UNIVERSIDADE FEDERAL DO AMAPÁ
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INANA FAURO DE ARAÚJO

Avaliação da atividade larvicida do extrato hidroetanólico de *Acmella oleracea* (L.) R. K. Jansen sobre *Aedes aegypti* e *Culex quinquefasciatus* (Diptera: culicidae)

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientador: Dr Raimundo Nonato Picanço Souto

Co-orientador: Dr Irlon Maciel Ferreira

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SUMÁRIO

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SÍMBOLOS, SIGLAS E ABREVIATURAS

Ae.	*Aedes*
CG-EM	Cromatografia Gasosa – Espectroscopia de Massas
CL	Concentração Letal
Cx.	*Culex*
DPPH	2,2-diphenyl-1-picryl-hydrazyl
DMSO	Dimetilsulfóxido
HCl	Ácido clorídrico
NaCl	Cloreto de sódio
OMS	Organização Mundial da Saúde
PPM	Parte por milhão
WHO	World Health Organization
%	Por cento
<	Menor que

AVALIAÇÃO DA ATIVIDADE LARVICIDA DO EXTRATO HIDROETANÓLICO DE *Acmella oleracea* (L.) R. K. JANSEN SOBRE *Aedes aegypti* E *Culex quinquefasciatus* (DIPTERA: CULICIDAE).

Introdução: Atualmente, a busca por substituintes inovadores e renováveis, principalmente derivados de plantas, que apresentem atividades biológicas significativas, estão ganhando mais espaço na comunidade científica, pois estes são obtidos de fontes renováveis e ambientalmente rentáveis. A espécie *Acmella oleracea* popularmente conhecida como jambú, é uma espécie rica em isobutilamidas, onde a principal molécula desta espécie é o alcalóide *N*-isobutilamida. **Objetivo:** Desta forma o objetivo deste trabalho foi avaliar atividade larvicida do extrato bruto hidroetanólico das folhas de *Acmella oleracea* frente larvas de *Aedes aegypti* e *Culex quinquefasciatus.* **Metodologia:** O extrato foi preparado utilizando álcool 70%, este foi rotoevaporado e liofilizado. Em seguida este foi dissolvido em dimetilsulfóxido (DMSO) em diferentes concentrações, (2.5, 5, 7.5, 10 e 15 ppm) para *Ae. aegypti,* (5, 10, 20, 30 e 40 ppm) para *Cx. quinquefasciatus*. O teste foi realizado em quintuplicata utilizando Becker de 100 mL com 10 larvas em cada. Para o teste foi utilizado como controle negativo água destilada e DMSO (1%). A taxa de mortalidade foi determinada após 24 horas**,** onde foram consideradas mortas larvas que não respondiam a estimulação. **Resultados e Discussões:** No bioensaio a mortalidade em 24 horas para *Ae. aegypti* em 15 ppm foi de 58% e em 2,5 ppm foi 18%, sendo os valores estimados da CL_{50} 11.41ppm e CL_{90} 23,23 ppm. A ação larvicida para *Cx. quinquefasciatus* demonstrou 54% de mortalidade em 40 ppm e 13% para 5 ppm, demonstrando valores de CL_{50} 32,40 ppm e CL_{90} 68,24 ppm. Desta forma observa-se que o extrato apresentou mortalidade elevada, mesmo em baixas concentrações, e que esta pode estar associada a presença da alquilamida espilantol, que foi identificada através de análise por cromatografia gasosa e espectroscopia de massa. **Conclusões:** Desta forma este estudo contribui com informações acerca de material de origem vegetal que podem vir a ser utilizados nas práticas integrativas no controle de vetores de doenças.

Palavras-Chave: Atividade larvicida; Jambu; *Aedes aegypti.*

Agradecimentos: CAPES, UNIFAP e FAPEAP.

EVALUATION OF THE LARVICIDE ACTIVITY OF THE HYDROETHANOLIC EXTRACT OF *Acmella oleracea* (L.) R. K. JANSEN ABOUT *Aedes aegypti* and *Culex quinquefasciatus* (DIPTERA: CULICIDAE).

