas são proteínas amplamente distribuídas na natureza, sendo encontradas em plantas, animais e fungos. São proteínas que possuem pelo menos um domínio não catalítico que se liga específica e reversivelmente a açúcares e glicoconjugados (MEJIA; PRISECARU, 2005; PAIVA et al., 2011; GONDIM et al., 2017). A ligação dessas proteínas a glicoconjugados presentes em superfícies celulares resulta em uma gama de propriedades biológicas, tais como: antibacteriana, antifúngica, antiviral, antitumoral e inseticida (PAIVA

et al., 2011; SANTOS et al., 2012; GOMES et al., 2013; SILVA et al., 2014; GUO et al., 2015; CARVALHO et al., 2015; OLIVEIRA et al., 2016; GONDIM et al., 2017; PROCÓPIO et al., 2017; OLIVEIRA et al., 2017).

A detecção da presença de lectinas em uma amostra é feita através do ensaio de atividade hemaglutinante, em que as lectinas presentes em uma solução se ligam aos carboidratos presentes na superfície dos eritrócitos, promovendo a hemaglutinação. A confirmação do caráter lectínico da aglutinação e a especificidade de ligação a carboidratos é feita através do ensaio de inibição da atividade hemaglutinante, na presença de diferentes monossacarídeos, dissacarídeos ou glicoproteínas (Figura 7) (PAIVA et al., 2011).

**Figura 7-** Detecção da presença de lectinas através do ensaio de hemaglutinação. (A) Representação da hemaglutinação promovida por lectinas. (B) Representação da inibição da atividade hemaglutinante de lectinas na presença de carboidratos livres.




Fonte: PAIVA et al. (2011)

Nas plantas, as lectinas têm sido isoladas a partir de folhas, entrecascas, raízes, rizomas, bulbos, vagens, sementes, frutos e flores (PAIVA et al., 2011). Lectinas de plantas estão abundantemente presentes em tecidos e órgãos de armazenamento (tubérculos, bulbos, rizomas, casca) e são produzidas de maneira constitutiva durante o desenvolvimento normal da planta. Por outro lado, podem ter sua expressão induzida em resposta a condições de estresse biótico ou abiótico, tais como ataque de insetos-praga, períodos de seca, baixas temperaturas, alto índice de salinidade do solo, lesões mecânicas e acumulam-se, geralmente, em folhas, raízes ou flores (VAN DAMME, 2008 a,b).

Lectinas de plantas mostram especificidade a glicoconjugados presentes em células, tecidos ou órgãos de organismos fora do reino vegetal, tais como animais, vírus e

microrganismos (HOPKINS & HARPER, 2001; RIPOLL, et al., 2003; WONG, et al., 2010, GONDIM et al., 2017). Diversas lectinas de plantas são tóxicas para insetos e as ações deletérias podem ser consequência de processos mediados por sensores gustativos (efeito pré-ingestão), manifestado pelo efeito de deterrência ou atração/estimulação alimentar, ou devido a efeitos pós-ingestão que estão associados, provavelmente, à resistência das lectinas à proteólise intestinal, possibilitando a interação destas com estruturas presentes na matriz peritrófica e células do intestino médio dos insetos provocando morte celular, alterações histológicas, morte de microrganismos da microbiota intestinal, alteração de atividade enzimática e distúrbios na absorção de nutrientes (SAUVION et al., 2004; MACEDO et al., 2007; COELHO, et al., 2009; MICHIELS et al., 2010; SPRAWSKA & GOLAWSKA, 2010; VANDENBORRE, et al., 2011; NAPOLEÃO et al., 2013; AGRA-NETO, et al., 2014; OLIVEIRA et al., 2016; OLIVEIRA et al., 2017).

Lectinas isoladas da casca (MuBL), cerne (MuHL) e folha de *Myracrodruon urundeuva* promoveram mortalidade de larvas de *A. aegypti* com $CL_{50}$ de 0,125, 0,04 e 0,202 mg/mL, respectivamente (SÁ et al., 2009; NAPOLEÃO et al., 2013). Coelho et al. (2009) e Oliveira et al. (2016) verificaram que lectinas isoladas de sementes íntegras (WSMoL) e da torta de sementes ($WSMoL_C$) de *M. oleifera* exerceram efeitos deletérios sobre larvas de *A. aegypti* ($CL_{50}$: 0,197 e 0,89 mg/mL, respectivamente) além de apresentarem atividade ovicida ($CE_{50}$: 0,1 e 0,14 mg/mL, respectivamente) e estimulante de oviposição (ambas a 0,1 mg/mL). WSMoL e $WSMoL_C$ foram capazes de alterar de enzimas intestinais das larvas. WSMoL também exerceu efeitos negativos sobre o desenvolvimento de *Anagasta kuehniella,* reduzindo o ganho de peso das larvas em 50%, alterando a atividade de enzimas digestivas e diminuindo em 90% a capacidade de digestão de proteínas (OLIVEIRA et al., 2017).

Napoleão et al. (2013) constataram que lectina de folhas de *M. urundeuva* (MuLL) exerceu forte efeito deterrente alimentar sobre adultos de *S. zeamais* quando incorporada em dieta artificial em concentrações de 30 e 150 mg/g, além de promover perda de biomassa devido a alteração de parâmetros nutricionais (diminuição da taxa relativa de ganho de biomassa e eficiência de conversão de alimentos ingeridos) e diminuição das atividades de protease, tripsina, fosfatase ácida e alcalina, amilase e endoglucanase no intestino dos insetos.

Com relação a *P. xylostella*, não foram encontrados na literatura, até o presente momento, estudos sobre o efeito de lectinas sobre esse inseto.

### 3.4.2 Metabólitos secundários

Metabólitos secundários são substâncias produzidas por plantas e que podem ser constitutivos e armazenados na forma inativa ou terem sua produção induzida por diversos fatores, tais como sazonalidade, altitude, temperatura, estímulos mecânicos, índice pluviométrico, radiação ultravioleta, composição atmosférica, ritmo circadiano, idade da planta, composição do solo e ataque de patógenos, como por exemplo os insetos fitófagos. Estes estímulos podem alterar tanto a quantidade quanto a natureza dos metabólitos secundários expressados. Estes constituintes químicos são diversos e cada família, gênero, e espécie produz uma categoria química característica ou uma mistura delas (GOBBO NETO; LOPES, 2007; MORANT et al., 2008; WAKSMUNDZKA-HAJNOS et al., 2008). Os metabólitos secundários podem ser divididos em três grandes classes: compostos fenólicos, terpenos e alcaloides (TAIZ; ZEIGER, 2009)

Os compostos fenólicos são substâncias que possuem pelo menos um anel aromático, onde pelo menos um hidrogênio é substituído por uma hidroxila. Grande parte dos compostos fenólicos estão complexados a carboidratos, proteínas e outros componentes vegetais (CARVALHO et al., 2002; ROBBINS, 2003). É um grupo quimicamente heterogêneo com cerca de 10.000 compostos diferentes. Flavonoides e taninos são compostos fenólicos com comprovada atividade inseticida. Os flavonoides são citotóxicos e se complexam com enzimas, alterando sua atividade. Protegem a planta influenciando comportamento, crescimento e desenvolvimento de insetos praga (WAR et al., 2012). Foi relatado que flavonoides agem como deterrentes alimentares contra *Sitophilus granarius*, *Tribolium confusum* e *Trogoderma granarium* (JACKOWSKI et al., 2017), afetam negativamente o desenvolvimento de *Spodoptera frugiperda,* aumentando período larval, diminuindo o peso de larvas e pupas e a viabilidade pupal (SILVA et al., 2016) e causam mortalidade em larvas de *A. aegypti* e *Anopheles stephensi* (GAUTAM et al., 2013).

Já os taninos são capazes de precipitar proteínas, diminuindo sua digestibilidade, inativando enzimas digestivas e reduzindo a absorção de nutrientes, além de diminuírem a palatibilidade das plantas, agindo como deterrentes alimentares, e causarem lesões no intestino médio de insetos (WAR et al., 2012; SHARMA et al., 2009; SHARMA & AGRWAL, 1983; BARBEHENN & CONSTABEL, 2011). Taninos apresentaram efeito larvicida sobre *A. aegypti* (SILVA et al., 2004), aumentaram o tempo de emergência de

insetos adultos, além de apresentarem ação deterrente sobre a alimentação de *Sitophilus oryzae, Sitotroga cerealella* e *Tribolium castaneum* (WONGO, 1998).

Os terpenos são produzidos a partir do ácido mevalônico (no citoplasma) ou do piruvato e 3-fosfoglicerato (no cloroplasto). São formados pela fusão de unidades isoprênicas de cinco carbonos $(C_5H_8)_n$ e são classificados de acordo com o número de unidades que se ligam entre si, no sentido cabeça-cauda: hemiterpenoides ($C_5H_8$), monoterpenoides $(C_5H_8)_2$, sesquiterpenoides $(C_5H_8)_3$, diterpenoides $(C_5H_8)_4$, triterpenoides $(C_5H_8)_6$ e carotenoides $(C_5H_8)_8$ (PERES, 2004; OLIVEIRA et al., 2003). Timoquinona, um monoterpeno presente em diversos óleos essenciais, apresentou ação tóxica por contato e fumigação em adultos de *S. zeamais* (HERRERA et al., 2015).

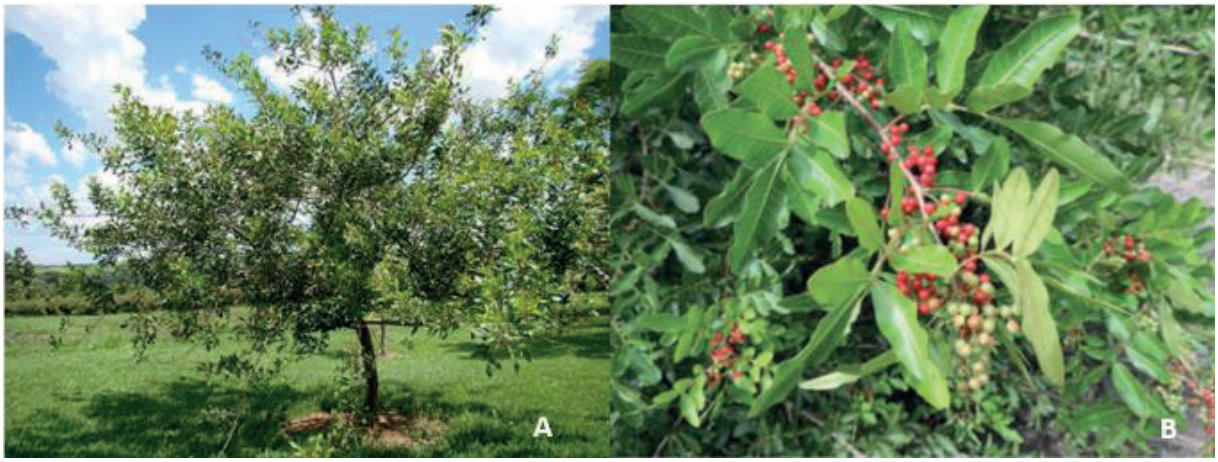
O óleo essencial de inflorescências de *Alpinia purpurata*, rico em monoterpenos e sesquiterpenos (β-pineno, α-pineno, trans-cariofileno, canfeno e 7-epi-α-selineno) causou alterações nos parâmetros nutricionais e teve efeito fumigante contra *S. zeamais* (LIRA et al., 2015). Bianco et al. (2013) relataram atividade larvicida contra *A. aegypti* do sesquiterpeno elatol isolado de extrato de alga marinha do Nordeste brasileiro. O terpinoleno isolado do óleo essencial de *Piper corcovadensis*, foi tóxico para larvas de *A. aegypti* e apresentou efeito deterrente de oviposição (SILVA et al. 2016). Saponinas, pertencentes à classe dos triterpenos, isoladas de *Pentaclethra macroloba* e *Cordia piauhiensis* apresentaram efeito larvicida sobre *A. aegypti* (SANTIAGO et al., 2005). Os compostos *α*-terpineno e *p*-cimeno tiveram influência negativa no desenvolvimento, apresentaram toxicidade por contato e efeito fumigante sobre *P. xylostella* (WEI et al., 2015).

Os alcaloides são provenientes de aminoácidos aromáticos (triptofano, tirosina), os quais são derivados do ácido chiquímico e de aminoácidos alifáticos (ornitina, lisina). São compostos cíclicos, de baixo peso molecular e que possuem pelo menos um átomo de nitrogênio em estado de redução negativa (ALVES, 2001; PERES, 2004; YANG; STOCKIGT, 2010). Alguns alcaloides são conhecidos por apresentarem efeito deterrente na alimentação de insetos (PETROSKI; STANLEY, 2009). Alcaloides isolados da casca de *Aspidosperma pyrifolium* foram tóxicos para larvas de *P. xylostella* (TRINDADE et al., 2008). Escopolamina, um alcaloide tropânico foi tóxico e atrasou o desenvolvimento larval de *Spodoptera frugiperda* (ALVES et al., 2007).

## 3.5 *Schinus terebinthifolia* Raddi

*Schinus terebinthifolia* é uma planta pertencente à família Anacardiaceae, sendo popularmente conhecida por aroeira-vermelha, aroeira-da-praia, aroeira-pimenteira, aroeira-negra, dentre outros (Figura 8A). É uma espécie perenifólia que, quando jovem, apresenta de 5 m a 10 m de altura e entre 20 cm e 30 cm de diâmetro. Os indivíduos adultos chegam a alcançar 15 m de altura e 60 cm de diâmetro (NEVES et al., 2016; MINISTÉRIO DA SAÚDE, 2014).

**Figura 8-** Aspecto geral da árvore de *Schinus terebinthifolius* (A) e detalhe dos ramos com folhas e frutos maduros (vermelho) e imaturos (esverdeados) (B)




Fonte: EMBRAPA (2016)

*S. terebinthifolia* possui distribuição tropical e subtropical, sendo originária da América do Sul, nativa do Brasil, Paraguai, Uruguai e leste da Argentina. No Brasil, pode ser encontrada nos estados de Sergipe, Paraíba, Alagoas, Pernambuco, Rio Grande do Norte, Bahia, Espirito Santo, Mato Grosso do Sul, Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Norte, Santa Catarina e São Paulo. Esta planta adapta-se a diversos habitats sendo comum em beira de rios, córregos e em várzeas úmidas e região litorânea, porém, cresce também em terrenos secos e pobres (SOUZA, 2005; LORENZI, 2008).

*S. terebinthifolia* é bastante utilizada na medicina popular, em forma de chás, infusões e tinturas e apresenta diversas atividades biológicas já descritas, tais como: anti-inflamatória, cicatrizante, antirreumática, antimicrobiana, antiparasitária antibiofilme, antialérgica, antitumoral, gastroprotetora, entre outras (QUEIRES et al. 2006; CAVALHER-MACHADO et al., 2008; MATSUO et al. 2011; BERNARDES et al. 2014; FEDEL-MIYASATO et al.

2014;BARBIERI et al., 2014; ROSAS et al. 2015; DANNEMBERG et al., 2016; SILVA et al., 2017).

Ácidos anacárdicos, monoterpenos, sesquiterpenos diterpenos, triterpenos, flavonoides e derivados do ácido gálico foram isolados de *S. terebinthifolia* (HERINGER, 2009). Uliana et al. (2016) identificaram δ-3-careno (68,78%), E-cariofileno (8,22%), mirceno (6,78%) e α-pineno (4,05%) como os principais compostos do óleo essencial de folhas de *S. terebinthifolia*, enquanto análises de espectrometria de massa revelaram que os ácidos ferulico e cafeico e a quercetina foram os principais componentes de extratos etanólicos. Santana et al. (2012) isolaram 6 compostos das folhas dessa espécie: ácido gálico, galato de etila, galato de metila, trans catequina, quercitrina e afzelina. Santos (2010) identificaram a partir das folhas de aroeira o ácido gálico com potencial alelopático

Uma lectina (SteLL) foi isolada de extrato salino de folhas de *S. terebinthifolia* através de cromatografia de afinidade em coluna de quitina SteLL apresentou atividade antimicrobiana contra *Escherichia coli, Klebisiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteritidis* e *Staphylococcus aureus* (GOMES et al., 2013) e atividade antitumoral *in vivo* no modelo de sarcoma 180 em camundongos (RAMOS et al., 2019).

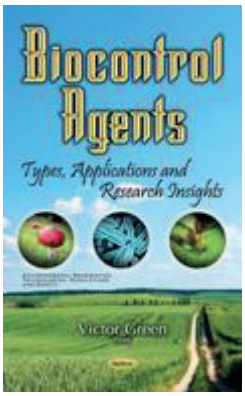
Procópio et al. (2015) detectaram proantocianidinas poliméricas, taninos hidrolisáveis, flavonoides heterosídeos e aglicônicos, derivados do ácido cinâmico e traços de esteroides em extrato salino de folhas de *S. terebinthifolia*. Esse extrato continha também lectina e apresentou efeito larvicida contra *A. aegypti*, promovendo desorganização intensa do epitélio do intestino médio das larvas, incluindo deformação e hipertrofia das células, ruptura de microvilosidades e vacuolização de citoplasmas, afetando células digestivas, enteroendócrinas, regenerativas e proliferativas. Além disso, células com DNA fragmentado foram observadas. Derivados do ácido cinâmico e flavonoides estão envolvidos com o efeito larvicida contra *A. aegypti.* Por outro lado, os autores demonstraram que, nesse caso, a lectina SteLL não está envolvida na ação larvicida contra esse mosquito.

# 4 RESULTADOS

Os resultados dessa pesquisa são apresentados na forma de artigos.

## 4.1 CAPÍTULO - PHYTOINSECTICIDES FOR CONTROLLING PESTS AND DISEASE VECTORS

Artigo de revisão publicado no livro **"Biocontrol Agents: Types, Applications and Research Insights"** (Chapter 5, Nova Science Publishers, New York, 2017 – ISBN 9781536105797)



In: Biocontrol Agents
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*Chapter 5*

# PHYTOINSECTICIDES FOR CONTROLLING PESTS AND MOSQUITO VECTORS OF DISEASES

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## ABSTRACT

Strategies to protect stored products from insect attacks are among the major concerns in the food industry and agriculture. In another aspect, the control of insect vector population is a key point in public health organizations to prevent diseases such as malaria, encephalitis, dengue, yellow fever, chikungunya, and zika. The use of synthetic insecticides still prevails as one of the main forms of insect management. However, the use of many synthetic insecticides is costly, pollutes the environment, is hazardous to non-target organisms, and promotes the emergence of resistant insect populations. In this regard, alternative strategies for insect control are of utmost importance. The integrated management of insects

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aims to maintain the pest or vector population density below the level of economic or social damage by using methods that are less hazardous to non-target organisms and to the environment in comparison with synthetic insecticides. Phytochemicals that are used in the protection of plants against herbivores and predators include secondary metabolites and defense proteins, which usually exhibit insecticidal activity. Furthermore, the use of phytoinsecticides may represent an important alternative to integrate control strategies that can be less aggressive and more ecologically beneficial, as these compounds usually show a more selective toxicity and a higher degree of biodegradability with respect to synthetic insecticides. In this respect, different parts of the plants have been evaluated for insect control in several studies. Plant extracts and tissue powders, wood ashes, fixed and essential oils, lectins, enzyme inhibitors, and a variety of secondary metabolites, among others, have been reported to show toxic, deterrent, attractive, and repellent effects. In addition, they are able to alter the insect development, reproduction, and behavior. This chapter is divided into four parts that review the state-of-the-art on the potential of phytoinsecticides, their action mechanisms, and their relative importance to integrated management strategies.

## 1. Agricultural Insect Pests

Insects (Kingdom Animalia, Phylum Arthropoda, Class Insecta) are considered as the most successful and diversified group in the animal kingdom. Insects play several roles in the environment acting as predators, parasites, pollinators, and decomposers, among others. They are economically and pharmacologically important to humans owing to their production of valuable materials or products such as honey, propolis, resilin, carmine, silk, wax, lacquer, and venoms (van Huis, 2003; Costa-Neto, 2004; Costa-Neto and Rezende, 2004; Elvin et al., 2005; Rodrigues, 2006). However, approximately 0.5% of the insect species (including some beetles, termites, and moths) are considered pests, which cause damage to several crops (Sallam, 2008).

The control of insect pests has been a major challenge since ancient times to the present, requiring substantial efforts from farmers, landowners, food industries, public health organizations, and environmental agencies (Food and Agriculture Organization, 2009; World Health Organization, 2012b). The predominance of agrosystems based on large monoculture plantations favors the dissemination of insect pests, particularly in the tropics and subtropics where the climate allows the development of a wide variety of insects (Deguine et al., 2008; Malézieux et al., 2009). In addition, in several

countries, harvest is stored under poor conditions (e.g., high temperature and humidity, oxygen availability, and presence of microorganisms), and agricultural technologies are not available, which leads to an easier establishment of pests (Antonello et al., 2009; Daglish et al., 2014).