Introduction: Currently, the search for innovative and renewable substituents, mainly derived from plants, that present significant biological activities, are gaining more space in the scientific community, as these are obtained from renewable and environmentally profitable sources. The species *Acmella oleracea* popularly known as jambu, is a species rich in isobutylamides, where the main molecule of this species is the alkaloid *N*-isobutylamide. **Objectives:** The objective of this work was to evaluate larvicidal activity of the crude hydroethanolic extract of the leaves of *Acmella oleracea* against larvae of *Aedes aegypti* and *Culex quinquefasciatus.* **Methodology:** The extract was prepared using 70% alcohol, this was rotoevaporated and lyophilized. This was then dissolved in dimethylsulfoxide (DMSO) in different concentrations, (2.5, 5, 7.5, 10 and 15 ppm) for *Ae. aegypti*, (5, 10, 20, 30 and 40 ppm) to *Cx. quinquefasciatus*. The test was performed in quintupleta using Becker of 100 mL with 10 larvae in each. For the test, distilled water and DMSO (1%) were used as controls. The mortality rate was determined after 24 hours, where larvae that did not respond to stimulation were considered dead. **Results and discussion:** In the bioassay the mortality in 24 hours for *Ae. aegypti* at 15 ppm was 58% and at 2.5 ppm it was 18%, with the LC_{50} values 11.41ppm and LC_{90} being 23.23 ppm. The larvicidal action for *Cx. Quinquefasciatus* showed 54% mortality at 40 ppm and 13% at 5 ppm, showing values of LC_{50} 32.40 ppm and LC_{90} 68.24 ppm. In this way, the extract showed high mortality, even in low concentrations, and this may be associated with the presence of the alkylamide spilanthol, which was identified by gas chromatography and mass spectroscopy. **Conclusions:** In this way this study contributes with information about material of vegetal origin that can be used in the integrative practices in the control of vectors of diseases.

Keywords: Larvicidal activity; Jambu; *Aedes aegypti.*

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1 INTRODUÇÃO

1.1 DIPTERA: CULICIDAE

Aedes aegypti e o *Culex quinquefasciatus* são mosquitos que apresentam grande dispersão em áreas urbanas e são importantes vetores de doenças como a dengue e filariose linfática, respectivamente, e tratam-se de vetores que acometem principalmente a população que mais sofre com problemas de saneamento básico.

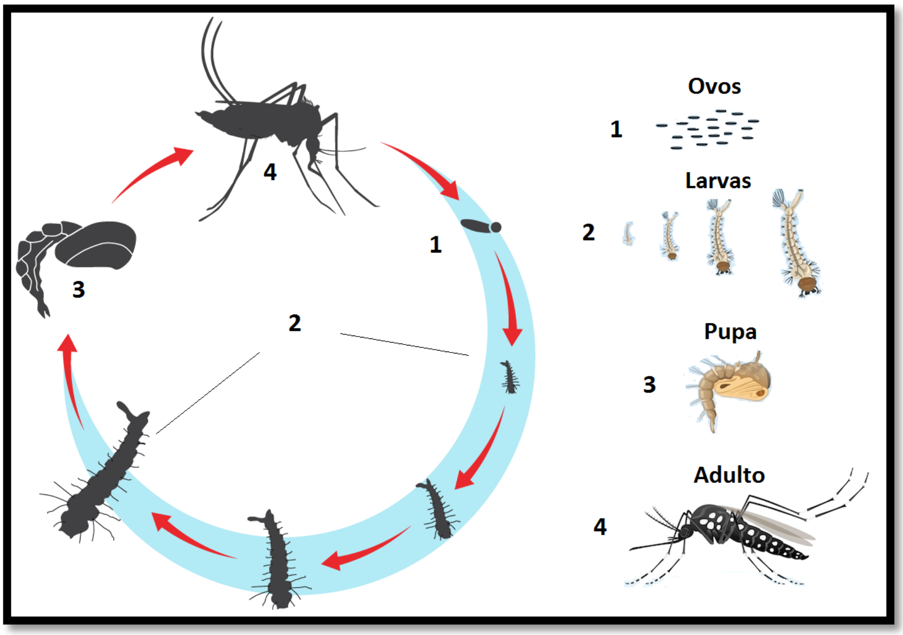
O *Ae. aegypti* (Linnaeus 1762), é o principal inseto transmissor do vírus zika, febre chikunguya e dengue nos países tropicais (WILSON; SCHLAGENHAUF, 2016), estando estas na lista nacional de notificação compulsória de doenças, agravos e eventos de saúde pública (BRASIL, 2017). O mosquito *Culex quinquefasciatus* (Say, 1823) é responsável pela transmissão da filariose linfática apresenta focos em áreas com elevada taxa populacional e baixa condições socioambientais (BRASIL, 2011).

Estes mosquitos pertencem ao filo arthropoda, classe insecta, ordem díptera e família Culicidae. "Os mosquitos são insetos holometábolos (desenvolvem-se por metamorfose completa) e apresentam ciclo biológico que compreende as fases de ovo, quatro estádios larvais (L1, L2, L3 e L4), pupa e adulto" (OLIVEIRA, 2014) (Figura 1).

A ordem diptera é um dos grupos mais diversos, compreendendo moscas, mosquitos, apresentando uma diversidade ecológica e uma riqueza de espécies. Os dipteros estão distribuídos por todos os continentes, colonizando vários tipos de hábitats, sobretudo o ambiente aquático que é onde ocorre seu estágio larval (PINHO, 2008; CONSOLI; OLIVEIRA, 1994).

Com a descoberta de que estes insetos eram responsáveis pela veiculação de arboviroses, como febre amarela, encefalites, dengue, estes passaram a receber uma atenção maior dos pesquisodores ao redor do mundo, onde estes se preocuparam em estudar sua biologia, a fim de descobrir pontos vulneráveis para assim combatê-los (CONSOLI; OLIVEIRA, 1994).

Figura 1 - Ciclo de desenvolvimento dipteros ovos (1), Larvas (2), pupa (3) e adulto (4).



Fonte: http://www.casadaciencia.com.br/o-fim-da-picada

O *Ae. aegypti* é um mosquito cosmopolita encontrado nas regiões tropicais e subtropicais, seus adultos podem permanecer vivos na natureza em média 30 a 35 dias (BRASIL, 2001). Os adultos de ambos os sexos se alimentam de seiva, néctar de vegetais e outras fontes de açucares, os machos não são hematófagos, entretanto, as fêmeas precisam tomar repasto de sangue de vertebrados para amadurecer os ovos de forma periodicamente.

O *Ae. aegypti* transmissor da dengue e da febre amarela urbana, é originário da África Tropical, foi introduzido no Brasil, provavelmente, através do processo de colonização, no século XVII, gerando a primeira epidemia de febre amarela no país, onde em 1686 no Estado da Bahia chegou a contaminar vinte e cinco mil pessoas e levando 900 a óbito no mesmo ano (BRASIL, 2001).

O vírus da dengue pertence ao gênero Flavivirus e à família Flaviviridae, são compostos por RNA de filamento único envelopados e possui quatro sorotipos: DEN 1, DEN 2, DEN 3 e DEN 4 (WESTAWAY, 1997). O primeiro caso de epidemia de dengue registrado no Brasil foi entre os anos de 1981-1982 no Estado de Roraima na cidade de Boa Vista em 1986 a epidemia chegou ao estado do Rio de Janeiro e em demais capitais do Nordeste.

Na década de 90 ocorreu aumento da dispersão do *Ae. aegypti* no território nacional, onde dos 26 estados da federação 20, registravam os sorotipos da dengue DEN 1 e DEN 2. No ano de 2004 vinte e três estados já apresentam os sorotipos DEN 1, DEN 2 e DEN 3

(BRASIL, 2009), em 2010 o sorotipo DEN 4, que há 28 anos não circulava no Brasil, foi isolado em Roraima (DIAS et al., 2010).

A proliferação do mosquito *Ae. aegypti* e o rápido crescimento demográfico associado a intensa desordenada urbanização, inadequada infraestrutura e o aumento da produção de resíduos não-orgânicos contribuem para o aparecimento de epidemias recorrentes (MONTEIRO, 2014).