An insect is considered a 'pest' when it reaches a population density that can cause damages of economic relevance (Imenes and Ide, 2002). The insect pests can attack all plant life stages and parts (e.g., buds, cotyledons, leaves, nodes, stems, petioles, seedlings, roots, seeds, and pods) as well as their products and by-products (Imenes and Ide, 2002; Santos, 2003). The damage caused by the insects on plants may be due to direct consumption, disease transmission, and competition for space and nutrients, reflecting a decrease in product yield or quality as well as impairing the normal development of the plant or even causing death (Santos, 2003). Some species that attack grains feed on the endosperm causing weight and quality losses, whereas others feed on the embryo resulting in inefficient seed germination and decreased viability (Caneppele et al., 2003; Antunes et al., 2011).

The attack by insect pests may also affect food safety (Food and Agriculture Organization, 2013). The deterioration of grains and the presence of insect fragments and/or excreta in foods may exert important effects on human and animal health. Perforations made by the insects in grains and fruits facilitate the entry and proliferation of pathogenic microorganisms (White, 1995; Faroni and Silva, 2000). Fungal presence can lead to contamination by highly toxic substances, known as mycotoxins (Faroni and Silva, 2000). Some insects may also favor the spread of bacterial and fungal infections due to the transmission of spores (Vega and Kaya, 2012).

The proper storage of seeds is important to preserve the quality of the grain in order to avoid losses as well as to supply the demand during the off-season (Alves et al., 2008). The risk of infestation by insect pests may also vary according to the kind of grain or seed, volume, and storage period, among other factors (Almeida et al., 2004). Estimates report that the cost related to damages in stored foods caused by insect pests may reach US$ 17.7 billion per year (Oliveira et al., 2014).

With increasing globalization, an increase of crop attack by invasive alien species, including insects, has been observed (Hulme, 2009). Alien insect pests pose a threat to biological diversity and may contribute to social and economical instability (Corey et al., 2016). In particular, in the United States, it was estimated in 2005 that losses of crop and forest production due to the action of invasive insects and pathogens were equivalent to approximately US$ 40 billion per year (Pimentel et al., 2005).

The insect pests that attack agricultural crops can be divided into two groups according to their mouthparts: sucking and chewing insects (Figure 1). The chewing insects act on different parts of the plants (e.g., leaves, flowers, flower buds, seeds, roots, and tubers). The destruction of the leaves by these insects reduces the light gathering area, thereby affecting photosynthesis and decreasing plant growth. When acting on stems, these insects may open galleries that interrupt sap flow. The attack of flowers leads to the reduction of seed production. Finally, the action of the chewing insects facilitates the invasion of microorganisms. Some of the most important chewing insects are mole crickets, crickets, grasshoppers and hopes (Orthoptera), beetles (Coleoptera), flies in the larval stage (Diptera), and caterpillars (Lepidoptera) (Marques et al., 1999; Imenes and Ide, 2002).

Insects can suck the sap from the roots, stems, branches, leaves, and fruits leading to darkening, chlorosis, wrinkling, deformation, and necrosis. In addition, they can inject toxins during suction, producing changes in normal tissue development. Other insects are vectors of diseases caused by viruses and fungi. The most important sucking insects are aphids, mealybugs, whiteflies, leafhoppers, bugs (Hemiptera), and thrips (Thysanoptera) (Imenes and Ide, 2002; Abro et al., 2004; Khalil et al., 2015).

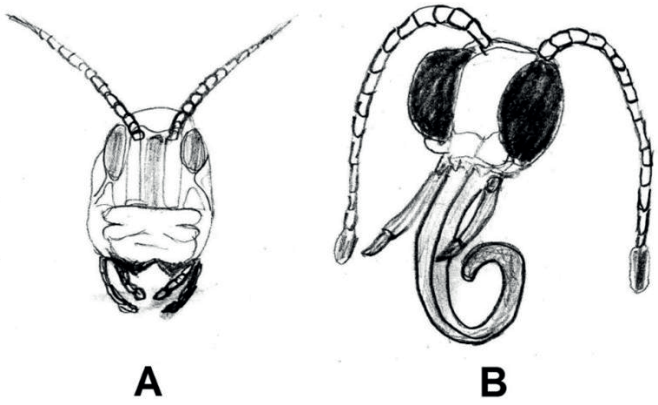


Figure 1. Representations of the mouthparts of chewing (A) and sucking (B) insects. Chewing insects possess a pair of mandibles, which are used to cut, tear, crush and/or chew the food. The maxillae are used to manipulate and masticate. Sucking insects possess a siphon-like structure that allows to pierce and to suck liquid from a plant or animal tissue.

In stored grains, attacking intact grains is a primary action of insect pests, such as those belonging to the genera *Sitophilus* and *Oryzaephilus* (Coleoptera) (Botton et al., 2005), whereas attacking grains previously damaged or byproducts is a secondary action of other insect pests, such as those belonging to the genus *Tribolium* (Coleoptera) (Sallam, 2008). The species *Sitophilus zeamais* (Coleoptera, Curculionidae), more commonly known as maize weevil, is a major pest of stored grains (e.g., corn, rice, wheat, sorghum, barley, and beans) and may attack industrialized foods (e.g., pasta, biscuits, chocolate, and dried fruits) and grape berries during the maturation phase (Gallo et al., 2002; Botton et al., 2005).

## 2. Mosquitoes as Vectors of Human Diseases

Many zoonoses are indirectly or directly transferred to the hosts by bloodsucking arthropods, such as some mosquitoes, flies, and bugs, which then act as vectors of diseases (Vinauger et al., 2016; World Health Organization, 2016a). Several viruses transmitted by arthropods (called arboviruses) circulate among wild and domestic animals as well as humans (Weaver and Reisen, 2010). Mosquitoes (Order Diptera) are found worldwide except in the coldest regions. Approximately 3,500 species of mosquitoes are known, and most of them are native from tropical and subtropical regions (Bee et al., 2009).

The number of deaths caused by diseases transmitted by mosquitoes is highly significant worldwide and more than 700 million people are annually affected (Kessler and Guerrin, 2008; World Health Organization, 2012b). Several species of the genera *Anopheles*, *Culex*, and *Aedes* (Culicidae family) are vectors of diseases of high epidemiological relevance such as malaria, filariasis, encephalitis, yellow fever, dengue fever, chikungunya, and zika, among others (Weaver and Reisen, 2009; Vasilakis et al., 2011; Yalcindag et al., 2012; European Centre for Disease Prevention and Control, 2016).

Vaccination programs against yellow fever have reduced the risk of outbreaks in some endemic regions. Vaccination is mandatory for visitors in some countries in Africa and tropical regions of America where there is a high risk of infection (World Health Organization, 2016e). Vaccines against malaria and dengue fever are not yet licensed and regulated, although there are promising advances in this direction (Capeding et al., 2014; Vannicea et al., 2016, World Health Organization, 2016b). When vaccines are not available, the control measures of mosquito-borne diseases mainly involve strategies for

the reduction of the vector density. The control of vector populations should minimize disease transmission without harming other organisms and the environment.

Mosquitoes belonging to the genus *Aedes* account for approximately 950 species and are found throughout the world, both in the tropics and in colder climates. The species *Aedes aegypti*, which is widely distributed in Asia, Africa, and Central and South America, is responsible for the transmission of viruses that cause yellow fever, dengue fever, and zika (all from the genus *Flavivirus*) as well as chikungunya (*Alphavirus*). In some areas, *Aedes* species are involved in the transmission of filariasis (Lenhart, 2007; Guo et al., 2016).

Dengue fever is currently classified as the most important disease transmitted by mosquitoes worldwide, but in spite of its relevance, it is still considered a neglected tropical disease. The incidence of dengue has increased 30 times in the last 50 years, and it is estimated that approximately 2.5 billion people live in countries where dengue is endemic. Approximately 390 million new cases are reported annually with 96 million manifesting the symptoms, and approximately 2.5% of the infected people die (World Health Organization, 2016b). The species *Aedes albopictus* can also transmit dengue (Conway et al., 2014).

Chikungunya virus (CHIKV) is the cause of an emerging infection that has spread along tropical and subtropical regions. It has been reported in more than 60 countries in Asia, Africa, Europe, and the Americas. During 2015, 693,489 suspected cases and 37,480 confirmed cases of chikungunya were reported in America (Prince et al., 2015; World Health Organization, 2016c).

The first outbreak of Zika virus disease occurred in 2007 in Micronesia. The next outbreak was recorded in French Polynesia in 2013 and 2014. In 2015, Zika virus spread in the Americas, and since then, it was reported in more than 40 countries of this continent (Duffy et al., 2009; European Centre for Disease Prevention and Control, 2015; Hennessey et al., 2016; World Health Organization, 2016d). The infection by Zika virus in pregnant women has gained notoriety recently owing to its strong association with Guillain-Barré syndrome and microcephaly in newborns (Johansson et al., 2016; Broutet et al., 2016; Miranda et al., 2016; Rasmussen et al., 2016).

Approximately 380 species of *Anopheles* mosquitoes are described as widely distributed around the world. Among these, 60 can act as malaria vectors, and some can also transmit filariasis and viral diseases to humans through bites. Malaria is caused by four protozoan species, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, which are widely distributed in Africa and Asia as well as Central and South

America. According to the World Health Organization (2015), 3.2 billion people live in areas at risk of infection. Approximately 200 million new cases of malaria occur annually, killing more than 500,000 people each year. Further, approximately 90% of the deaths occur in Africa followed by Asia (7%) and the eastern Mediterranean (2%). In the Americas, 14% of the population is at risk of transmission.

*Culex* is the largest genus of the family Culicidae comprising mosquitoes that are vectors of diseases such as epidemic encephalitis and lymphatic filariasis. *Culex pipiens* is a vector of viral encephalitis in North America, Rift Valley fever in Egypt, and lymphatic filariasis in East Asia. *Culex quinquefasciatus*, commonly known as 'stilt' or 'muriçoca', is considered a pest by the inhabitants of warmer regions of the Americas, Asia, Africa, and Oceania and an important vector of filariasis in the tropics (Behura et al., 2011; Harbach, 2011, 2013).

## 3. Control of Insect Pests and Vectors

Several types of practices have been employed aiming to control insects. Some methods involve agricultural techniques such as crop rotation, destruction of crop residues, pruning, and tillage, among others (Katan, 2000). The use of plants resistant to insects is considered the ideal control method owing to the possibility of maintaining the pest population at levels lower than that able to promote economic damage without the need of insecticide application. Physical methods include burning, drainage, flood, regulation of temperature and light, employment of sound, and ionizing radiation; however, all of them may be hazardous to the environment and/or humans. Mechanical control is usually employed to combat insect vectors and is based on the elimination or adequate protection of potential breeding sites. Biological control is based on regulating the size of pest populations using predators, parasitoids, pathogens, or competitors. Chemical control corresponds to the use of chemical compounds (insecticides) to reduce insect population through direct or indirect application at appropriate concentrations (Castro et al., 2012; Boyce et al., 2013; Grasswitz and Fimbres, 2013; Andorno and Lopez, 2014).

Insecticides are used to kill insects or interfere with their behavior, development, and fitness. An optimal insecticide should be effective at low concentrations, exhibit specificity to the target organism, and have a short half-life time, being easily degraded into non-toxic products. The effective application of an insecticide also depends on the economic viability (Sakkas

et al., 2002; Rosell et al., 2008; Castro et al., 2012). The use of chemical insecticides still prevails as one of the main forms of control owing to its immediate efficiency and low initial cost (Roubos et al., 2014). However, the indiscriminate use of synthetic pesticides pollutes the environment, may be hazardous to humans, animals, plants, and other non-target organisms, and leads to the selection of resistant individuals and establishment of resistant populations. In addition, some insecticides accumulate through the food chain (Benhalima et al., 2004; Kemabonta and Odebiyi, 2005; Corrêa and Salgado, 2011).

The organochlorines, organophosphates, carbamates, and pyrethroids, among others, are the compounds largely used for insect control (Ranson et al., 2010). All these compounds target the nervous system of insects. Organophosphates and carbamates act as acetylcholinesterase inhibitors, whereas pyrethroids and organochlorines act on voltage-dependent sodium channels (Ranson et al., 2010; Martins et al., 2009). Studies have shown that these pesticides can remain in the environment for a long period of time, causing adverse environmental effects (Borja et al., 2005; Skarphedinsdottir et al., 2010).

Organochlorines are used for a long time in agriculture and vector control programs without any concern about the possible damages to human health and to the environment (Skarphedinsdottir et al., 2010; Silva et al., 2016). These insecticides are present in many lists of pollutants due to their high persistence in the environment and easy accumulation in the adipose tissues of animals, causing bioaccumulation and biomagnification effects along the food chain (Amr et al., 1995; Borja et al., 2005; Skarphedinsdottir et al., 2010). The unspecific toxicity of organochlorines also promotes the disruption of the natural insect control by natural enemies leading to insect pest outbreaks and other biological imbalance problems (Kim et al., 2003; Costa et al., 2004; Menezes, 2005; Silva et al., 2016).

Organophosphates and carbamates bind to the active site of the acetylcholinesterase enzyme, thus inhibiting the physiological action of acetylcholine hydrolysis at the neuromuscular junctions of the nervous system of insects. The resulting accumulation of acetylcholine leads to paralysis and death of the insect (Hemingway and Ranson, 2000; Ranson et al., 2010). Organophosphate insecticides have been widely used as an alternative to replace organochlorine compounds owing to facile synthesis, lower cost and toxicity, higher biodegradability, and lower bioaccumulative degree. However, the intensive application of these insecticides has caused adverse impacts on

agrosystems and resulted in a large number of resistant insect populations (Ahmad, 2007; Kliot and Ghanim, 2012).

The indiscriminate and prolonged use of any insecticide results in the selection of resistant individuals, decreasing the frequency of susceptible insects and reducing the variability in the mosquito population (Valle et al., 2015). This resistance can be due to several mechanisms, such as the expression of modified/insensitive target and increased capacity of xenobiotic detoxification (Li et al., 2007; Bellinato et al., 2016). Several mutations in genes encoding voltage-dependent sodium channels were identified in resistant individuals of *Anopheles gambiae*, *Anopheles arabiensis*, *Culex pipiens* and *Aedes aegypti* (Brengues et al., 2003; Bahnck and Fonseca, 2006; Chang et al., 2009; Siller et al., 2011; Yanola et al., 2011; Jones et al., 2012; Ochomo et al., 2012).

The detoxification of xenobiotics is divided into two phases. The first corresponds to the chemical modification of substrates by the action of mixed-function oxidases and esterases. The second stage involves the conjugation enzymes, such as glutathione-S-transferases. An overexpression of these enzymes or expression of modified forms with higher catalytic efficiency is usually linked with insecticide resistance (Yang et al., 2001; Yu, 2008). Studies have demonstrated the resistance of *A. aegypti* populations to organophosphates related to alterations of the levels and functioning of esterases, mixed-function oxidases, and glutathione-S-transferase (Montella et al., 2007; Strode et al., 2008; Araújo et al., 2013; Poupardin et al., 2014).

The most recent legislations in several countries have placed greater restrictions on pesticide use, mainly on products with broad action spectrum. This stimulated the adoption of more environmentally friendly insecticides and the development of integrated pest management strategies using chemical and biological controls (Hillocks, 2012). Diflubenzuron and novaluron (chitin synthesis inhibitors) and pyriproxyfen (juvenile hormone analogue) are among the alternative chemical insecticides introduced for mosquito control (Dhadialla et al., 2005; Jaffer et al., 2015). Alternative products also recommended by the World Health Organization for mosquito control include biolarvicides Bti (*Bacillus thuringiensis* var. *israelensis*) and Bs (*Bacillus sphaericus*) and the natural insecticide spinosad (World Health Organization, 2016f). The development of new insecticides, preferably with natural origin, is important to expand the list of available alternatives.

## 4. PHYTOINSECTICIDES

There are several problems related to the use of synthetic insecticides as mentioned in the previous section. This has caused an increased interest for using alternative compounds in insect control, with particular importance being delegated to phytochemical/botanical insecticides (Sauvion et al., 2004; Macedo et al., 2007; Coelho et al., 2009). A large number of plants with insecticidal activity have been studied. The use of natural insecticides constitutes important alternatives to meet the demand for less aggressive control strategies, since they usually rapidly degrade, resulting in a low persistence and residual action, and present a more selective toxicity (Pontual et al., 2014; Benelli, 2015).

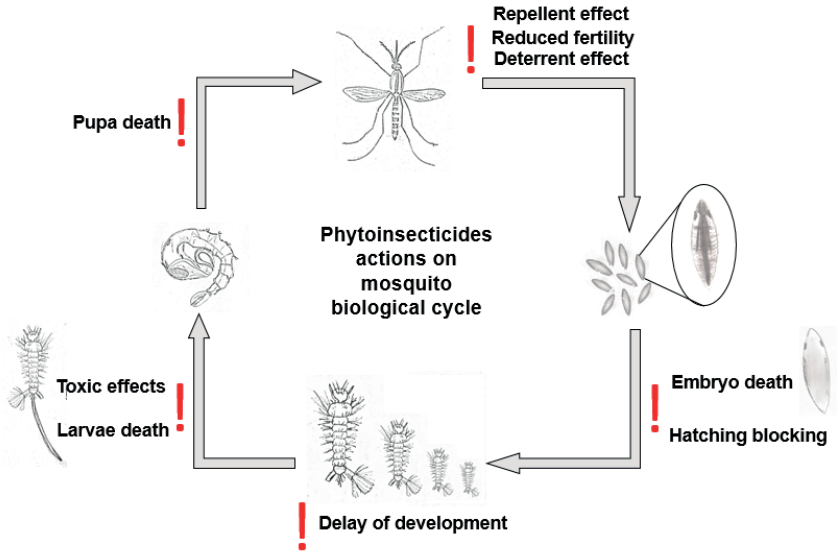


Figure 2. Effects of phytoinsecticides on different moments during the life cycle of mosquitoes. These agents may interfere with embryo development or cause the death of the larvae inside the egg, blocking the hatching (Santos et al., 2012b). Phytoinsecticides also may cause delay of larvae metamorphosis and promote toxic effects (such as induction of gut content elimination and midgut disorganization), resulting in larvae death (Procópio et al., 2015). On adults, phytoinsecticides may have repellent effect and cause death as well as disturb the reproduction by exerting deterrent effect or impairing the fertility (due to problems during metamorphosis of pupa to adult).

Plants contain compounds produced for self-defense against herbivores and predators. These agents have been widely evaluated as insecticides. Phytoinsecticides can promote mortality of insects at all stages, interfere with the metamorphosis, cause morphological changes, and exert irritant, repellent, attractive, and deterrent effects. In addition, the fertility, reproduction, and behavior may be affected (Cavalcante et al., 2006; Costa et al., 2012; Deletre et al., 2013; Navarro et al., 2013; Pontual et al., 2014; Procópio et al., 2015; Mohankumar et al., 2016). Some effects of phytoinsecticides on the life stages of mosquitoes are shown in Figure 2.

Phytoinsecticides may correspond to their own plant material (usually powdered), derived products (wood ash) or mixtures/compounds obtained by extraction in aqueous solutions or organic solvents (Menezes, 2005). The active principles present in phytoinsecticides may be classified as primary metabolites, such as proteins, or secondary metabolites. Most active compounds in phytoinsecticides are secondary metabolites, which are extremely diverse in structure (Kim et al., 2003; Bernhoft, 2010). Each plant family, genus, and species can produce a specific chemical category or a different mix of these metabolites (Waksmundzka-Hajnos et al., 2008). The synthesis of secondary metabolites is influenced by several factors, such as seasonality, circadian rhythm, developmental stage, temperature, water availability, UV incidence, available nutrients, altitude, atmospheric pollution, mechanical damage, and pathogen/predator/herbivore attack (Gobbo Neto and Lopes, 2007).