Neste contexto, a ordem diptera requer uma atenção especial pelo fato de muitas espécies serem hematófagas e atuarem como vetores de doenças ao homem, tendo em vista que o *Ae. aegypti* desenvolveu em sua trajetória evolutiva um comportamento sinantrópico e antropofílico (NATAL, 2002), com atividade hematofágica diurna utilizando de depósitos artificiais de água limpa para depositar seus ovos individualmente.

Segundo Natal (2002) os fatores ambientais, especialmente chuva e temperatura, são marcante na dinâmica populacional e que essa espécie de mosquito, pela complexa bioecologia, desenvolveu incríveis adaptações ao modo de vida do homem, onde vem demonstrando características de rápida adaptação ecológica a ambientes urbanos cada vez mais complexos (BESERRA et al., 2014).

O vetor da filária, *Cx. quinquefasciatus*, é um mosquito doméstico, antropofílico, com hábito hematófago normalmente ao entardecer pode causar incômodo, alergias e transmitir doenças no momento da sua picada. Seus ovos são depositados sobre a água parada e de preferência poluída, como esgotos e valões, pois apresentam condições favoráveis para o desenvolvimento das larvas, visto que apresentam matéria orgânica necessária para alimentação das mesmas até a fase adulta (SCUDELER, 2013).

No Brasil o agente da filariose possuía ampla distribuição nos anos de 1950 e 1960, atualmente os focos desta doença estão relacionados com a abundância de seu transmissor *Cx. quinquefasciatus*, a elevada taxa de densidade populacional e as péssimas condições ambientais que contribuem para sua proliferação (BRASIL 2011). De acordo com a Organização Mundial da Saúde (OMS), esta doença é endêmica em 73 países na Ásia, África e Américas, com um percentual de 120 milhões de pessoas afetadas.

O agente etiológico da filariose *Wuchereria bancrofit* é um nematódeo transmitido para o homem através da picada da fêmea no momento do repasto sanguíneo. A filariose linfática é um problema de saúde pública, pois estes vermes quando adultos nos vasos linfáticos provocam um quadro clínico típico da doença, como deformação e edema a qual pode levar a incapacidade física temporária ou permanentes (OLIVEIRA, 2014; BRASIL, 2016). Também é vetor do vírus Oropouche que causa febre Oropouche, sendo uma arbovírose presente principalmente na região da Amazônia, além de também ser o transmissor do arbovirus do gênero *Flavivirus* responsável pela Febre do Nilo Ocidental (BRASIL 2011; OLIVEIRA 2014).

A partir de medidas de controle do vetor que foram adotadas houve um descrescimento nas taxas de infecção por filariose, e devido a preocupação do agente *Cx. quinquefasciatus* se apresentar como vetor de diversas aborviroses ou doenças tropicais, em 2012 foi lançado o plano integrado de ações para a eliminação de hanseníase, filariose, esquistossomose e ancocercose, sendo todos estes tratados pelo governo como um problema de saúde pública (BRASIL, 2016).

1.2 DIVERSIDADE QUÍMICA DAS PLANTAS E ATIVIDADE REPELÊNCIA E INSETICIDA SOBRE CULICIDAE

Sabe-se que as plantas produzem metabolitos primários, onde estes se encontram em todas as plantas e são responsáveis pelo desenvolvimento, tais como, os ácidos graxos, e os metabolitos secundários, que exercem proteção para as plantas contra predadores ou mudanças trágicas no clima. (RAVEN; EVERT; EICHHORN, 2001). Devido a este potencial de reação a alguns organismos, essas substâncias vêm ganhado espaço nas pesquisas acadêmicas para que se encontrem substâncias ou conjuntos delas com atividade antimalárica, inseticida e antimicrobiana (SANTOS et al., 2014).

O aumento da transmissão de doenças, tendo os insetos como vetores, tem levado a comunidade científica a uma contínua busca por novos bioinseticidas. As plantas através de seus metabólicos primários e secundários são fonte importantes de diferentes substâncias com diversas atividades contra os insetos com resultados satisfatórios, entretanto, seu uso direto ou de seus extratos brutos se limita a aplicações domésticas (VIEIRA; FERNANDES; ANDREI, 2007).