The plant secondary metabolites are divided into three main categories: terpenes and terpenoids, alkaloids, and phenolic compounds. Phenolic compounds are synthesized by the routes of shikimic acid and mevalonic acid and can be classified as simple phenols (phenolic acids and cinnamic acids) and polyphenols (flavonoids, tannins, and others). Flavonoids are important agents in defense against insects and pathogens, in addition to protecting plants against the incidence of UV rays and attracting animals for pollination (Yao-Lan et al., 2002; Zuanazzi and Montanha, 2004; Angelo and Jorge, 2007; Simões et al., 2010). Tannins are associated with the resistance of plants to herbivores (Haukioja, 2003). Terpenes are produced from mevalonic acid (in the cytoplasm) or pyruvate and 3-phosphoglycerate (in the chloroplast) and are classified into hemiterpenoides, monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetraterpenoids according to the number of isoprene units (Oliveira et al., 2003; Peres, 2004). They can be volatile compounds acting as attractants or repellents to insects and are usually found in the constitution of essential oils. In addition, they have antimicrobial, anti-

herbivory, hormonal, and pesticide activities (Croteau et al., 2000; Oliveira et al., 2003; Niero and Malheiros, 2007). Alkaloids are derived from aromatic amino acids (tryptophan and tyrosine), which are derivatives of shikimic acid or aliphatic amino acids (ornithine and lysine). These compounds are pharmacologically active and found predominantly in angiosperms (Henriques et al., 2002); some examples include nicotine, caffeine, and vincristine (Alves, 2001; Peres, 2004).

Plant extracts and essential oils contain different types of metabolites that exert deleterious effects on insects. The use of plant extracts or other kinds of compound mixtures is advantageous owing to the fact that the presence of several active principles may hinder insect resistance causing different insecticidal mechanisms to be involved (Pontual et al., 2012). Pyrethrins are phytoinsecticides broadly used worldwide as aerosols and are usually commercially available in formulations associated with synthetic compounds that increase the time of action and delay the expiration date (Veer and Gopalakrishnan, 2016). The alkaloid nicotin has insecticidal effects similar to those of organophosphates and carbamates, being usually used as fumigant; however, it shows toxicity to mammals and has limited insecticidal efficacy (Slotkin, 2004; Veer and Gopalakrishnan, 2016). Tannins have proved insecticidal activity by affecting growth and survival of insects due to formation of complexes with proteins at the intestinal tract (including digestive enzymes), reducing the digestibility of nutrients (Mello and Silva-Filho, 2002). Flavonoids are also able to exert antinutritional effects on insects (Salvador et al., 2010; Tavares et al., 2014).

Essential oils are mixtures of lipophilic and volatile substances, which are usually odoriferous and liquid (De la Rosa et al., 2010). They are usually composed of not only molecules with terpene nature but also aldehydes, ketones, phenols, esters, oxides, peroxides, furans, organic acids, lactones, and coumarins (Simões and Spitzer, 2004; De la Rosa et al., 2010). Essential oils can be found in all plant organs, and are related to several functions necessary for plant survival, playing a key role in the defense against pathogens (Siqui et al., 2000).

Essential oils have been applied in distinct agricultural areas owing to their insecticidal, fungistatic, and herbicidal effects. In insects, they exert toxicity through contact or fumigation, repellent property, feeding and oviposition deterrent effects, impairing the effects on fecundity and fertility, and inhibitory effect on development and growth. A few examples of essential oils with these properties are listed in Table 1.

**Table 1. Plant sources of essential oils with insecticidal activity against agricultural pests**

| Plants | Species affected | Effects |
|---|---|---|
| *Allium sativum* | *Choristoneura rosaceana* | Larvicidal |
| *Alpinia purpurata* | *Sitophilus zeamais* | Fumigant toxicity and feeding deterrent |
| *Anethum graveolens*<br>*Carum carvi*<br>*Cuminum cyminum* | *Sitophilus oryzae* | Fumigant toxicity |
| *Apium graveolens*<br>*Citrus sinensis*<br>*Eucalyptus globulus*<br>*Juniperus oxycedrus*<br>*Laurus nobilis*<br>*Lavandula hybrida*<br>*Mentha microphylla*<br>*Mentha viridis*<br>*Ocimum basilicum*<br>*Origanum vulgare*<br>*Pistacia terebinthus*<br>*Rosmarinus officinalis*<br>*Thuja orientalis* | *Acanthoscelides obtectus* | Fumigant toxicity, repellent and impairing effects on fecundity and fertility |
| *Cananga odorata*<br>*Lepechinia betonicifolia*<br>*Lippia alba*<br>*Rosmarinus officinalis*<br>*Tagetes lucida* | *Tribolium castaneum* | Repellent |
| *Cinnamomum glaucescens* | *Callosobruchus chinensis* | Fumigant toxicity, antifeedant, oviposition deterrent, impairing effects on fecundity and fertility |
| *Citrus latifolia*<br>*Citrus reticulate*<br>*Citrus sinensis*<br>*Citrus paradise* | *Callosobruchus maculatus* | Contact toxicity, fumigant toxicity, impairing effects on fecundity and fertility |
| *Crithmum maritimum* | *Spodoptera exigua* | Larvicidal, impairing effects on development and growth |
| *Crithmum maritimum* | *Sitophilus oryzae*<br>*Oryzaephilus surinamensis* | Fumigant toxicity, contact toxicity |
| *Cuminum cyminum* | *Callosobruchus chinensis*<br>*Sitophilus oryzae* | Fumigant toxicity, repellent, oviposition deterrent, ovicidal, larvicidal and pupicidal. |
| *Cymbopogon winterianus* | *Spodoptera frugiperda* | Impairing effects on fecundity and fertility |

**Table 1. (Continued)**

| Plants | Species affected | Effects |
|---|---|---|
| *Cymbopogon winterianus*<br>*Eucalyptus citriodora*<br>*Eucalyptus staigeriana*<br>*Foeniculum vulgare* | *Callosobruchus maculatus* | Contact toxicity, fumigant toxicity, repellent, impairing effects on fecundity and fertility, |
| *Elettaria cardamomum* | *Cydia pomonella* | Antifeedant and oviposition deterrent |
| *Lavandula stoechas* subsp. *stoechas*<br>*Mentha spicata*<br>*Origanum onites*<br>*Thymbra spicata* subsp. *spicata* | *Tetranychus cinnabarinus* | Contact toxicity |
| *Litsea cubeba* | *Lasioderma serricorne*<br>*Liposcelis bostrychophila* | Fumigant toxicity, contact toxicity, repellent |
| *Mikania micranta* | *Plutella xylostella* | Repellent, oviposition deterrent |
| *Vitex trifolia*<br>*Vitex agnus-castus* | *Spilosoma obliqua* | Impairing effects on development and growth |

References: Papachristos et al. (2002); Zhang et al. (2004); Kiran et al. (2006); Tandon et al. (2008); Sertkaya et al. (2010); Machial et al. (2010); Caballero-Gallardo et al. (2011); Kim et al. (2013); Prakash et al. (2013); Gusmão et al. (2013); Yang et al. (2014); Kedia et al. (2015); Lira et al. (2015); Dutra et al. (2016); Polatoglu et al. (2016); Kovanci (2016); Silva et al. (2016)

Essential oils extracted from several plant species have shown considerable effectiveness as toxic and repellent agents against various blood-sucking arthropods, especially mosquitoes (Table 2). Phytochemicals present in essential oils such as thymol, linalool, citronellol, limonene, carvacrol, α- and β-pinene, 3-carene, myrcene, camphene, thymoquinone, carvone, ihydrocarvone, menthone, verbenone, ocimenone, camphor, thujone and piperitenone were described as responsible for larvicidal and adulticide effects against mosquitoes (Phillis and Appel, 2010; Govindarajan et al., 2013).

The toxic effect of essential oils on insects and other arthropods may be due to the neurotoxic action of their components, which act as acetylcholinesterase inhibitors or blockers of octopamine receptors (Isman and Machial, 2006; Kim et al., 2013). Essential oils may also have several other targets, such as GABA receptors coupled to chloride channels, tyramine receptors, nicotinic acetylcholine receptors, and sodium channels (Tong and Coats, 2010). It is important to highlight that several essential oils did not have their action mechanisms defined.

**Table 2. Plant sources of essential oils with insecticidal activity against mosquitoes**

| Plant | Species affected | Activity |
|---|---|---|
| *Acantholippia seriphioides*<br>*Achyrocline satureioides*<br>*Aloysia citriodora*<br>*Anemia tomentosa*<br>*Anethum graveolens*<br>*Baccharis spartioides*<br>*Chenopodium ambrosioides*<br>*Eucalyptus saligna*<br>*Hyptis mutabilis*<br>*Kaempferia galanga*<br>*Minthostachys mollis*<br>*Rosmarinus officinalis*<br>*Tagetes minuta*<br>*Tagetes pusilla*<br>*Zanthoxylum piperitum* | *Aedes aegypti* | Repellent |
| *Alpinia purpurata*<br>*Piper corcovadensis* | *Aedes aegypti* | Larvicidal and oviposition deterrent |
| *Chromolaena odorata* | *Aedes albopictus* | Repellent |
| *Cinnamomum zeylanicum* | *Anopheles stephensi*<br>*Aedes aegypti*<br>*Culex quinquefasciatus* | Repellent and oviposition deterrent |
| *Cinnamosma madagascariensis* | *Culex quinquefasciatus* | Larvicidal |
| *Citrus aurantium*<br>*Citrus sinensis* | *Anopheles labranchiae* | Larvicidal |
| *Eucalyptus camaldulensis*<br>*Mentha piperita*<br>*Ocimum basilicum*<br>*Pimpinella anisum* | *Culex pipiens* | Repellent |
| *Juniperus macropoda*<br>*Pimpinella anisum* | *Anopheles stephensi*<br>*Aedes aegypti*<br>*Culex quinquefasciatus* | Larvicidal and ovicidal |
| *Juniperus procera* | *Anopheles arabiensis* | Repellent |
| *Mentha microcorphylla*<br>*Myrtus communis*<br>*Origanum syriacum*<br>*Pistacia lentiscus*<br>*Lavandula stoechas* | *Culex pipiens molestus* | Larvicidal |
| *Rosmarinus officinalis*<br>*Zingiber officinale* | *Anopheles stephensi*<br>*Aedes aegypti*<br>*Culex quinquefasciatus* | Ovicidal and repellent |
| *Tagetes minuta* | *Anopheles gambiae* | Larvicidal |
| *Zingiber officinalis* | *Culex quinquefasciatus* | Larvicidal and repellent |

References: Traboulsi et al. (2002); Tawatsin et al. (2006); Prajapati et al. (2005); Erler et al. (2006); Choochote et al. (2007); Gillij et al. (2008); Pushpanathan et al. (2008); Santos et al. (2012a); Kyarimpa et al. (2014); Karunamoorthi et al. (2014); El-Akhal et al. (2015); Silva et al. (2016); Pavela et al. (2016)

Lectins and the enzyme inhibitors are among the entomotoxic proteins produced by plants. Lectins are carbohydrate-binding proteins widely found in

nature; in plants, they have been reported in the leaves, barks, roots, rhizomes, bulbs, pods, seeds, fruits, and flowers (Paiva et al., 2011a). These proteins possess at least one non-catalytic domain that binds specifically and reversibly to a carbohydrate or glycoconjugate.

It has been proposed that plant lectins play a role in the overall protection of plants against phytopathogenic microorganisms, nematodes, or insect pests. In addition, they may act as storage proteins for growth and development of the plant (Vandenborre et al., 2011). The binding of these proteins to glycoconjugates present on cell surfaces results in a range of biological properties and are related to the deleterious effects of lectins on phytopathogens and insects (Paiva et al., 2011a).

The insecticidal action of plant lectins against many insect pests and mosquitoes has been reported in Table 3. These proteins may interfere with the development, reproduction, and survival of insects at different stages of life (Sadeghi et al., 2006; Coelho et al., 2007; Macedo et al., 2007; Oliveira et al., 2011; Paiva et al., 2013). The action of lectins on insects may result from processes mediated by gustatory sensors (pre-ingestion effect), usually expressed as deterrent or attractive stimuli, and/or poisoning action (post-intake effect), which is usually associated with the binding of lectin to the digestive tract components (Sauvion et al., 2004; Michiels et al., 2010; Sprawska and Golawska, 2010). Some examples of the mechanisms involved in the insecticidal activity of lectins are listed in Table 4.

Several studies have shown that the incorporation of lectins in artificial diets affects negatively the performance of insects from different orders, such as Lepidoptera, Coleoptera, Diptera, Isoptera, and Hemiptera (Zhou et al., 1999; Sauvion et al., 2004; Subramanyam et al., 2008; Yarasi et al., 2008; Sá et al., 2009; Shahidi-Noghabi et al., 2009; Napoleão et al., 2013; Lima et al., 2016). Insecticidal lectins are usually resistant to degradation by intestinal proteases of insects and can also interact with the digestive enzymes, modulating their activity and promoting metabolic imbalance (Albuquerque et al., 2012; Napoleão et al., 2012, 2013; Paiva et al., 2013; Agra-Neto et al., 2014).

Chitin-binding lectins have the ability to interact with the components of the peritrophic matrix, causing abnormalities in its structure and function. In addition, lectins may disturb the structure of the intestinal microvilli. The binding of these proteins to glycosylated molecules present at the surface of cells at the intestinal tract may induce cellular responses, including caspase activation and DNA fragmentation, for example (Harper et al., 1998; Zhu-Salzman et al., 1998; Hopkins and Harper, 2001; Carlini and Grossi-de-Sá, 2002; Sauvion et al., 2004; Vandenborre et al., 2011; Paiva et al., 2013; Vishwanathreddy et al., 2014; Lima et al., 2016).

**Table 3. Plant sources of lectins with insecticidal action against pests and vectors**

| Lectin (plant) | Source tissue | Insect affected |
|---|---|---|
| ACLEC (*Annona coriacea*) | Seeds | *Corcyra cephalonica* |
| BmoLL (*Bauhinia monandra*) | Leaves | *Anagasta kuehniella*<br>*Callosobruchus maculatus*<br>*Zabrotes subfasciatus* |
| *BmoRoL (Bauhinia monandra*) | *Roots* | *Nasutitermes corniger* |
| CEA (*Colocasia esculenta*) | Tuber | *Bemisia tabaci*<br>*Lipaphis erysimi* |
| cMoL (*Moringa oleifera*) | Whole seeds | *Anagasta kuehniella* |
| ConA (*Canavalia ensiformis*) | Seeds | *Acyrthosiphon pisum*<br>*Lacanobia oleracea*<br>*Rhopalosiphum padi* |
| CrataBL (*Crataeva tapia*) | Bark | *Nasutitermes corniger* |
| DB1 (*Dioscorea batatas*) | Tubers | *Helicoverpa armigera* |
| GNA (*Galanthus nivalis*) | Bulbs | *Lacanobia oleracea*<br>*Nilaparvata lugens* |
| GSII (*Griffonia simplicifolia*) | Leaves | *Callosobruchus maculatus* |
| HHA (*Hippeastrum hybrid*) | Bulbs | *Spodoptera littoralis* |
| Labramin (*Labramia bojeri*) | Seeds | *Ephestia kuehniella* |
| MuBL and MuHL (*Myracrodruon urundeuva*) | Bark and heartwood | *Aedes aegypti*<br>*Nasutitermes corniger* |
| MuLL (*Myracrodruon urundeuva*) | Leaf | *Aedes aegypti*<br>*Nasutitermes corniger*<br>*Sitophilus zeamais* |
| *MvRL (Microgramma vaccinifolia*) | Rhizome | *Nasutitermes corniger* |
| *OfiL (Opuntia ficus indica)* | *Cladodes* | *Nasutitermes corniger* |
| PF2 (*Olneya tesota*) | Seeds | *Zabrotes subfasciatus* |
| PHA (*Phaseolus vulgaris*) | Seeds | *Sitobion avena* |
| SNA-I, SNA-II (*Sambucus nigra*) | Seeds | *Tribolium castaneum* |
| TEL (*Talisia esculenta*) | Seeds | *Diatraea saccharalis* |
| WGA (*Triticum vulgaris*) | Seeds | *Ostrinia nubilalis* |
| WSMoL, WSMoL$_C$ (*Moringa oleifera*) | Whole seeds and seed cake | *Aedes aegypti* |

References: Harper et al. (1998); Powell et al. (1998); Zhu-Salzman et al. (1998); Fitches et al. (2001); Sauvion et al. (2004); Coelho et al. (2007); Macedo et al. (2007); Sá et al. (2008, 2009); Coelho et al. (2009); Lagarda-Diaz et al. (2009); Ohizumi et al. (2009); Sprawska and Goławska (2010); Paiva et al. (2011b); Souza et al. (2011); Napoleão et al. (2011); Oliveira et al. (2011); Araújo et al. (2012); Albuquerque et al. (2012); Caccia et al. (2012); Freire et al. (2012); Martinez et al. (2012); Santos et al. (2012b); Napoleão et al. (2013); Roy et al. (2014); Sprawka et al. (2014); Walski et al. (2014); Oliveira et al. (2016)

Some lectins can cross the intestinal barrier by transcytosis and reach the hemolymph and insect tissues and organs (Powell et al., 1998; Fitches et al., 2001; Roy et al., 2014). Lectins may also alter the expression of some genes in the intestinal epithelial cells, such as genes associated with cytoskeletal organization, chitin metabolism, digestive enzymes, detoxification reactions, and energy metabolism (Li et al., 2009; Vandenborre et al., 2011).

The proteinaceous protease inhibitors are another class of defensive plant proteins with insecticidal properties against pests and vectors (Table 5). These proteins are able to interact with proteolytic enzymes in different ways (e.g., modifying the enzyme structure or preventing the access of the substrate to the active site) leading to the reduction of the catalytic activity (García-Carreño, 1996; Haq et al., 2004). Protease inhibitors are widely distributed among plants and animals being capable of inhibiting proteolytic enzymes from different organisms. The plants express these proteins in the reproductive and storage organs as well as in vegetative tissues (Falco and Silva-Filho, 2003; Lopes et al., 2004; Oliveira et al., 2013.

**Table 4. Examples of mechanisms involved in the insecticidal activity of some lectins**

| Lectins | Mechanism of action |
|---|---|
| MuBL, MuHL, MuLL | Termiticidal activity: resistance to insect digestive enzymes, antibacterial effect on gut symbionts, disruption of midgut organization, induction of oxidative stress and apoptosis in midgut cells, disruption of peritrophic matrix. |
| MuLL | Against *Sitophilus zeamais*: antinutritional and deterrent effects, inhibition of gut endoglucanase and alkaline phosphatase activities. Against *Aedes aegypti*: resistance to proteolysis at larval gut, inhibition of protease and trypsin activities and stimulation of α-amylase activity. |
| WSMoL$_C$ | Against *Aedes aegypti*: resistance to proteolysis by larval enzymes; stimulation of protease and α-amylase activities. |
| WSMoL | Against *Aedes aegypti*: disruption of larval midgut epithelium, stimulation of larval protease, trypsin-like and α-amylase activities, inhibition of larval β-esterase activity, induction of embryo death by penetration inside eggs. |
| ConA | Against *Rhopalosiphum padi*: death of the gut epithelial cells and effects on feeding behavior |
| *MvRL* | Against *Nasutitermes corniger*: inhibition of trypsin-like activity and stimulation of acid phosphatase activity at midgut. |

References: Coelho et al. (2009); Napoleão et al. (2011); Albuquerque et al. (2012); Santos et al. (2012b); Napoleão et al. (2013); Agra-Neto et al., (2014); Sprawka et al. (2014); Lima et al. (2016); Oliveira et al. (2016).

Protease inhibitors mainly act on the digestive system of insect pests, impairing their physiology. They can be classified according to the type of enzymes which they inhibit, such as serine-, cysteine-, aspartic-, or metallo-protease inhibitors (Prasad et al., 2010). The insect pests usually express serine and cysteine proteases. Protease inhibitors usually do not exert acute toxic effects. However, their chronic ingestion leads to the inhibition of protein digestion, decreasing the bioavailability of amino acids and consequently delaying growth and development as well as affecting insect survival (Falco and Silva-Filho, 2003; Pompermayer et al., 2001).

The potential of protease inhibitors and lectins for controlling insect pests has led to the development of transgenic plants resistant to the action of phytophagous insects by incorporation of genes encoding these proteins (Guo et al., 2013; Fernandez-del-Carmen et al., 2013; Jadhav et al., 2016).

**Table 5. Examples of plant protease inhibitors with insecticidal activity**

| Protease inhibitor (plant) | Plant tissue | Insects affected |
|---|---|---|
| ApTI (Adenanthera pavonina) | Seeds | Aedes aegypti |
| BmPI (Butea monosperma) | Seeds | Helicoverpa armigera |
| CanPIs (Capsicum annuum) | Leaves | Chilo partellus |
| ILTI (Inga laurina) | Seeds | Diatraea saccharalis<br>Heliothis virescens |
| IVTI (Inga vera) | Seeds | Anagasta kuehniella<br>Corcyra cephalonica<br>Heliothis virescens<br>Spodoptera frugiperda<br>Helicoverpa zea |
| LbAPI (Lupinus bogotensis) | Seeds | Hypothenemus hampei |
| MoFTI (Moringa oleifera) | Flowers | Aedes aegypti |
| PFTI (Plathymenia foliolosa) | Seeds | Anagasta kuehniella |

References: Ramos et al. (2012); Molina et al. (2014); Pontual et al. (2014); Jamal et al. (2015); Sasaki et al. (2015); Bezerra et al. (2016); Jadhav et al. (2016).