Os extratos são preparações com consistência líquida, sólida ou intermediária, obtida a partir de um material vegetal ou animal, este pode ser preparado por percolação, maceração ou outro método adequado e validado, onde este utiliza solvente, como, álcool etílico, água ou outro solvente adequado (FARMACOPÉIA, 2010). Óleos essências de acordo com Simões e Spitzer (2010) são produtos obtidos através da destilação por arraste a vapor d'água tal como os produtos obtidos por expressão dos pericarpos de frutos cítricos, a composição química de extratos, e óleos essenciais provenientes de plantas, é complexa e pode chegar a centenas de compostos com funções diferentes.

Substituto para os inseticidas sintéticos tem estimulado os pesquisadores ao estudo dos bioinsenticidas, viabilizando a utilização de óleos, extratos ou constituintes ativos provenientes de plantas (GUISSONI et al., 2013). Compostos a partir de plantas são apresentados como alternativa no controle de vetores, por ser de fácil obtenção e em alguns casos apresentar menor toxicidade ao homem.

Tradicionalmente, as plantas e seus derivados sempre foram usadas para matar os mosquitos e outras pragas domésticas, desta forma, a utilização de diferentes partes das plantas e seus produtos secundários no controle dos mosquitos vem sendo bem estabelecida por várias investigações (SUBRAMANIAM et al., 2012).

Satput et al. (2015) define repelente como sendo uma substância química volátil capaz de repelir o arthropoda a partir da sua fonte, ou seja, impede o contato do vetor com a pele do homem e estes podem ser formulados de várias formas como loções, aerossóis, sprays, dentre outros.

A utilização de plantas como repelente contra os insetos tem sido relatada desde a época greco-romana, em algumas regiões da Índia as mulheres aplicam diariamente cúrcuma (*Curcuma longa* L., Zingiberaceae) em óleo para proteção contra mosquitos, assim como desde o ínicio do século XX, muitos produtos foram empregados como repelente contra os insetos, entre eles se destacam a citronela, piretro e a andiroba (BUENO; ANDRADE, 2010).

Bioensaios realizados para repelência contra espécies de dipteros, em particular aqueles pertencentes ao gênero *Aede*s, *Anopheles* e *Culex* que são vetores de doenças, responsáveis por problemas de saúde públicas, como dengue, malária, febre amarela, vem crescendo no meio científico (NERIO et al., 2010).

Satput et al. (2015) relataram vinte e quatro espécies de plantas que apresentam potencial repelente contra mosquitos, dentre elas a *Citrus hystrix e Cymbopogon Martini* apresentou 100% de proteção por três horas contra mosquito *Ae. aegypti* e 100% de proteção contra o mosquito *Cx. quinquefasciatus* durante um período de doze horas.

O óleo essencial de cinco espécies de plantas: *Acorus calamus, Mentha arvensis, Ocimum basilicum, Saussrea lappa* e *citratos Cymbopogan* também foram investigados para avaliar o potencial larvicida, onde larvas de terceiro instar de *Aedes* e *Culex* foram expostas a diferentes concentrações e sua mortalidade foi avaliada após 24 horas sob condições de laboratório, no qual os resultados demonstraram que todos os óleos apresentavam mortalidade larval significativa, tanto para *Ae. aegypti* como para *Cx. quinquefasciatus* (FURTADO et al., 2005).

A atividade larvicida de extratos também vem sendo investigada com resultados promissores, como foi observado por Imam Zarnigar e Sofi (2014), que avaliaram atividade larvicida do extrato de éter de petróleo e álcool etílico de *Acorus calamus* frente às larvas de terceito e quarto instar do mosquito *Ae. aegypti.* Outrora, em pesquisas realizadas com a família anacardeaceae, espécie *Anacardicum ocidentale,* apresentou atividade larvicida contra o mosquito da dengue através do liquído da castanha do caju (GUISSONI et al., 2013).

Trabalhos com plantas oriundas do Brasil também tem sido testada para avaliação da atividade biológica, principalmente larvicida contra *Ae. aegypti*, como o extrato bruto hexânico de *Ottonia ansisum,* obteve 100% de mortalidade após 24 horas de exposição com 200 µg/mL, ao isolar o metabolito 1-butil-3,4-metilenodioxibenzeno do extrato, foi possível observar mortalidade de 100% das larvas com concentração de 30 µg/mL (MARQUES et al., 2017). Plantas como *Anacardium occidentalis, Cymbopogon winterianus*, *Copaifera langsdorffii*, *Ageratum conyzoides* e *Carapa guianensis* também já foram testadas contra *Ae. aegypti* obtendo resultados satisfatórios como larvicidas (DE MENDONÇA et al., 2005).

O extrato metanólico de *Spermacoce latifólia,* também foi testado para larvas de terceiro instar de *Ae. aegypti,* onde este apresentou uma toxicidade para larvas e uma CL_{50} de 0,625 mg/mL (-1), onde esta atividade foi associada a sua composição química pela presença de componentes fenólicos e flavonóides, indicando assim o seu potencial para o controle deste vetor (COSMOSKI et al., 2015).

1.3 *Acmella oleracea* (L.) R. K. JANSEN

Dentre várias espécies, destaca-se a *Acmella oleracea* (L.) R. K. Jansen popularmente conhecida como jambú, agrião-do-pará, agrião-bravo, agrião-do-brasil, botão-de-ouro ou jabuaçu (GILBERT; FAVORETO, 2010), pertence à família Asteraceae, esta família apresenta distribuição cosmopolita, sendo encontrada em todos os continentes, com exceção da Antártica. Várias dessas espécies são utilizadas como produtos alimentícios, na produção de cosméticos ou como plantas ornamentais (ROQUE; BAUTISTA, 2008).

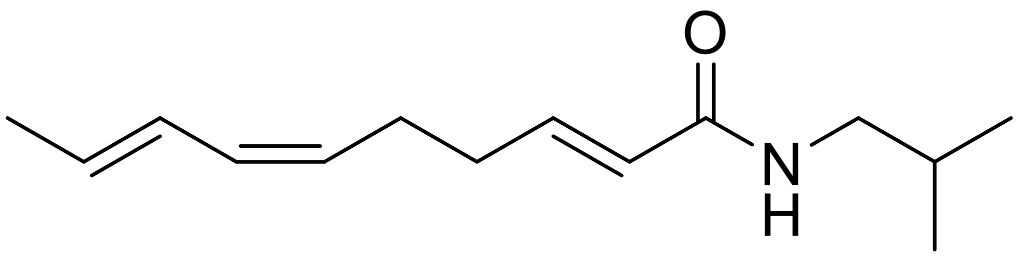
Esta espéce é encontrada em regiões tropicais próximas a linha do equador e a ausência de grandes populações selvagens sugere que esta planta não é nativa do Brasil, e sendo encontrada apenas em residências adjacentes em forma doméstica (GILBERT; FAVORETO, 2010).

Jambu é uma herbácea de pequeno porte com hastes rastejantes e ramificadas, com porte de 20-40 cm de altura, quase rasteira, com caule cilindro, carnoso e de ramos decumbentes, geralmente sem raíz nos nós (GILBERT; FAVORETO, 2010). Suas folhas são longo-pecioladas, de disposição oposta ovadas, dentadas e de ápice agudo. (CARDOSO; GARCIA; 1997, BARBOSA et al., 2016). Na região amazônica suas folhas e talos são utilizados na culinária local (CARDOSO; GARCIA, 1997), como também tem sido muito utilizada como remédio para a dor de dente, gripe, inseticida entre outras atividades.

Abeysiri et al. (2013) ao estudarem a composição fitoquímica das partes diferentes (folhas, flores e caule) de *A. oleracea* identificaram como principais compostos alcaloides, flavonoides, saponinas, glicosídeos esteroides e taninos. O óleo essencial das flores foi estudado por Baruah e Leclercq (1993), onde identificaram 20 constituintes através da cromatografia gasosa por espectrometria de massas, sendo o limoneno (23,6%), β-cariofileno (20,9%), (Z)-β-ocimeno (14,0%), germacreno D (10,8%) e merceno (9,5%) os compostos marjoritários.