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## 4.2 ARTIGO 1- *Sitophilus zeamais* ADULTS HAVE SURVIVAL AND NUTRITION AFFECTED BY *Schinus terebinthifolius* LEAF EXTRACT AND ITS LECTIN (SteLL)

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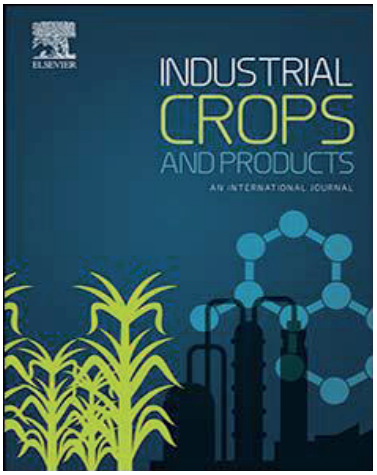


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# *Sitophilus zeamais* adults have survival and nutrition affected by *Schinus terebinthifolius* leaf extract and its lectin (SteLL)

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ABSTRACT

Alternative methods for controlling insect pests are required because of the hazards of synthetic chemicals to people and the environment. Lectins are proteins that have been reported as insecticidal agents; however, only one study on the effects of these proteins on *Sitophilus zeamais* Motsch. (maize weevil) has been performed. In the present study, we evaluated the effects of ingestion of artificial diets containing a saline extract from *Schinus terebinthifolius* Raddi leaves (LE) or its lectin (SteLL, *S. terebinthifolius* leaf lectin) on the survival and nutritional parameters of *S. zeamais* adults. The in vitro effects of LE and SteLL on the activity of insect digestive enzymes were also investigated. In addition to SteLL, the LE contained hydrolysable tannins (including gallic acid at 0.559 g%) and flavonoids. Ingestion of LE (100, 200, and 250 mg of extract per g of *Triticum aestivum* L. flour) impaired the survival of the *S. zeamais* adults, with mortality rates ranging between 94% and 97% after 12 days of incubation. A strong deterrent effect was detected, and the insects lost biomass during the assay. However, more than 60% of the insects in the SteLL (1–5 mg/g) treatments remained alive during the 34 days of the experiment. The lectin did not show a deterrent effect, but the biomass and efficiency in conversion of ingested food decreased in a dose-dependent manner. The LE was able to inhibit in vitro the protease activity of the insect gut, while SteLL inhibited protease activity and stimulated amylase activity. In conclusion, the leaf extract had insecticidal properties against *S. zeamais*, which may be due to starvation induction in consequence of the deterrent effect and interference with proteolytic enzymes. Although SteLL did not cause the mortality of the insects, it may be useful as an additive or synergistic agent that reduces pest fitness by affecting the food conversion into biomass.

## 1. Introduction

Hundreds of insect species have been reported to be capable of attacking stored products of agricultural and animal origin (Rajendran and Sriranjini, 2008). The infestation of grains by insects during cultivation, storage, and transport can seriously damage production and cause significant economic losses as well as threaten food safety (Tefera, 2012; Kumar and Kalita, 2017). Annual losses of 10–15% in grain production have been estimated to be caused by insect pest attacks (Casini and Santajulia, 2015).

*Sitophilus zeamais* Motschulsky, known as the maize weevil, is one of the main stored grain pests. It attacks mainly maize but also rice, wheat, barley, oat, cotton, and derived products, and it reduces the weight, nutritional value, germination ability, and market value of the grains (Goñi et al., 2017). Together with other insect pests, *S. zeamais* is responsible for losses in maize production (14–50%) (Yuya et al., 2009; Tefera et al., 2011; Ojo and Omoloye, 2012), which can reach 90% in the case of unprotected grains (Nwosu et al., 2015a,b).

According to the Department of Agriculture of the United States of America, the global production of maize in 2017/2018 will be around 1,043.9 million tons, and Brazil will be responsible for the production of 95,000.000 tons (USDA, 2017). There is great concern about the protection of maize production since it is the source of many human and animal nutritional products as well as it has many industrial uses

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(Herrera et al., 2017). However, despite all the advances in agriculture techniques and technologies, maize production still faces losses, mainly because of the action of pests (Kumar and Kalita, 2017).

Chemical (insecticides), physical (e.g. heat and radiation) and biological (use of predators, parasitoids, pathogens, or competitors) methods have been used for controlling pest insects (Zhou et al., 2014; Yun et al., 2016; Coelho et al., 2017; Porto et al., 2017; Malaikozhundan and Vinodhini, 2018). The use of conventional synthetic insecticides is effective, but there are several problems such as toxicity to non-target organisms, residual contamination of the products, high toxicity to the manipulators, and emergence of resistant populations due to intensive and indiscriminate application (Camaroti et al., 2017). The resistance of *S. zeamais* to pyrethroids has been described since the 1990s (Ribeiro et al., 2003; Fragoso et al., 2003, 2005, 2007). In Brazil, the resistance of populations of *S. zeamais* to organophosphates such as malathion and fenitrothion has been reported (Guedes et al., 1994, 1995), and an increase in the emergence of resistant populations is expected because of the excessive use of synthetic insecticides to combat this pest (Zhang et al., 2015; Freitas et al., 2016).

The co-evolution of plants and predators/herbivores resulted in the selection of plants that possess the best arsenals of defensive biomolecules produced in response to aggression. Thus, plants may provide potential alternatives to insect control. Plant extracts contain several types of secondary metabolites, bioactive proteins (e.g., lectins and enzyme inhibitors), and essential oils that have been reported to be insecticidal agents (Camaroti et al., 2017). These compounds may affect the survival, nutrition, development, locomotion, and behavior of insect pests (Mouhouche et al., 2009; Wale and Assegie, 2015; Lira et al., 2015; Correa et al., 2015; Herrera et al., 2015). For example, a lectin from the leaves of *Myracrodruon urundeuva* Allemão was found to have a deterrent effect on *S. zeamais* and caused death because of starvation (Napoleão et al., 2013).

*Schinus terebinthifolius* Raddi (Anacardiaceae) is a plant commonly known as “aroeira-da-praia” in Portuguese or “Brazilian pepper tree” in English. It is known for its medicinal properties such as healing, anti-inflammatory, antioxidant, anticancer, and antimicrobial activities (Queires et al., 2006; Matsuo et al., 2011; Bernardes et al., 2014; Fedel-Miyasato et al., 2014; Costa et al., 2015; Rosas et al., 2015). The bark of this plant is commonly commercialized in public markets in Brazil, mainly for therapeutic use (Miranda et al., 2016). The leaves of this plant contain a chitin-binding lectin (SteLL) with an antimicrobial effect against human pathogenic bacteria and fungus (Gomes et al., 2013). A leaf extract obtained using saline solution was reported to be a larvicidal agent against *Aedes aegypti* Linnaeus, causing damage to the midgut of the larvae and interfering with their development (Procópio et al., 2015). This extract also contained SteLL, but this protein was not active against the mosquito larvae.

In the present study, it was evaluated the effects of ingestion of artificial diets containing the saline extract from *S. terebinthifolius* leaves (LE) or SteLL on the survival and nutritional parameters of *S. zeamais* adults. The in vitro effects of the extract and lectin on the activity of insect digestive enzymes were also investigated.

## 2. Materials and methods

### 2.1. Plant material

Leaves of *S. terebinthifolius* were collected from different specimens found in an area (8°02′55.9″S 34°56′48.4″W) of the campus of the *Universidade Federal de Pernambuco* at Recife, Brazil. The leaves were dried for 3–5 days at 28 °C and then powdered using a blender. The powder was stored at −20 °C. The collection of plant material was performed with authorization (36301) from the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) of the Brazilian Ministry of Environment. A voucher specimen has been archived in the herbarium of the *Instituto Agronômico de Pernambuco* (IPA), Recife, Brazil, under the registration number 73,431.

### 2.2. Insects

A colony of *S. zeamais* is maintained at the *Laboratório de Bioquímica de Proteínas* of the *Universidade Federal de Pernambuco* with authorization (36301) of the ICMBio. The insects are reared in glass vessels (capacity of 1 L) containing maize grains (100 g), sealed with unwoven fabric, and maintained in a BOD chamber at 25 °C, relative humidity of 70%, and 12:12 light:dark. The maize grains (non-GMO) were obtained from crops for which agrochemicals were not used. Insects of 30–40 days of age were used in the assays.

### 2.3. S. terebinthifolius leaf extract (LE)

The LE was prepared in 0.15 M NaCl, since it has been previously reported that saline solution is effective in solubilizing both SteLL and hydrophilic secondary metabolites from *S. terebinthifolius* leaves (Gomes et al., 2013; Procópio et al., 2015). Ten grams of the leaf powder was homogenized for 16 h at 28 °C with 100 mL of 0.15 M NaCl by using a magnetic stirrer. Next, the suspension was passed through a filter paper, centrifuged (3000*g* for 15 min at 4 °C), and dialyzed against distilled water for 4 h (one change of water after 2 h). The extract was then freeze-dried in a lyophilizer (LIOTOP L101; Liobras, São Carlos, Brazil) at −45 °C and vacuum of 300 µm Hg below atmospheric pressure. The material was stored at −20 °C until further use.

### 2.4. Phytochemical characterization and lectin detection assay

The LE and standards listed in Table 1 were analyzed using thin-layer chromatography (TLC) in 60-$F_{254}$ silica gel plates (Macherey-Nagel®, Germany). The plates were developed in chambers after saturation with the mobile phase (Table 1) for 15 min at 28 °C. After elution, the plates were dried at 28 °C and observed under UV light (254 and 365 nm) and visible light. Next, the plates were analyzed with specific reagents for each metabolite class (Table 1). The bands were compared with the standards.

For high-performance liquid chromatography (HPLC) analysis, LE (5 mg) was transferred to a volumetric flask and diluted in 5 mL of ultrapure water (PureLab Classic UV, Elga). The solution was then placed in an ultrasound bath (Ultracleaner®) for 15 min and then filtered with a 0.45 µm PVDF filter. The extract was analyzed using the HPLC system Ultimate 3000 (Thermo Fisher Scientific, USA) coupled to a photodiode array detector (DAD; Thermo Fisher Scientific) and equipped with a binary pump (HPG-3x00RS; Thermo Fisher Scientific), degasser, and automatic sampler with a 20 µL loop (ACC-3000; Thermo Fisher Scientific). The wavelength was fixed at 270 and 350 nm.

Chromatographic separation was performed at 26 °C in an NST $C_{18}$ column (250 mm × 4.6 mm d.i., 5 µm) equipped with a Phenomenex

**Table 1**
Elution systems, revealers, and standards used in the phytochemical analysis of the saline extract from *Schinus terebinthifolius* leaves with thin-layer chromatography (TLC).

| Metabolite class | System | Reagent | Standard |
|---|---|---|---|
| Hydrolysable tannins | 90:5:5 | Iron(III) chloride | Gallic acid |
| Condensed tannins | 90:5:5 | Chloridric vanillin | Catechin |
| Flavonoids | 100:11:11:27 | NEU + PEG | Quercetin and rutin |
| Cinnamic derivatives | 100:11:11:27 | NEU + PEG | Caffeic acid |
| Terpenes and steroids | 70:30 | Lieberman-Burchard + Δ | β-sitosterol |

Systems: 90:5:5, ethyl acetate:formic acid:water; 70:30, toluene:acetate; 100:11:11:27, ethyl acetate:acetic acid:formic acid:water. NEU: Neu's reagent. PEG: polyethylene glycol. Δ: heating. The analysis was performed according to Wagner and Bladt (1996).

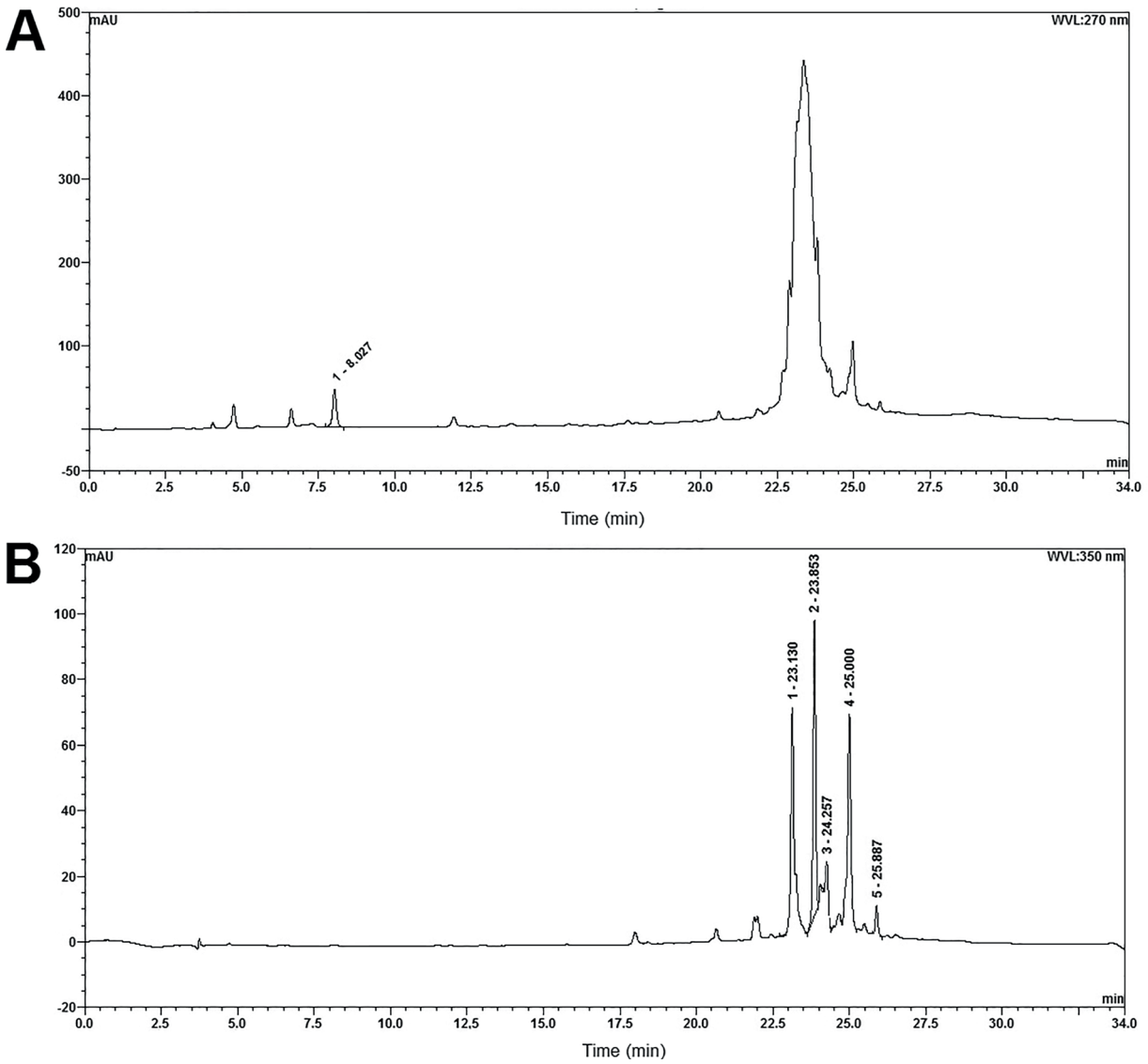


**Fig. 1.** HPLC-DAD analysis of *Schinus terebinthifolius* leaf extract (LE). (A) The chromatogram profile monitored at 270 nm revealed the presence of gallic acid (peak 1) on the basis of the standard retention time. (B) The profile at 350 nm showed five main peaks that corresponded to flavonoids.

**Table 2**
Retention times and wavelengths of maximum absorption for the compounds present in the peaks detected using HPLC analysis of the saline extract from *Schinus terebinthifolius* leaves monitored at 350 nm.

| Peak[a] | Retention time (min) | Maximum absorption wavelength (nm) |
|---|---|---|
| 1 | 23.13 | 212.1, 269.9, 352.8 |
| 2 | 23.85 | 206.8, 255.1, 352.8 |
| 3 | 24.25 | 204.6, 256.4, 358.6 |
| 4 | 25.00 | 203.6, 255.3, 353.4 |
| 5 | 25.88 | 202.5, 264.5, 342.0 |

[a] The peaks are numbered as indicated in Fig. 1B. All of them show the characteristics of flavonoids.

pre-column ($C_{18}$; 4 mm × 3.9 µm). The mobile phase was composed of ultrapure water (A) and methanol (B), both acidified with 0.05% (w/v) trifluoroacetic acid, and the flow rate was adjusted to 0.8 mL/min. The following gradient program was used: 0–10 min, 5–20% B; 10–13.5 min, 20–25% B; 13.5–20 min, 25–40% B; 20–25 min, 40–80% B; 25–30 min, 80% B; 30–34 min, 80–5% B. The data were analyzed and processed using the software Chromeleon 6.8 (Dionex/Thermo Fisher Scientific, USA). Gallic acid, quercetin, and rutin (1 mg/mL; Sigma-Aldrich, USA) in ultrapure water and filtered through a 0.45 µm PVDF filter were used as the standards. The gallic acid content was calculated on the basis of the calibration curve ($y = 1.1534\,x - 0.1667$) obtained using chromatography of this standard at different concentrations.

In addition, the presence of lectins in LE was evaluated by the hemagglutinating activity (HA) assay, which was performed as described by Procópio et al. (2017). The number of hemagglutinating activity units (HAU) was determined as the reciprocal of the highest dilution of the sample that promoted full agglutination of erythrocytes. The specific HA was defined as the ratio between the units and the protein concentration (mg/mL).

### 2.5. Purification of SteLL

SteLL was isolated from LE according to the method described by Gomes et al. (2013). The LE was re-suspended in 0.15 M NaCl and loaded onto a chitin (Sigma-Aldrich, MO, USA) column (7.5 × 1.5 cm) previously equilibrated with saline solution at a flow rate of 20 mL/h. After washing with 0.15 M NaCl to remove the extract components that did not adsorb to the matrix, SteLL was eluted with 1.0 M acetic acid. Fractions of 2 mL were collected, and protein elution was monitored by checking the absorbance at 280 nm. The lectin was dialyzed against

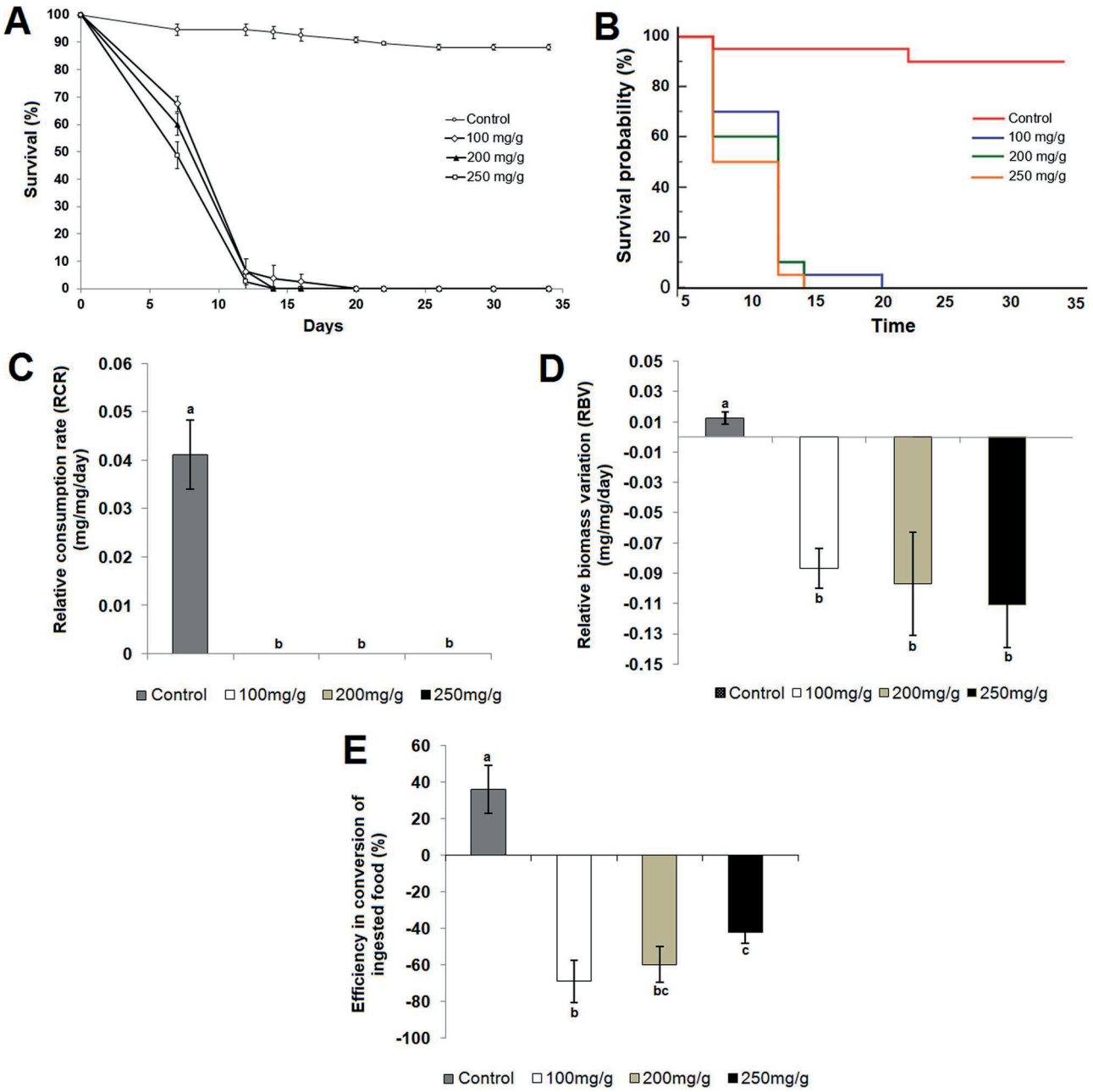


**Fig. 2.** Effects of *Schinus terebinthifolius* leaf extract (LE) on *Sitophilus zeamais* adults. (A) Survival rates of insects reared for 34 days on an artificial diet composed of wheat flour disks containing or not containing the extract. (B) Kaplan-Meier curves of control and LE treatments. Survival log-rank test indicated a significant trend of reduction in median survival times in LE treatments compared with control. The following nutritional parameters were determined: relative consumption rate (C), relative biomass variation (D), and efficiency in conversion of ingested food (E). Each bar corresponds to the mean ± SD of five replicates. Different letters indicate significant ($p < 0.05$) differences between treatments by Tukey's test.

distilled water (6 h, two changes of water) to eliminate the eluent. Purified SteLL was then evaluated for protein concentration and HA as described below. The concentration of proteins in the SteLL samples was estimated using the method described by Lowry et al. (1951).

### 2.6. Insecticidal assay

The toxicity by ingestion of LE and SteLL to *S. zeamais* adults was evaluated using an adaptation of the Xie et al. (1996) method described by Napoleão et al. (2013). For each assay, a suspension composed of 2.0 g of wheat (*Triticum aestivum* L.) flour (Bunge Alimentos S.A., Benevides, Brazil) homogenized with 5.0 mL of the sample solution was prepared. Next, 200-µL aliquots of the suspension were transferred to sterile petri plates (90 × 100 mm) to form wheat flour disks (five disks per plate) after incubation for 16 h at 56 °C. The weight of the plates containing the dried disks was recorded. Then, groups of 20 adult insects were transferred from the colony to plastic vessels, their weight was recorded, and they were then transferred to a petri plate containing the disks. The plates were then maintained in the BOD chamber at 25 °C, relative humidity of 70%, and 12:12 light:dark. The mortality was evaluated daily until the death of all the insects. The insects were considered dead when their appendage did not move and no other reaction was observed when touched with tweezers. The weights of the flour disks and insects were recorded on Day 7 after the start of the experiment. The following treatments were performed, all in quintuplicate: LE at 100, 200, and 250 mg/g (mg of extract per g of wheat flour in the disks); SteLL at 1.0, 3.0, and 5.0 mg/g (mg of protein per g of wheat flour); and sterile distilled water (control).

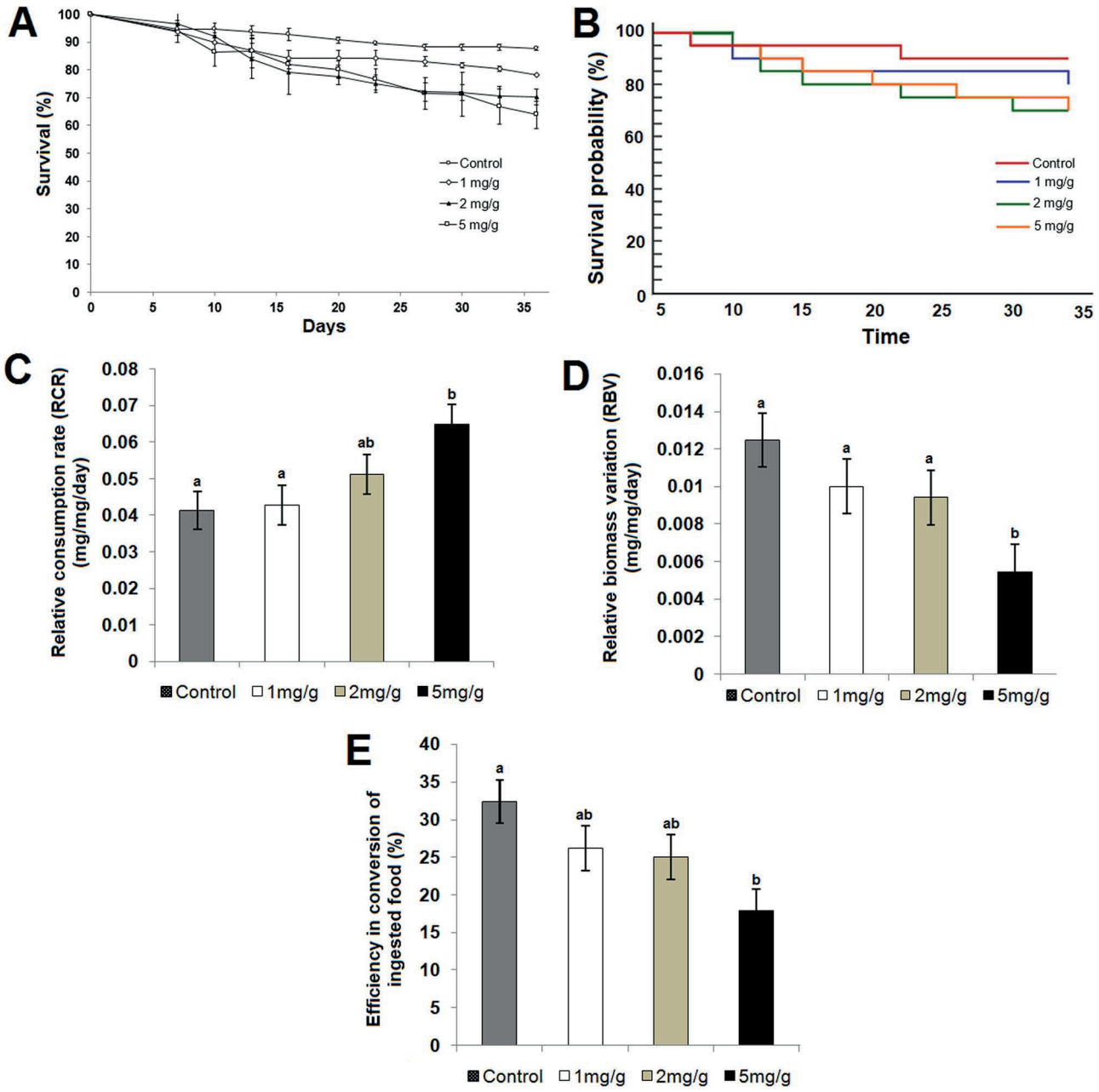


**Fig. 3.** Effects of *Schinus terebinthifolius* leaf lectin (SteLL) on *Sitophilus zeamais* adults. (A) Survival rate of insects reared for 34 days on an artificial diet composed of wheat flour disks containing or not containing the lectin. (B) Kaplan-Meier curves of control and SteLL treatments. Survival log-rank test did not indicate a significant trend of reduction in median survival times in SteLL treatments compared with control. The following nutritional parameters were determined: relative consumption rate (C), relative biomass variation (D), and efficiency in conversion of ingested food (E). Each bar corresponds to the mean ± SD of five replicates. Different letters indicate significant ($p < 0.05$) differences between treatments by Tukey's test.

In addition to mortality, the deterrent effect and nutritional parameters were evaluated using the values obtained after 7 days of the experiment. The feeding deterrence index (FDI) was calculated as follows: FDI (%) = 100 × $(A - B)/(A)$, where $A$ is the mass of diet ingested by insects from the control and $B$ is the mass ingested by insects from the treatments with LE or SteLL. On the basis of the FDI value, the treatment was classified as non-deterrent (FDI < 20%), weak deterrent (50% > FDI ≥ 20%), moderate deterrent (70% > FDI ≥ 50%), or strong deterrent (FDI ≥ 70%) (Isman et al., 1990; Liu et al., 2007). The following nutritional indexes were calculated: relative consumption rate (RCR) = $C/(D \times \text{days})$, where $C$ is the ingested mass (mg) and $D$ is the initial biomass (mg) of the insects; relative biomass variation (RBV) = $E/(D \times \text{days})$, where $E$ is the biomass (mg) acquired or lost by the insects; and efficiency in conversion of ingested food (ECIF) = $E/(C \times 100)$ (Xie et al., 1996).

### 2.7. Enzyme preparations from the guts of S. zeamais adults

Gut extracts from *S. zeamais* adults were prepared as described by Napoleão et al. (2013). The adults were removed from the colony and immobilized at 4 °C for 10 min. Then, the guts of the insects were dissected using a needle by pulling at the end of the abdomen after the removal of the elytra. The dissected guts were maintained in an ice bath. Then, 50 guts were homogenized with 1 mL of a buffer solution (0.1 M sodium acetate pH 5.5 or 0.1 M Tris–HCl pH 8.0, both containing 0.02 M calcium chloride) by using a tissue grinder. The homogenates were centrifuged (9000*g* for 15 min at 4 °C), and the supernatant corresponded to the gut extracts (enzyme preparations). The protein concentration in the gut extracts was determined using the method described by Lowry et al. (1951).

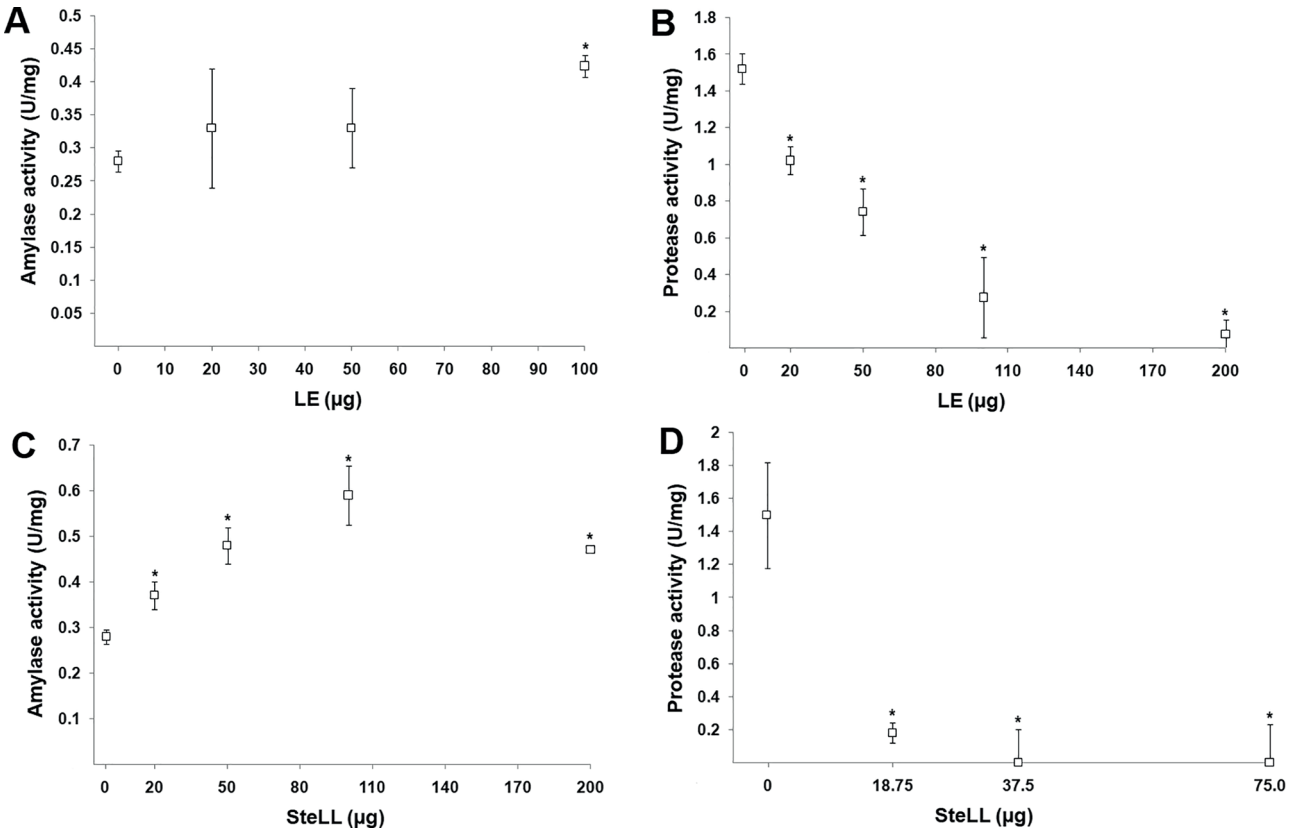


**Fig. 4.** Effects of *Schinus terebinthifolius* leaf extract, LE (A and B), and SteLL (C and D) on the activities of amylase (A and C) and protease (B and D) present in *Sitophilus zeamais* gut extracts. (*) indicates significant ($p < 0.05$) differences when compared with the control (absence of extract and lectin) by Tukey's test.

### *2.8. Determination of the effects of leaf extract (LE) and SteLL on the activity of digestive enzymes*

The activities of digestive enzymes from *S. zeamais* gut extracts were determined in the absence or presence of LE or SteLL. The following samples were evaluated in the assays: 50 µL of gut extract plus 50 µL of distilled water (control); 50 µL of gut extract plus 50 µL of LE or SteLL (test); 50 µL of distilled water plus 50 µL of LE or SteLL (blank). All the samples were incubated for 15 min at 28 °C before evaluation of enzyme activity. Assays were performed in quintuplicate.

The α-amylase activity was evaluated according to Bernfeld (1955) by using the gut extract in acetate buffer. The sample (control, test, or blank) was incubated at 56 °C for 10 min with 400 µL of soluble starch (1%, w/v, in acetate buffer). The reaction was stopped by the addition of 500 µL of 3,5-dinitrosalicylic acid (DNS) reagent. Next, the solution was heated to 100 °C in boiling water for 6 min and immediately cooled on ice. The absorbance at 540 nm was recorded, and, after discounting the values found in the control and blank, the amount of reduced sugars released was determined using a standard curve of the reaction of glucose ($y = 0.7216x + 0.0216$, where $x$ is the glucose concentration in µM and $y$ is the absorbance) with DNS. One unit of amylase activity was defined as the amount of enzyme-containing-extract required to generate 1 µmol of glucose per minute. The amount of proteins in the gut extract aliquot used in the assays (50 µL) was 240 µg. Different amounts of LE (25–100 µg of protein) and SteLL (25–200 µg) were evaluated.

Protease activity was evaluated using the method described by Azeez et al. (2007) and gut extracts prepared in Tris buffer. The control, test, or blank sample was added to 300 µL of 0.1 M sodium phosphate, pH 7.5, and 50 µL of 0.6% (w/v) azocasein. Then, 100 µL of 0.1% (w/v) Triton X-100 was added, and the solution was incubated at 37 °C for 3 h. The reaction was stopped by adding 200 µL of 10% (w/v) trichloroacetic acid, and the solution was incubated at 4 °C for 30 min. Then, the solution was centrifuged (9000*g* for 10 min), and the absorbance of the supernatant was evaluated at 366 nm. The values found for the control and blank were discounted, and each 0.01 value of absorbance corresponded to one unit of protease activity. The amount of proteins in the extract aliquot used in the assays (50 µL) was 610 µg. Different amounts of LE (25–200 µg of protein) and SteLL (18.75–75 µg) were evaluated.

### *2.9. Statistical analysis*

The survival data were analyzed by survival log-rank test ($p < 0.05$) using the MedCalc version 17.9.7 (MedCalc Software bvba, Belgium). This program was also used to generate the Kaplan-Meier curves and to calculate the mean survival times ( ± standard errors). Data of nutritional parameters and enzyme activities were submitted to one-way fixed-effects ANOVA followed by Tukey's test (significance at $p < 0.05$) conducted using the Action 2.8.29.357.515 software (Estatcamp, São Carlos, Brazil). These data were expressed as mean of replicates ± standard deviation values.

## 3. Results and discussion

The use of natural or botanical chemicals as alternative to traditional synthetic insecticides is a common practice in Integrated Pest Managent (IPM), frequently minimizing the hazards to people and environment. In the present study, it was evaluated *S. terebinthifolius* leaves as a source of insecticidal agents against *S. zeamais*, a pest of great economic importance that is resistant to several of the few allowed/registered insecticides.

LE showed hemagglutinating activity (256 HAU), confirming the extraction of the lectin. The TLC analysis showed the presence of hydrolysable tannins (probably gallic acid because of the gray-blue color of the band) and flavonoids (orange bands). No bands corresponding to condensed tannins, terpenes, steroids, and cinnamic derivatives were

observed. On the basis of these results, the HPLC profiles of LE at 270 and 350 nm were obtained for detection of hydrolysable tannins (that absorb UV light at 270 nm) and flavonoids (that absorb at both wavelengths).

The chromatogram at 270 nm showed a peak with a retention time of 8.027 min (Fig. 1A), which was confirmed as gallic acid when compared with the standard retention time and absorption spectrum. The gallic acid content calculated was 0.559 ± 0.0071 g%. The other peaks observed at ~23–26 min in the profile at 270 nm were better resolved in the chromatogram at 350 nm (Fig. 1B): it can be observed five peaks of compounds. Indeed, the maximum absorption wavelengths for these five peaks were characteristic of flavonoids (Table 2). However, these compounds did not correspond to the standards quercetin and rutin.

The mortality rates of *S. zeamais* adults in the treatments with LE are shown in Fig. 2A. On Day 7, mortality rates of 32%, 40%, and 51% were detected for the LE treatments at 100, 200, and 250 mg/g, respectively. On Day 12, the percentage of dead insects after these treatments varied between 94% and 97%. On Day 20, all the insects that received the SE treatments died, while on Day 34, more than 90% of the control insects were still alive. The Kaplan-Meier curves can be seen in Fig. 2B and the survival log-rank test indicated a significant trend of reduction in median survival times in LE treatments compared with control ($\chi^2$ = 8.6089; df: 3; $p$ = 0.0001). The mean survival times were 11 ± 0.72, 10.2 ± 0.61, and 9.6 ± 1.41 days in treatments at 100, 200 and 250 mg/g, respectively.

The nutritional parameters of the insects were calculated after 7 days of treatment with LE. No reduction in the mass of the artificial diet was detected, and, thus, RCR was nil for all the LE treatments (Fig. 2C; $F_{3,16}$: 215.775; $p$ = 0.0000). Consequently, FDI was 100%, indicating a strong deterrent effect. Unlike the control, RBV was negative for all the LE treatments (Fig. 2D; $F_{3,16}$: 39.133; $p$ = 0.0000), showing that the weight of the insects was lower at that point than at the start of the assay. ECIF was also negative (Fig. 2E; $F_{3,16}$: 191.099; $p$ = 0.0000) once there was no food ingestion and the insects showed loss of biomass. This indicates that the insects metabolized energy reserves to survive. Deterrent agents are particularly interesting as potential grain protectants.

The data described above demonstrate the presence of deterrent compounds in LE, which contains gallic acid, flavonoids, and lectins. Flavonoids were detected in the extracts of *Tagetes erecta* Linnaeus and *Tagetes patula* Linnaues, and they were toxic to *S. zeamais* adults (Santos et al., 2016). The flavonoid meliternatin (3,5-dimethoxy-3′,4′,6,7-bismethylendioxyflavone) was the active compound of a methanolic extract from *Melicope subunifoliolata* (Stapf) T.G. Hartley leaves that, similar to our extract, had a strong feeding deterrent effect on *S. zeamais* (Ho et al., 2003). The deterrent effect of the *M. subunifoliolata* extract was also reflected in the reduced growth, food consumption, and efficiency of food conversion of *S. zeamais*. According to Simmonds (2001), flavonoids may act as feeding inhibitors or phagostimulants, depending on the concentration. Gallic acid may also be involved in the effects of LE, although it is present in a small concentration. It was reported that gallic acid (in powder form) was able to induce behavior alterations and mortality of *Acanthoscelides obtectus* Say adults (Regnault-Roger et al., 2004).

To test the hypothesis that SteLL would be an active principle of LE against *S. zeamais*, the lectin was isolated and incorporated into the artificial diets. The tested concentrations were selected by estimating the lectin content in the LE. SteLL showed a specific HA of 16,384, confirming its carbohydrate-binding ability. Low reduction (~5%) in the survival rates of the insects in the control and SteLL treatments was observed on Day 7. After this day, a slightly higher reduction in survival in comparison with the control was observed, but, at the end of the experiment, more than 60% of the insects remained alive in all the treatments (Fig. 3A). The Kaplan-Meier curves (Fig. 3B) showed no significant trend of reduction in mean survival times in SteLL treatments, compared with control ($\chi^2$ = 2.9099; df: 3; $p$ = 0.4057). This result shows that SteLL is not a major active principle of LE against *S. zeamais* adults.

Unlike the saline extract, SteLL had no deterrent effect, and the RCR in the treatment with the highest concentration was even significantly higher than that in the control group (Fig. 3C; $F_{3,16}$: 7.373; $p$ = 0.0025). However, the RBV rate (Fig. 3D) decreased with increasing lectin concentration in the diet ($F_{3,16}$: 10.969.; $p$ = 0.0004), demonstrating the anti-nutritional effects of the lectin. The ECIF variation in control and SteLL treatments was significantly different (Fig. 3E; $F_{3,16}$: 4.336; $p$ = 0.0204) and Tukey's test revealed significant decrease ($p$ = 0.012) in the treatment with highest concentration compared with control. These results are interesting because they suggest that SteLL would be useful as an additive or synergistic agent able to reduce pest fitness by affecting the food conversion into biomass.

Lectins are known for their toxic and anti-nutritional effects on insects. The coagulant *Moringa oleifera* Lamarck seed lectin (cMoL), when incorporated into an artificial diet, had anti-nutritional effects on *Anagasta kuehniella* larvae by decreasing the efficiency of food conversion and biomass gain and then affecting both growth and survival (Oliveira et al., 2011). The proteins of this class may also affect insect metabolism by modulating the activity of the gut enzymes; for example, the lectins from *M. oleifera* seeds (cMoL, WSMoL, and $WSMoL_C$) and *M. urundeuva* leaves (MuLL) are able to alter the activity of gut amylase, protease, and trypsin-like enzyme of *A. aegypti* larvae (Napoleão et al., 2012; Agra-Neto et al., 2014; Oliveira et al., 2016). Napoleão et al. (2013) reported that ingestion of MuLL led to reduction in the activity of the digestive enzymes in the gut of *S. zeamais* adults, which was probably linked to a post-ingestion deterrent effect.

The effects of LE and SteLL on the activity of the digestive enzymes of *S. zeamais* were analyzed. The extract showed some stimulatory effect on amylase activity (Fig. 4A; $F_{3,16}$: 5.915; $p$ = 0.0065) and was able to inhibit proteolytic enzymes (Fig. 4B; $F_{4,20}$: 89.867; $p$ = 0.0000). The deterrent effect of LE may be, in part, the consequence of the interference of the action of digestive enzymes and results in starvation. Thus, a post-ingestion effect caused by minimal ingestion of LE may be responsible for the rejection of the diet. According to Maceljski and Korunić (1973), *S. zeamais* adults died in 11 days when subjected to total starvation, a time period similar to that in which the death of insects that had contact with LE was observed.

In contrast, SteLL stimulated the amylase activity (Fig. 4C; $F_{4,20}$: 49.553; $p$ = 0.0000) but strongly inhibited the protease activity (Fig. 4D; $F_{3,16}$: 52.850; $p$ = 0.0000). This suggests that the reduction in growth and ECIF in the insects is due to the disturbance caused by the lectin at an absorption or systemic level. Chitin-binding lectins, like SteLL, may interact with the components of the peritrophic matrix, causing abnormalities in its structure and function and interfering with the absorption of nutrients; in addition, lectins can interact with digestive enzymes, modulating their activity and promoting metabolic imbalance (Camaroti et al., 2017).

Our findings show that the extract of *S. terebinthifolius* leaves evaluated here is a natural source of insecticidal agents against *S. zeamais* adults and the lectin SteLL, when ingested, is capable of promoting an imbalance in the digestive metabolism of these insects.

## 4. Conclusion

Ingestion of the leaf extract from *S. terebinthifolius* induced the death of *S. zeamais* adults. A strong deterrent effect and inhibition of gut proteolytic enzymes may be due to the presence of flavonoids. The lectin SteLL probably did not have a major role in the toxic action of the extract but contributed to the anti-nutritional effects observed. The rejection of the diet caused by the extract promotes its use as a grain protector agent.

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## 4.3 ARTIGO 2- *Schinus terebinthifolia* LEAF EXTRACT IS A LARVICIDAL, PUPICIDAL AND OVIPOSITION-DETERRENT AGENT AGAINST *Plutella xylostella*

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**Abstract**

BACKGROUND: *Plutella xylostella* is one of the main pests of brassicas worldwide and the main strategy for controlling this pest is the use of synthetic insecticides. However, the indiscriminate use is associated with environmental problems and arising of resistant populations. This scenario stimulates the search for plant-derived compounds for control of *P. xyostella*. This work evaluated the insecticidal effects of the *Schinus terebinthifolia* leaf extract and a lectin (SteLL) isolated from it on *P. xylostella*. RESULTS: The extract did not affect egg hatching but caused larvae mortality with $LC_{50}$ of 14.49% and 11.74% for 96 and 144 h, respectively. The survival log-rank test indicated a significant trend of reduction in median survival times in treatments with the leaf extract. The percentage of individuals that died at larval or pupal stage ranged between 32.5% and 90% in treatments with the extract (2.0–15.0%, w/v), while in control (0.15 M NaCl) this value was 12.5%. Treatment of larvae with the extract at 10% reduced the fertility of adults; in addition, the eggs produced showed decreased viability. On the other hand, *P. xylostella* females laid their eggs preferentially in oviposition sites treated with 0.15 M NaCl (control) compared to the extract. Deterrent indexes of 63.42% and 68.02% were recorded for the extract at 2% after 24 and 48 h,

respectively. SteLL (0.2 mg/mL) was not toxic to larvae being not an active principle of the extract against *P. xylostella*. CONCLUSION: The leaf extract of *S. terebinthifolius* is a new insecticidal agent against *P. xylostella* by killing immature stages and exerting a strong oviposition-deterrent effect.

**Keywords:** natural insecticide; Brazilian pepper tree; agricultural pest; diamondback moth.

## 1. Introduction

*Plutella xylostella* (L.) (Lepidoptera: Plutellidae), popularly known as diamondback moth or cabbage moth in English as well as "traça-das-brássicas" in Portuguese, is an oligophagous insect that is considered the main pest of brassica plants worldwide (Zaluck et al., 2012; Furlong et al., 2013; Shen et al., 2017). The ability of larvae to feed voraciously since the hatching, the short life cycle length and the high reproductive potential contribute to outbreaks of this pest, which can cause loss in 100% of the production of unprotected plantations (Takelar and Shelton, 1993; Castelo-Branco and Gatehouse, 2001; Ulmer et al., 2002; Torres et al., 2006; Zhou et al., 2011).

The cultivation of brassicas requires the use of a large amount of insecticides throughout the growing season mainly in the tropics and subtropics, where problems with *P. xylostella* reach large proportions (Ribeiro et al., 2017). It has been estimated that the total cost for prevention and control of *P. xylostella* worldwide can reach 5 billion dollars per year (Zaluck et al. 2012; Furlong et al 2013). In addition to the economic impact, the indiscriminate use of synthetic insecticides for controlling agricultural pests is associated with several problems, such as contamination of the products (compromising the health of the manipulators), environmental toxicity, and selection of resistant organisms (Camaroti et al., 2017). *P. xylostella* has a high capacity to develop resistance and populations resistant to about 95 active principles of synthetic insecticides have been described (Zhang et al., 2016). In Brazil, it has been reported the resistance of *P. xylostella* to pyrethroids, avermectins, indoxacarb, benzoylurea, and diamides (Santos et al., 2011, Ribeiro et al., 2017).

The control of insects by using plant-derived compounds represents an important alternative in integrated pest management. Plants produce a wide variety of bioactive compounds with insecticidal properties, such as secondary metabolites and proteins (lectins and protease inhibitors) capable of interfering with nutrition, development, reproduction and survival (Lingathurai et al., 2011; Gao et al., 2011; Poonsri et al. ,2015; Wei et al., 2015;

Camaroti et al., 2017; Sangha et al., 2017). *Mutingia calabura* extracts showed toxic effects to larvae and pupae of *P. xylostella* (Bandeira et al., 2013) and extracts of *Acalypha fruticosa* and saponins from the bark of *Catunaregam spinosa* showed antifeedant effects on *P. xylostella* larvae (Gao et al., 2011; Lingathurai et al., 2011).

*Schinus terebinthifolia* Raddi (Anacardiaceae), popularly known as Brazilian pepper tree, is mainly known due to its medicinal properties, such as healing and anti-inflammatory effects (Queires et al., 2006; Matsuo et al., 2011; Bernardes et al., 2014; Fedel-Miyasato et al., 2014; Rosas et al., 2015; Costa et al., 2015). Procópio et al. (2015) reported the larvicidal activity of a saline extract from *S. terebinthifolia* leaves against *Aedes aegypti*, which was linked to drastic damages caused at the gut epithelium of the larvae. The authors attributed this insecticidal activity to the presence of cinnamic derivatives and flavonoids. This extract also contains a chitin-binding lectin called SteLL, which showed antimicrobial activity (Gomes et al., 2013) but was not involved in the larvicidal effect mentioned above (Procópio et al., 2015).

This study aimed to evaluate the insecticidal activity of saline extract from *S. terebinthifolia* leaves on *P. xyllostella*, evaluating the following aspects: egg hatchability, larval survival and development, and oviposition behavior. In addition, it was investigated whether SteLL would be an active principle of the leaf extract against *P. xylostella*.

## 2. Materials and methods

### *2.1. Plant material*

Leaves of *S. terebinthifolia* were collected at the campus of the *Universidade Federal de Pernambuco* (8°02'55.6"S 34°56'48.3"W) at Recife, Brazil, and put to dry during 3–5 days at 28 ºC. Then, the material was powdered using a blender and stored at -20 ºC. The collection of leaves was performed with authorization (36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from the Brazilian Ministry of Environment and was recorded (AC551B2) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). A voucher specimen (73,431) is archived in the herbarium from the *Instituto Agronômico de Pernambuco* (IPA), Recife, Brazil.

### *2.2. Insects*

Eggs, larvae and adults of *P. xylostella* were obtained from colonies reared in leaves of organic cabbage (*Brassica oleracea* var. *acephala*) in the *Laboratório de Biologia de Insetos* from the *Departamento de Agronomia* of the *Universidade Federal Rural de Pernambuco*. The colonies are reared under controlled conditions (25 ºC, 70% relative humidity, 12L:12D photoperiod) according to Carvalho et al., (2010).

### *2.3. Schinus terebinthifolia leaf extract*

The saline extract from *S. terebinthifolius* leaves was prepared by homogenizing 10 g of leaf powder in 100 mL of 0.15 M NaCl during 16 h at 28 ºC, using a magnetic stirrer. Next, the suspension was passed through filter paper, centrifuged (3,000 g, 15 min, 4 ºC) and dialyzed against distilled water for 4 h (Procópio et al., 2015). The leaf extract was then lyophilized at -45ºC and vacuum of 300 μm Hg below atmospheric pressure and stored at -20 ºC.

### *2.4. Isolation of SteLL*

SteLL was isolated from the saline extract according to Gomes et al. (2013). The extract was loaded onto a chitin (Sigma-Aldrich, MO, USA) column (7.5 × 1.5 cm) previously equilibrated with the saline solution at a flow rate of 20 mL/h. After washing with 0.15 M NaCl, SteLL was eluted with 1.0 M acetic acid. Fractions of 2 mL were collected, and protein elution was monitored by checking the absorbance at 280 nm. The lectin was dialyzed against distilled water (6 h, two changes of water) in order to eliminate the eluent. The concentration of SteLL was estimated using the method described by Lowry et al. (1951). Bovine serum albumin (31.25–500 μg/mL) was used as standard. Carbohydrate-binding ability of the isolated lectin was monitored by the hemagglutinating activity (HA) assay (Procópio et al., 2017) using rabbit erythrocytes treated with glutaraldehyde (Bing et al., 1967). The erythrocytes were collected employing a method approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Pernambuco (process 23076.033782/2015-70).

*2.5. Effects of the extract on egg hatchability*

To evaluate the effect of the extract on the hatchability of *P. xylostella* eggs, it was used the leaf immersion method described by Tabashnik and Cushing (1987). Cabbage leaf disks (5-cm diameter) containing 10 eggs (laid in less than 10 h) were immersed in extract solutions (1.0, 3.0, and 5.0%) or in 0.15 M NaCl (control) for 30 s. After drying at 28 °C for 40 min, the disks were transferred to Petri dishes (9-cm diameter, 1.5-cm) and the eggs were checked daily during 96 h to assess the hatchability. Hatched larvae were removed from the plates at the end of each evaluation to prevent cannibalism. All the assays were performed in quadruplicates. The eggs that did not hatch at the end of the experiment were observed in a Leica KL300 stereomicroscope in order to evaluate the embryo integrity.

*2.6. Evaluation of toxicity by ingestion*

Leaf immersion method was used to evaluate the toxicity of the extract or SteLL to larvae by ingestion. Disks of fresh cabbage leaves (3-cm diameter) were punched with a needle and immersed in 5 mL of extract solution (2.0%, 5.0%, 10.0%, or 15.0% w/v), SteLL (0.2 mg/mL) or in 0.15 M NaCl (control) for 30 s. After immersion, the disks were dried at 28 °C for 40 min and transferred to petri dishes (9-cm diameter, 1.5-cm depth). Groups of 10 larvae in the first instar were transferred from the colony to each plate. The treated discs were replaced daily and the mortality of larvae was monitored until all surviving larvae reached the pupal stage. Larval viability was recorded as the percentage of larvae that reached the pupa stage. Pupal viability was also determined, corresponding to the percentage of larvae that survived and reached the adult stage. All assays were performed in four replicates.

*2.7. Evaluation of reproductive capacity*

The adults originated at the end of the previous assay were separated and four couples (for each treatment) were maintained in separate cages (14 × 14 × 15 cm) containing a cabbage leaf disk (5-cm diameter). The treatment with the extract at 15% was not evaluated due to the low number of emerged adults due to mortality of larvae and pupae. After 48 h, the disks were removed to count the eggs and then transferred to Petri plates (9-cm diameter, 1.5-cm depth) to check the hatching during the next 96 h.

*2.8. Evaluation of oviposition-deterrent effect*

To evaluate whether the extract would have a deterrent effect on oviposition, it was used a one-choice test where sixteen adult couples were placed in eight plastic cages (14 × 11 × 5 cm), so that each cage received two couples. Two disks of cabbage leaves (5-cm diameter) were immersed in 5 mL of the extract (2.0%, w/v) or 0.15 M NaCl (control) for 30 s. After immersion, the disks were dried (28 °C) for 40 min and one disk treated with the extract and one control disk were placed in each cage. The position of the disks was alternated in each cage to cancel out any trend linked to spatial preferences that may exist. After 24 h and 48 h of the experiment start, the disks were removed to count the number of laid eggs. The oviposition deterrence index (ODI) was calculated as follows: ODI (%) = [1–(Et/Ec) × 100], where Et corresponds to the number of eggs in the treated disk and Ec the number of eggs laid in control disk. The assays were performed in duplicate.

*2.9. Statistical analysis*

The survival data were analyzed by survival log-rank test ($p < 0.05$) and the $LC_{50}$ values (with 95% confidence limits) were established for 96 and 144 h after the start of the assay through probit analysis using the MedCalc version 17.9.7 (MedCalc Software bvba, Belgium). This program was also used to generate the Kaplan-Meier curves and to calculate the mean survival times.

## 3. Results and discussion

The saline extract from *S. terebinthifolia* leaves has been the subject of recent studies focusing the search for new insecticidal agents and its toxicity to *A. aegypti* larvae (Procópio et al., 2015) and *Sitophilus zeamais* adults (Camaroti et al., 2018) was demonstrated. In the present work, this extract was assayed for the effects against the insect pest *P. xylostella*.

The extract sample using in the present work was the same used by Camaroti et al. (2018), which showed insecticidal activity against *S. zeamais*. These authors reported the presence of hydrolysable tannins (including gallic acid at 0.559 g%) and flavonoids and that the extract did not contain condensed tannins, terpenes, steroids, and cinnamic derivatives. Gallic acid and flavonoids have been also reported as insecticidal agents against *Acanthoscelides obtecus* (Regnault-Roger et al., 2004).

The effects of leaf extract on the egg hatchability are shown in Figure 1A. There was a delay in the hatching in the first 24 h in all treatments with the extract, However, no lethal effect on the eggs was observed since, after 96 h of treatment, all larvae emerged in all treatments. The extract was able to kill *P. xylostella* larvae that ingested cabbage leaves treated with it. The survival log-rank test indicated a significant trend of reduction in median survival times (Table 1) in treatments with the leaf extract (5% to 15%) compared with control ($\chi^2$ = 198.1074; df: 4; $p$=0.0001). The mean survival times were 6.979±0.112, 6.857±0.116, and 6.162±0.122 days in treatments at 5, 10 and 15 %, respectively. The Kaplan-Meier curves can be seen in the Figure 1B. The $LC_{50}$ values were 14.49 [12.73-17.06] % and 11.74 [8.87-14.62] % for 96 and 144 h, respectively (Table 2).

Leaf extract was also able to disrupt the development of *P. xylostella* larvae since there was a decrease in the number of larvae that became pupae (larval viability) in a dose dependent way (Table 3). Together, the data indicate the efficiency of the leaf extract as an alternative tool for management of *P. xylostella* since it is the larval phase that causes the most damage to the brassica culture (Castelo Branco et al., 2001). Boiça Junior et al. (2005) also found similar results using aqueous plant extracts against *P. xylostella*. These authors reported total or almost total mortality of larvae treated with extracts from *Enterolobium contortisilliquum*, *Nicotiana tabacum*, *Sapindus saponaria* (fruits), *Trichilia pallida* (branches and leaves), *Azadirachta indica*, *Symphytum officinale*, *Bougainvillea glabra*, *Achillea millefolium* (leaves) and *Chenopodium ambrosioides* (leaves, branches and fruits).

At the end of the assay of toxicity by ingestion, the larvae that survived to the treatment were kept for follow-up any alterations during the pupal stage. It was observed pupal mortality and cocoon deformation, which led to death or resulted in defective-winged adults. These results shows that the leaf extract exerted deleterious effects that impaired *P. xylostella* metamorphosis. The pupal viability varied between 90% and 50% in treatments with the extract (2–15%), values significantly ($p < 0.05$) lower than that found in control (Table 3). When the total number of dead individuals is analyzed, it is possible to see that the *S. terebinthifolia* leaf extract was able to cause the death of 90% of the individuals, in the larval and pupal stages, in the treatment at the highest concentration (Table 3). De Jesus et al. (2011) also found deformities in *P. xylostella* pupae that were treated with aqueous extracts from *Aspidosperma indica*, *Sapindus saponaria*, *Dimorphandra mollis* and *Stryphnodendron adstringens*. Procópio et al. (2015) reported a significant decrease in the emergence of *A. aegypti* adults derived from larvae treated with saline extract from *S. terebinthifolia* leaves obtained using the same method employed in the present work.

Aiming to evaluate the effects of the ingestion of leaf extract by *P. xylostella* in adult reproduction, the adults derived from larvae that survived to the treatments were separated to form couples and maintained in cages containing cabbage leaf disks. After 48 h, the number of laid eggs was counted and is shown in Table 4. There was a decrease of 71% in the number of eggs laid per female in the treatment with the extract at 10% while slight reduction was detected in the groups exposed to the extract at 2% and 5%, in comparison with control. It was also observed reduction in the viability of eggs laid by insects that were exposed to the extract at 10% (49.30%) in regard to control (93.15%).

The *P. xylostella* females laid their eggs preferentially in control disks, in comparison with those treated with the extract at 2.0% (Figure 1C), indicating an oviposition-deterrent effect. The ODI values were 63.42% and 68.02% for 24 and 48 h, respectively. Similarly to our results, Medeiros et al. (2005) reported that aqueous extracts from aerial parts of several plants (*Achillea millefolium*, *Azadirachta indica*, *Bidens pilosa*, *Bougainvillea glabra*, *Chenopodium ambrosioides*, *Datura suaveolens*, *Enterolobium contortisilliquum*, *Mentha crispa*, *Nicotiana tabacum*, *Piper nigrum*, *Plumbago capensis*, *Pothomorpheum bellata*, *Sapindus saponaria*, *Solanum cernuum*, *Symphytum officinale*, *Trichilia catigua* and *Trichilia pallida*) exerted oviposition-deterrent effect on *P. xylostella* females. Aqueous extracts from *Melia azedarach* and *Aspidosperma pyrifolium* also showed deterrent effect on diamondback moth (Torres et al., 2006).

To evaluate whether SteLL is an active principle from the leaf extract, the lectin (0.2 mg/mL) was tested for larvicidal activity but no toxicity by ingestion was detected. This datum is similar to that previously found for *A. aegypti* larvae, to which SteLL was also not toxic (Procópio et al., 2015). For *S. zeamais*, SteLL induced the mortality of adults but in a lower level than the saline extract (Camaroti et al., 2018). Together, these results indicate that this lectin did not have a major role in the insecticidal effects of *S. terebinthifolia* leaves.

## 4. Conclusion

The saline extract of *S. terebinthifolia* leaves represents a new insecticidal agent against *P. xylostella* by killing larvae and pupae and impairing the reproductive ability of the emerged adults. In addition, the extract is an oviposition-deterrent agent. The insecticidal activity can be due to the action of gallic acid and flavonoids since the lectin present in the extract did not show activity when isolated.

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**Figure captions**

**Figure 1.** Insecticidal activity of *Schinus terebinthifolia* leaf extract on *Plutella xylostella*. (A) Effect of the extract (1 to 5 %, w/v) on egg hatchability. Different letters indicate significant differences ($p < 0.05$) between the treatments. (B) Kaplan-Meier curves of control and extract treatments (2 to 15 %, w/v). Survival log-rank test indicated a significant trend of reduction in median survival times in leaf extract treatments compared with control. (C) Oviposition-deterrent effect of the extract (2%, w/v) on *P. xylostella* females. (*) indicate significant differences regarding the control.

**Figure 1**

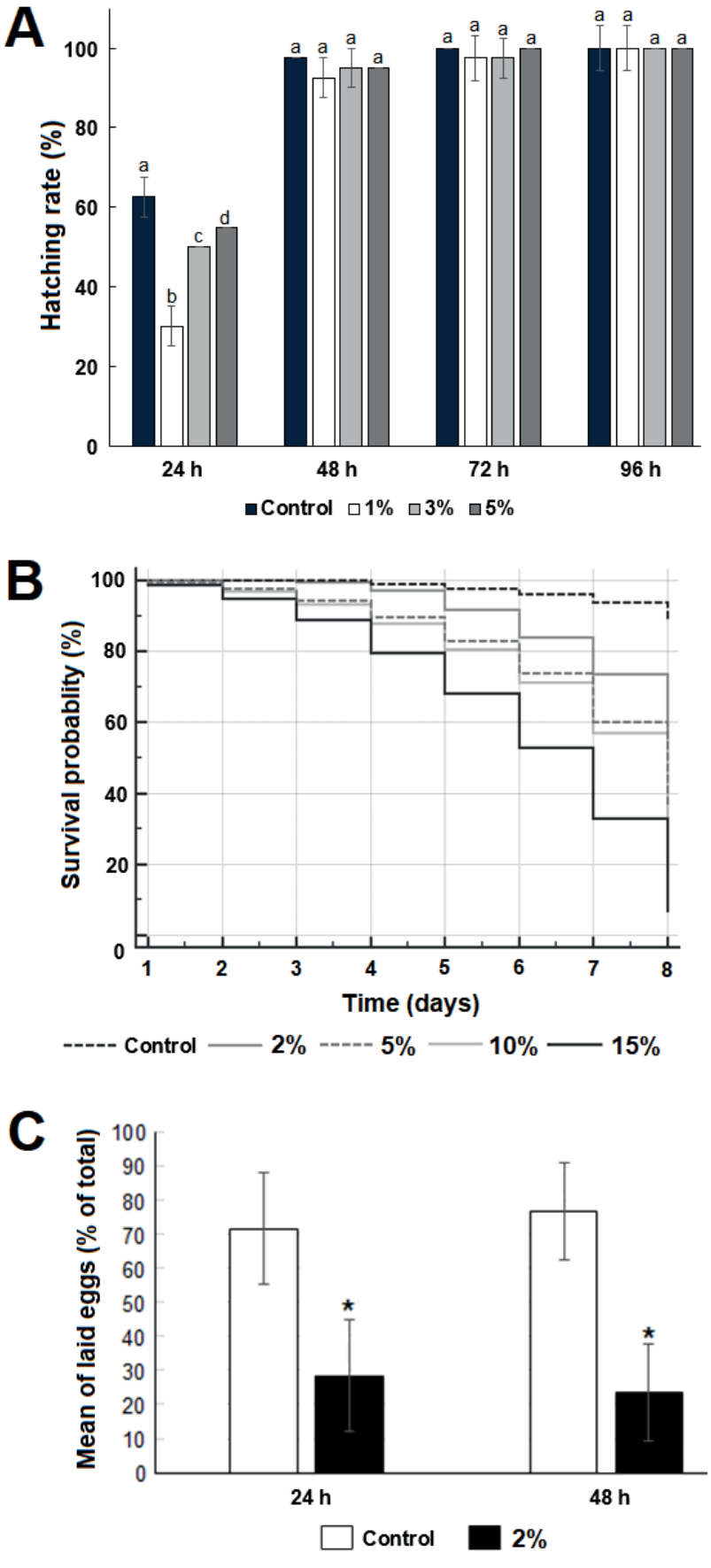
A
Hatching rate (%)
100
80
60
40
20
0
a
b
c
d
a a a a
a a a a
a a a a
24 h
48 h
72 h
96 h
Control
1%
3%
5%
B
Survival probablity (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8
Time (days)
Control
2%
5%
10%
15%
C
Mean of laid eggs (% of total)
100
90
80
70
60
50
40
30
20
10
0
*
*
24 h
48 h
Control
2%

**Table 1.** Median survival times in treatments with the leaf extract (2 to 15 %, w/v) or control solution (0.15 M NaCl).

| **Treatment** | **Survival time** | **CI 95%*** | **Median** |
|---|---|---|---|
| Control | 7.866 ± 0.0494 | 7.769–7.963 | --- |
| Leaf extract | | | |
| 2 % | 7.458 ± 0.0848 | 7.292–7.624 | --- |
| 5 % | 6.979 ± 0.112 | 6.759–7.198 | 8.000 |
| 10 % | 6.857 ± 0.116 | 6.629–7.085 | 8.000 |
| 15 % | 6.162 ± 0.122 | 5.923–6.401 | 7.000 |

*Confidence interval at 95%. Chi-square value: 198.1074, Significance at $p < 0.0001$.

**Table 2.** Toxicity by ingestion of the *S. terebinthifolius* leaf extract against *P. xylostella* larvae.

| Time of treatment (h) | Lethal concentrations (w/v)[a] | | |
|---|---|---|---|
| | $LC_{20}$ | $LC_{50}$ | $LC_{99}$ |
| 96 | 5.85 [4.28-7.17] | 14.49 [12.7-17.06] | 38.38 [32.34-48.17] |
| 144 | 3.74 [0.86-6.61] | 11.74 [8.87-14.62] | 33.87 [30.87-36.88] |

[a]Lethal concentrations of leaf extract required to kill 20% ($LC_{20}$), 50% ($LC_{50}$), and 99% ($LC_{99}$) of *P. xylostella* larvae in 96 and 144 h calculated by probit regression. The values in square brackets correspond to the confidence intervals at 95%.

**Table 3.** Viability of *P. xylostella* larvae and pupae from control and treatments with the *S. terebinthifolius* leaf extract.

| Treatment | Larval viability (%)* | Pupal viability (%)** | Total mortality (%)*** |
|---|---|---|---|
| Control | 95 ± 4.0 [a] | 92.1 ± 4.0 [A] | 12.5 |
| Leaf extract | | | |
| 2% | 75 ± 4.0 [b] | 90 ± 5.0 [AB] | 32.5 |
| 5% | 60 ± 0.0 [b] | 80 ± 6.0 [B] | 50.0 |
| 10% | 53 ± 3.0 c | 52.38 ± 7.0 [C] | 72.5 |
| 15% | 20 ± 0.0 [d] | 50 ± 0.0 [C] | 90.0 |

*Percentage of larvae that reached the pupa stage. **Percentage of larvae that reached the adult stage. ***Total mortality was calculated by the sum of the percentage of individuals that dead at larval or pupal stages. Different uppercase or lowercase letters indicate significant differences ($p < 0.05$) between the treatments by Student's t test.

**Table 4.** Effect of the ingestion of *S. terebinthifolius* leaf extract by *P. xylostella* larvae on the fertility of adults.

| Treatment | Eggs laid per female | Viable eggs (%)** |
|---|---|---|
| Control | 37.5 ± 3.6 [a] | 93.15 ± 1.97 [A] |
| Leaf extract | | |
| 2% | 36.75 ± 3.5 [a] | 91.26 ± 2.49 [A] |
| 5% | 34.25 ± 2.7 [a] | 80.95 ± 1.49 [B] |
| 10% | 10.75 ± 1.3 [b] | 49.30 ± 4.96 [C] |

The number of couples in each treatment was 4. The treatment with the extract at 15% was not evaluated since the number of emerged adults was not enough to perform the assay. *Percentage of viable eggs laid by females in each treatment. Different uppercase or lowercase letters indicate significant differences ($p < 0.05$) between the treatments by Student's t test.

## 4.4 ARTIGO 3 - LARVICIDAL PREPARATIONS FROM *Schinus terebinthifolia* LEAVES INTERFERE WITH MIDGUT DEVELOPMENT OF *Aedes aegypti*

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### Abstract

The control of the vector *Aedes aegypti* is the main prophylactic measure to control the spread of dengue fever, Chikungunya, and Zika virus diseases. In face of the environmental toxicity and the resistance development associated with the use of synthetic pesticides, plant-derived insecticides have been studied as potential alternatives. The saline extract from *Schinus terebinthifolia* leaves and its fraction rich in cinnamic derivatives (called F1) were reported be larvicidal against *A. aegypti*. This work evaluated the impacts of the exposure of *A. aegypti* larvae (third instar) to sub-lethal concentrations of these preparations on the midgut development. The larvae were incubated with the extract (0.6%, w/v), F1 (0.2%, w/v) or distilled water (control) for 24 h. After this period, they were transferred to distilled water and the development was followed until they became adults. Larvae, pupae and adults were collected and had their midguts dissected and stained. The treatment with the extract and F1 resulted in cellular deformation, hypertrophy, and vacuolization in the midgut epithelium in all stages (larvae, pupae and adults). The number of proliferative cells (phosphohistone H3-

positive) was lower ($p < 0.05$) in larvae and pupae treated with the extract and F1, when compared to control, indicating problems in midgut regeneration and remodeling. Differently from the control, proliferating cells were detected in adult midguts from individuals exposed to the extract. Caspase-3-stained cells were observed in larvae and pupae exposed to the extract and F1. In conclusion, the exposure of *A. aegypti* larvae to the saline extract and F1 chronically affected the midgut development, leading to drastic morphological alterations in the subsequent life stages. These changes were associated with occurrence of apoptosis and alterations in the process of cellular regeneration.

**Keywords:** dengue mosquito; midgut epithelium; botanical insecticide.

## 1. Introduction

The mosquito *Aedes aegypti* is the main vector of the viruses that cause dengue fever, Chikungunya, yellow fever, and Zika virus diseases (Bhatt et al., 2013; Wheetman et al., 2018). The World Health Organization (WHO) considers dengue fever one of the main health problems since it affects about 390 million people per year worldwide and is present in about 128 countries (Bhatt et al., 2013; Olliaro et al., 2018; WHO, 2018a, 2018b). In 2018, it was estimated 446,150 cases of dengue fever, an incidence of 45.9/100,000 people (WHO, 2018b). Chikungunya outbreaks have been reported in Argentina, Bolivia, Brazil, Colombia, India, Kenya, Pacific islands, and Senegal (WHO, 2017). Zika virus infection has been reported in 86 countries and territories and a large outbreak of Zika occurred French Polynesia in 2013 and in Brazil in 2015. In this last country, Zika virus infection was found to be associated with Guillain-Barré syndrome and microcephaly (WHO, 2018c).

Vaccines have been developed against dengue virus, but some restrictions have been pointed out, such as greater efficacy in individuals already seropositive for DEN virus and risk of developing severe disease in seronegative individuals (Aguiar et al., 2016, 2017). There are no vaccines for Chikungunya and Zika viruses (WHO, 2017, 2018c). In this scenario, the main prophylactic measures consist of vector control, with the use of insecticides, improvement of basic and environmental sanity, and community education aiming to eliminate breeding sites (Freitas et al., 2014; Sarwar, 2014; Bahrati et al., 2018a). The prolonged and indiscriminate use of synthetic insecticides has polluted the environment, affected non-target organisms, and led to the selection of resistant individuals, resulting in failure of control programs (Braga and Valle, 2007; Corbel and N'Guessan, 2013; Bahrati et

al., 2018b). Plant-derived insecticides represent potential alternatives because they are more environment-friendly due to more selective toxicity and high degree of biodegradability; in addition, the emergence of insect resistance may be minimized when using plant preparations containing a mixture of active principles (e.g. extracts) or whether the botanical insecticides are included as an additional option for rotation programs (Pontual et al., 2014; Benelli, 2015; Camaroti et al., 2017).

Plant secondary metabolites and defense proteins exhibit insecticidal activity causing morphological changes, interfering with behavior and metamorphosis, and inducing mortality; many of the phytochemicals with insecticidal activity are toxic by ingestion, being their targets present at the midgut (Camaroti et al., 2017; Napoleão et al., 2019). During the life cycle of *A. aegypti*, occurs a remodeling of the gut epithelium through coordinated processes of cell death and proliferation during the ecdyses. Thus, damages at the midgut of mosquitoes can affect their survival, development and reproductive capacity (Fernandes et al., 2014).

*Schinus terebinthifolia* is a plant belonging to the family Anacardiaceae, being popularly known as Brazilian Pepper tree. It is widely used in popular medicine in the form of teas, infusions and tinctures (Silva et al., 2018; Uliana et al., 2016) and has been reported as source of insecticidal agents. Saline extract from its leaves caused the death of *Sitophilus zeamais* adults by induction of starvation in consequence of feeding-deterrent effect and inhibition of protease activity at insect gut; these effects were suggested to be linked to the presence of hydrolysable tannins and flavonoids (Camaroti et al., 2018). In another work, the leaf saline extract and its fraction rich in cinnamic derivatives (called F1) were reported to exert larvicidal activity against *A. aegypti*. It was reported that the extract promoted intense disorganization of larval midgut epithelium, including deformation and hypertrophy of cells, disruption of microvilli, and vacuolization of cytoplasms, affecting digestive, enteroendocrine and regenerative cells (Procópio et al., 2015).

In view of these previous findings the present work was designed in order to evaluate the effects of the exposure of larvae of these preparations (extract and F1) at sub-lethal conditions on the development of midgut until the adult stage.

## 2. Materials and methods

### *2.1. Plant material*

The leaves of *S. terebinthifolius* were collected in the campus of the *Universidade Federal de Pernambuco* (Recife, Brazil). Plant collection was performed with authorization (no. 36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from the Brazilian Ministry of Environment and the access was recorded (AC551B2) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). A voucher specimen is archived under number 73,431 at the herbarium from the *Instituto Agronômico de Pernambuco* (IPA), Recife, Brazil. The leaves were left to dry at 28 °C during 72 h, powdered using a blender, and stored at -20°C.

*2.2. Insects*

The insects (PPCampos strain) were reared in insectary of the *Departamento de Biologia Geral* of the *Universidade Federal de Viçosa* at 26±1 °C, relative humidity 75±10%, and photoperiod 12L:12D. Larvae were hatched in dechlorinated water containing turtle food (ReptoLife, São Paulo, Brazil) and used in the assays when reaching the third instar (L3).

*2.3. Leaf extract and fraction (F1)*

Leaf extract and F1 were obtained as described by Procópio et al. (2015). The leaf powder (10 g) was suspended in 0.15 M NaCl (100 mL) and homogenized for 16 h at 28 °C using a magnetic stirrer. The suspension was passed through filter paper, centrifuged (3,000 × *g*, 15 min, 4 °C) and dialyzed against distilled water (4 h, three liquid changes). The resulting extract was then lyophilized to dryness in a freeze-dryer (LIOTOP L101, Liobras, São Carlos, Brazil) and stored at -20 ºC.

To obtain F1, the extract was submitted to solid-phase extraction using Chromabond C18 (500 mg/3 mL) cartridges (Macherey-Nagel, Düren, Germany) coupled to a vacuum pump. The cartridges were equilibrated with 1 mL of methanol followed by 3 mL of 0.1 M Tris-HCl pH 9.0. The extract was dissolved in the Tris buffer to obtain a concentration of 25 mg/mL and passed through a 0.45 µm syringe filter. Next, 1 mL was loaded in the cartridge and F1 was obtained by elution with Tris buffer. The fraction was lyophilized and stored at -20 ºC.

*2.4. Effect of leaf extract and F1 on midgut development*

*2.4.1. Bioassay*

The extract and F1 were dissolved in distilled water to obtain test solutions at 0.6% and 0.2% (w/v), respectively. The assay was performed at sub-lethal conditions since the exposure time was 24 h and these values correspond to the $LC_{50}$ for 8 days (Procópio et al., 2015). One hundred L3 were incubated with 100 mL of the test solution or distilled water (negative control). After the 24-h period, the larvae were transferred to another recipient containing 100 mL of distilled water and the development was followed until they became adults. Individuals who died during the test were removed from the medium every 24 h. The assay was performed in triplicate. For each replicate, 10 larvae (L3) were collected after 24 h of treatment, 10 pupae were collected 48-h after pupation, and 10 adults were collected shortly after emergence. The assays were maintained at 26±1 °C, relative humidity of 75±10% and photoperiod 12L:12D. The collected individuals had their midguts dissected in physiological solution (0.1 M NaCl, 20 mM $KH_2PO_4$, 20 mM $Na_2HPO_4$) and fixed in Zamboni's solution (formaldehyde and picric acid).

*2.4.2. Histological analysis*

The fixed midguts were washed with 1% PBST (phosphate buffered saline with 1% Tween; Sigma-Aldrich, USA), dehydrated in a graded series of ethanol (70–100%), and embedded in Historesin (Leica, Heidelberg Mannhein, Germany). The tissue was cut into 3-µm sections, stained with 1% (w/v) toluidine blue, and mounted in Eukitt medium (Fluka, USA). The stained midguts were observed under an optical microscope (Olympus BX53, Olympus America, Inc., NY, USA) and photographed using a digital camera (Olympus DP 73, Japan).

*2.4.3. Midgut analysis by fluorescence microscopy*

The midguts were washed three times for 30 min with 1% PBST and incubated for 24 h at 4 °C with the following primary antibodies, diluted in PBS: anti-phosphohistone H3 (1:100) (Cell Signaling, USA) and anti-caspase-3 (1:500) (Sigma-Aldrich, USA). After incubation, the midguts were washed three times with PBST and incubated for 24 h at 4 °C with a solution (1:500) of anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, USA) diluted in PBS. Cell nuclei were stained with

diamidino-2-phenylindole (DAPI) (Biotium, USA). Next, the midguts were washed three times, cut into 7 μm-thick histological sections, mounted with Mowiol antifading solution (Sigma-Aldrich, USA), and observed under an epifluorescence microscope (Olympus BX53 coupled with an Olympus DP 73 digital camera). The stained cells were counted using six fields of longitudinal DAPI-stained sections per midgut, which were visualized with a 40× objective lens (total area: 0.414 $mm^2$) (Fernandes et al., 2010). The morphometric analyses were performed with the image analysis program Image Pro Plus 4.0 for Windows (Media Cybernetics).

*2.5. Statistical analysis*

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) and data have been expressed as mean of replicates ± SD. Significant differences between treatments were analyzed by ANOVA (significance at $p < 0.05$).

## 3. Results and discussion

In a previous study, saline extract from *S. terebinthifolia* leaves was evaluated for larvicidal activity against *A. aegypti* presenting $LC_{50}$ values for 3 and 8 days of 1.05% and 0.62%, respectively. The authors reported the elimination of gut content together in the peritrophic matrix by the larvae exposed to the extract as well as the midgut histological changes mentioned in the 'Introduction'. In addition, on the eighth day of the experiment, the number of larvae that become pupae as well as the number of emerged adults were lower in treatments with the extract than in the control, which could be associated with the midgut damages caused at larval stage (Procópio et al., 2015). In the present work, we monitored possible changes in the midgut of pupae and adults derived from larvae exposed to this extract at sub-lethal conditions. Additionally, it was evaluated the effect of the F1 fraction, rich in active principles of the extract with larvicidal activity (Procópio et al., 2015).

Histological analysis revealed a remarkable disorganization of the midgut epithelium in all the stages (larvae, pupae and adults) of individuals exposed to the extract and F1, in comparison with control (Figure 1). It was observed intense cellular deformation and hypertrophy, several spaces between the cells and vacuolated cytoplasm. Cell disruption and presence of debris in the lumen were also observed. These aspects were most strikingly

evident in F1 treatment. These morphological changes are similar to those found by Procópio et al. (2015) in *A. aegypti* larvae treated with the extract. Interestingly, the alterations persist in the subsequent life stages.

During the metamorphosis of *A. aegypti*, a remodeling of the midgut occurs from the proliferation of regenerative cells. This process occurs in a coordinated way to maintain the epithelial integrity of the insect gut. Thus, interferences in this remodeling process can compromise the metamorphosis and the survival of the individuals even if they reach adulthood (Jiang and Edgar, 2012; Fernandes et al., 2014). The results obtained here demonstrate that the damaging effects of extract and F1 at the larval stage reverberate throughout the development of the insect. Fernandes et al. (2015) also reported deformations of digestive cells and presence of vacuoles in the gut of larvae, pupae and adults of *A. aegypti* exposed to imidacloprid, one of the most used larvicides in the world (Elbert et al., 2008).

Proliferative cells (PH3-positive) were observed in the gut of larvae and pupae from control and both extract and F1 treatments (Figure 2). However, the number of PH3-positive cells was significantly ($p < 0.05$) lower in larvae and pupae exposed to extract and F1 (Figure 3A). This impairment of cell proliferation at the midgut of pupae can be a reflection of difficulties in the regeneration response to the damages caused still in the larval phase, which also reflected in the morphological alterations observed also at the adult stage.

No proliferating cells were detected in the midgut of adults from control group (Figure 3A) which was expected since, under normal conditions, mitosis of the regenerative cells should not be occurring in adult mosquitoes (Brown et al., 1985; Hecker, 1977). On the other hand, there was a discrete labeling in the midgut of adults derived from larvae exposed to the extract (Figures 2 and 3A), which can be due to activation of regenerative processes in response to the damages occurring since the larval stage. Janeh et al. (2017) reported that cell proliferation was induced at the midgut of *A. albopictus* after bacterial or chemical lesions.

Cells labelled for caspase-3 were observed in larvae and pupae exposed to the extract and F1 but not from control; no staining was observed in adult midguts (Figure 4). Caspase-3 is an intracellular enzyme that participates in the effector processes of apoptotic pathways and has its increased activity in cellular aggression processes. The number of caspase-labeled cells was similar in extract and F1-treated insects (Figure 3B) and the damages caused by these preparations can be due to a cytotoxic effect. Procópio et al. (2015) reported the presence of cells positive for TUNEL reaction (indicative of DNA fragmentation) in the midgut of larvae treated with the saline extract from *S. terebinthifolia*. The data obtained here confirm the activation of apoptotic death of midgut cells in consequence of

treatment with extract and also with F1. Fernandes et al. (2015) also reported the presence of TUNEL-positive cells in the midgut of all stages of *A. aegypti* development when the insects were exposed to imidacloprid.

## 4. Conclusion

The exposure of *A. aegypti* larvae to the saline extract and F1 from *S. terebinthifolia* leaves chronically affected the midgut development, leading to drastic morphological alterations in the subsequent life stages (pupa and adult). These changes were associated with occurrence of apoptosis and alterations in the process of cellular regeneration.

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**Figure captions**

**Figure 1.** Longitudinal histological sections of the midgut of *A. aegypti* larvae, pupae and adults from the control and exposed for 24 h during the third larval instar to saline extract from *Schinus terebinthifolia* leaves and its fraction (F1) at 0.6% and 0.2%, respectively. Sections were stained with toluidine blue. Scale bars correspond to 20 μm. l, gut lumen; ep, epithelium.

**Figure 2.** Histological sections of the midgut of *A. aegypti* larvae, pupae and adults from the control and exposed for 24 h during the third larval instar to saline extract from *Schinus terebinthifolia* leaves and its fraction (F1) at 0.6% and 0.2%, respectively. The nuclei of all epithelium cells were stained with DAPI (blue fluorescence). The nuclei of proliferating stem cells were green-stained as phosphohistone H3-positive (PH3). Scale bars correspond to 20 μm.

**Figure 3.** Number of mitosis/proliferating (A) and apoptotic (B) cells in the midguts of *A. aegypti* larvae, pupae and adults from the control and exposed for 24 h during the third larval instar to saline extract from *Schinus terebinthifolia* leaves and its fraction (F1) at 0.6% and 0.2%, respectively, determined by phosphohistone H3 and caspase-3 stainings. Different uppercase or lowercase letters indicate significant differences ($P < 0.05$) between treatments.

**Figure 4.** Histological sections of the midgut of *A. aegypti* larvae, pupae and adults from the control and exposed for 24 h during the third larval instar to saline extract from *Schinus terebinthifolia* leaves and its fraction (F1) at 0.6% and 0.2%, respectively. The nuclei of all

epithelium cells were stained with DAPI (blue fluorescence). The nuclei of apoptotic cells were red-stained as caspase 3-positive. Scale bars correspond to 20 µm.

**Figure 1**

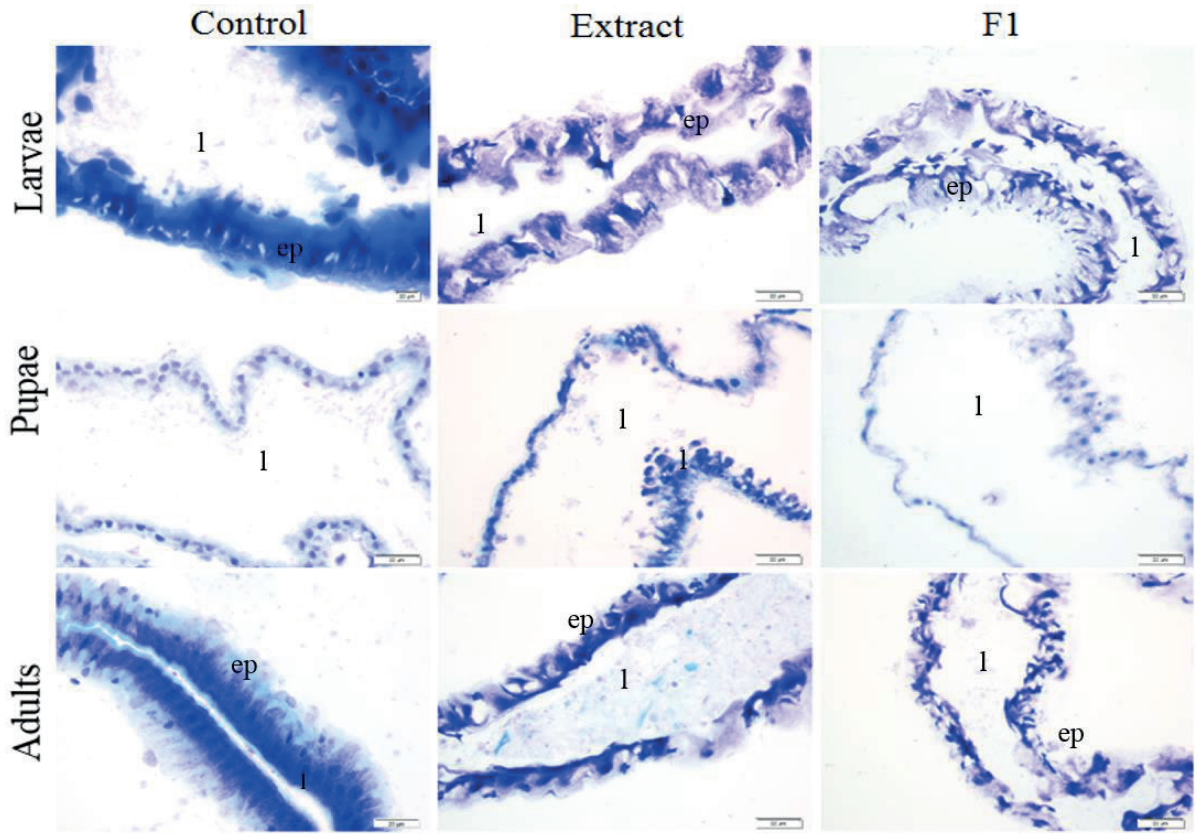


**Figure 2**

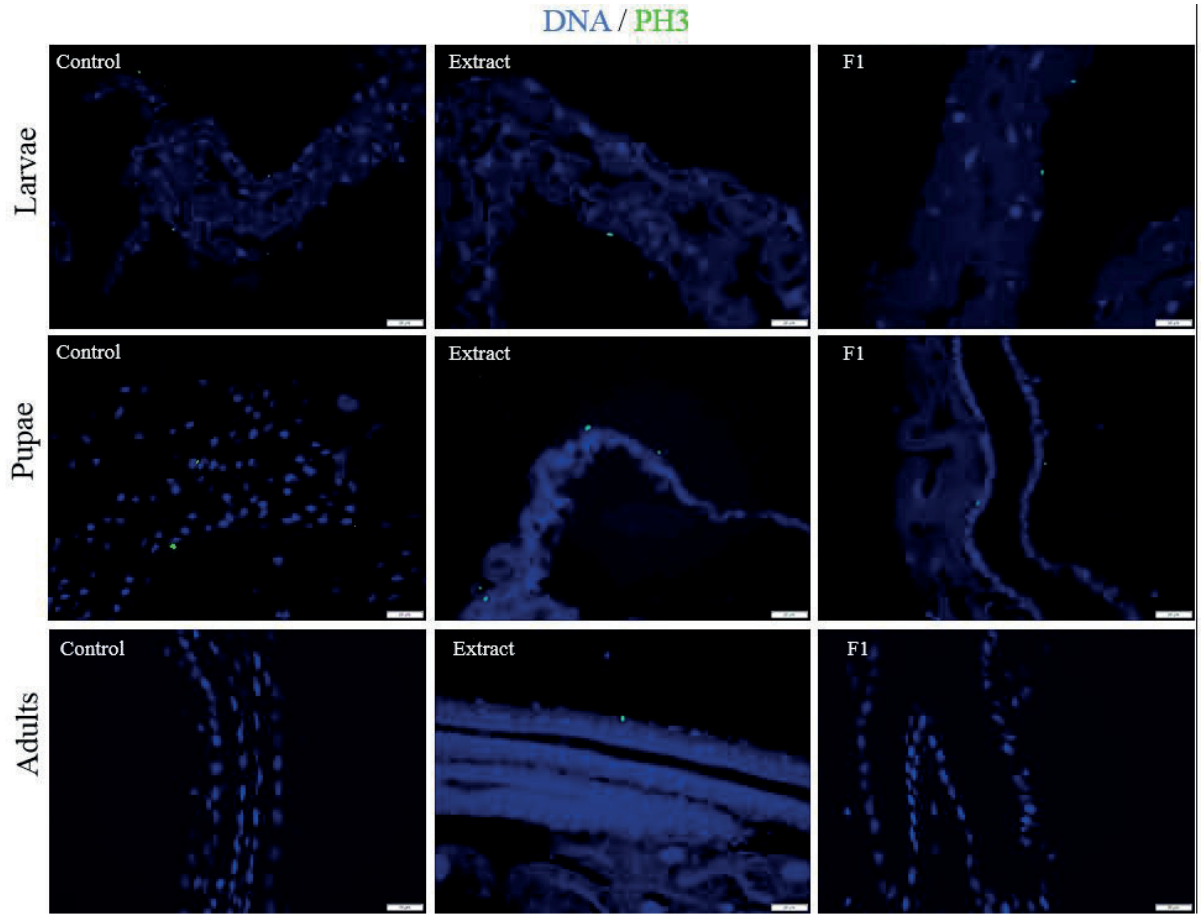
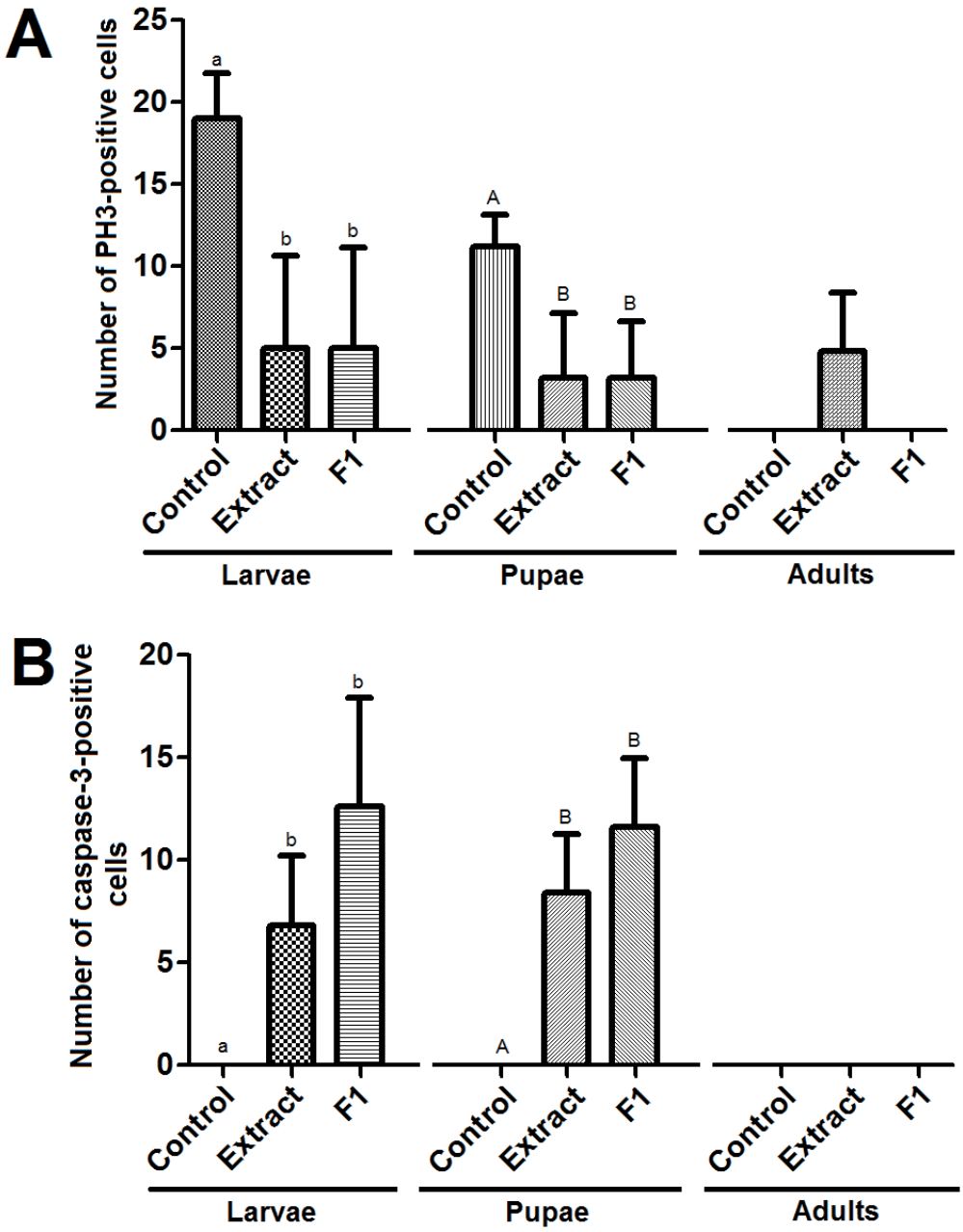

DNA / PH3
Control
Extract
F1
Larvae
Control
Extract
F1
Pupae
Control
Extract
F1
Adults

**Figure 3**


A
Number of PH3-positive cells
25
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15
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b
A
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B
Control
Extract
F1
Larvae
Pupae
Adults
B
Number of caspase-3-positive cells
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a
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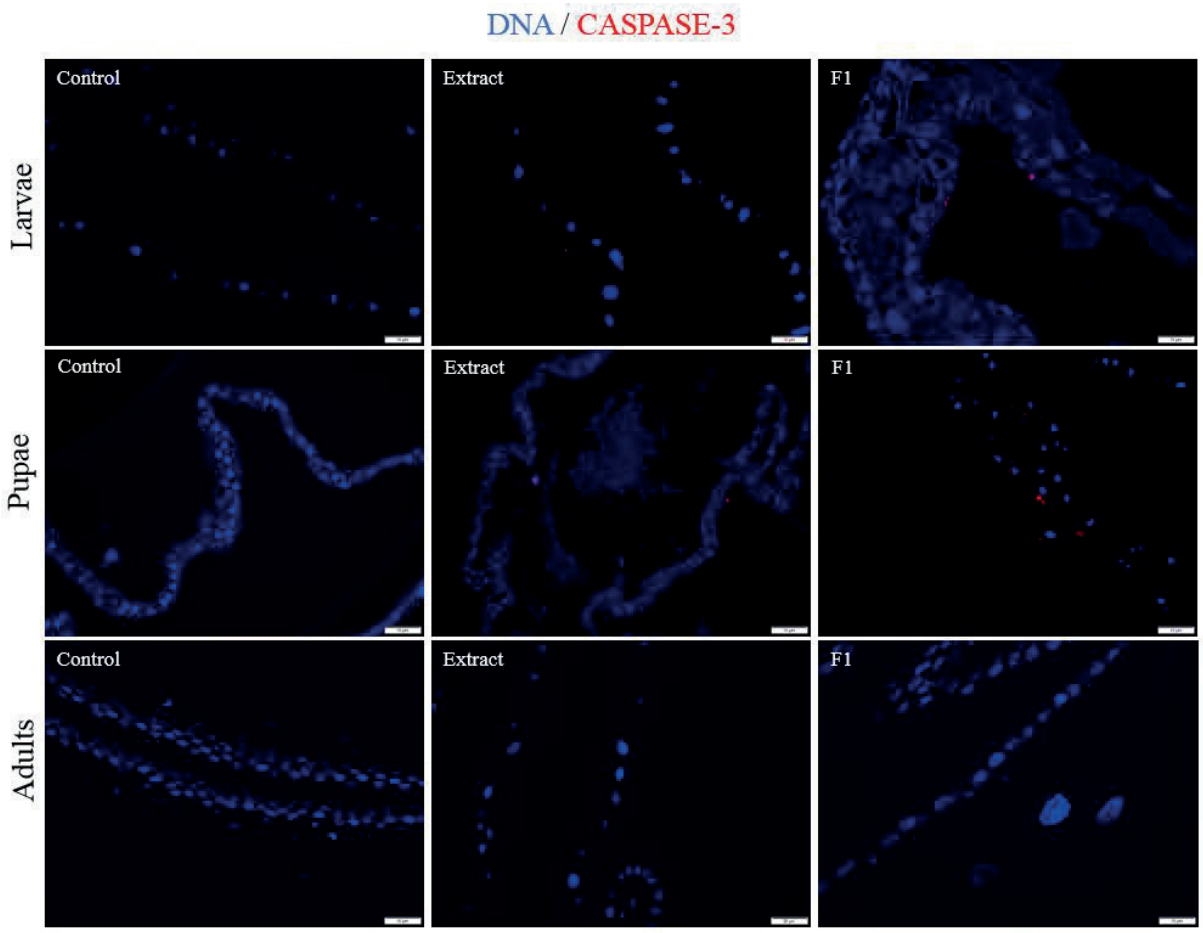
b
A
B
B

**Figure 4**


DNA / CASPASE-3
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# 5 CONCLUSÕES

A ingestão do extrato salino de folhas de *S. terebinthifolia* (contendo ácido gálico e flavonoides) causou a morte de adultos de *S. zeamais*, bem como exerceu um forte efeito deterrente. O extrato foi capaz de alterar a atividade de enzimas intestinais *in vitro*. A lectina (SteLL), provavelmente, não teve um papel importante na ação tóxica do extrato, mas contribuiu para os efeitos antinutricionais observados. A rejeição da dieta causada pelo extrato promove seu uso como um agente protetor de grãos armazenados.

O extrato salino de folhas também é um novo agente inseticida contra *P. xylostella*, matando lagartas e pupas e prejudicando a capacidade reprodutiva dos adultos emergidos das lagartas tratadas. Além disso, o extrato é um agente deterrente de oviposição. A atividade inseticida pode ser devida à ação do ácido gálico e dos flavonóides, uma vez que a lectina presente no extrato não apresentou atividade quando isolada.

A exposição de larvas de *A. aegypti* ao extrato salino e F1 (fração rica em derivados cinâmicos) afetou cronicamente o desenvolvimento do intestino médio dos insetos, levando a alterações morfológicas drásticas nos estágios subsequentes da vida (pupa e adulto). Essas alterações foram associadas à ocorrência de apoptose e alterações no processo de regeneração celular.

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