A *A. oleracea* é uma espécie rica em isobutilamidas bioativas, onde a principal molécula desta espécie é o alcaloide *N*-isobutilamida do ácido (2*E*, 6*Z*, 8*E*)-deca-2,6,8-trienóico, conhecido como espilantol (GILBERT; FAVORETO, 2010). A presença desta substância e derivados faz a planta apresentar inúmeras aplicações em produtos farmacêuticos, alimentares e de saúde, sendo a atividade anestésica mais estudada (PANDEY; AGRAWAL, 2009).

Figura 2 - Estrutura Química do espilantol



Fonte: Autor

Considerando o potencial biológico do espilantol muitos trabalhos estão sendo desenvolvidos na busca por novas atividades biológicas que este composto ou outros presentes na espécie *A. oleracea* possam apresentar. Entre algumas dessas atividades podemos citar atividade inseticidade do extrato hexânico no controle de *Tuta absoluta* (Lepidoptera), onde espilantol foi isolado e demonstrou ser mais potente (MORENO et al., 2011). Atividade anti-inflamatória do espilantol também já foi descrita na literatura (WU et al., 2008; DIAS et al., 2012).

Estudos sobre atividade diurética das flores de *Spilanthes Acmella* foi estudada, apresentando resultados promissores, onde a ação diurética começou após 1 hora da admistração do extrato de aquoso, obtendo um efeito durante todo o período de estudo (RATNASOORIYA et al., 2004). Estudos voltados para impregnação do extrato de *A. oleracea* em filme para aplicação tópica na mucosa oral também obteve resultados positivos, considerando a eficácia anestésica observada (DE FREITAS-BLANCO et al., 2016).

Extrato etanólico de *A. oleracea* também foi testado para atividade acaricida contra *Amblyomma cajennense,* onde este apresentou mortalidade de 100% após um período de 72 horas de exposição e apresentando CL_{50} para os machos de 29,45 mg/mL e CL_{50} 17,63 para as fêmeas (ANHOLETO et al., 2017), este resultado também foi observado por Castro et al., (2014) contra *Rhipicephalus microplus* apresentando uma CL_{50} 79,7 mg/mL contra fêmas.

Estudo voltado para atividade antioxidante também vem chamando a atenção da classe científica (ABEYSIRI et al., 2013), estudos para inibição da enzima tirosinase também já foram realizados demonstrando que extrato hexano foi capaz de estimular atividade oxidante da enzima, demonstrando que este pode vir a ser utilizado para formulações de uso tópicos, podendo prevenir hiperpigmentação da pele (BARBOSA et al., 2016).

Considerando que certos compostos químicos provenientes de fontes naturais geralmente apresentam menor toxicidade para o meio ambiente e para o homem, que a Organização Mundial da Saúde recomenda o controle de vetores que transmitem doenças de importância para saúde pública, a espécie *A. oleracea* apresenta grande potencial para ser investigada, podendo ser apresentada como uma alternativa no mercado para o controle de vetores como *Ae. aegypti* e *Cx. quinquefasciatus*.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar atividade larvicida do extrato hidroetanólico de *Acmella oleracea* (L.) R. K. Jansen frente larvas de *Aedes aegypti* e *Culex quinquefasciatus.*

2.2 OBJETIVOS ESPECÍFICOS

a) Obter extrato hidroetanólico de *Acmella oleracea;*

b) Analisar composição fitoquímica do extrato por Cromatografia Gasosa – Espectroscopia de Massas (CG-EM);

c) Avaliar ação larvicida e estimar concentração letal (CL_{50} e CL_{90}) do extrato hidroetanólico de *Acmella oleracea* frente larvas de *Aedes aegypti* e *Culex quinquefasciatus;*

d) Avaliar ecotoxicidade do extrato contra um organismo não alvo, o fungo endofítico *Trichoderma ssp.*

3 ARTIGO 1

Submetido na revista*: South African Journal of Botany (ISSN: 0254-6299)*

LARVICIDAL EFFECT OF HYDROETHANOLIC EXTRACT FROM THE LEAVES OF *Acmella oleracea* L. R. K. Jansen IN *Aedes aegypti* and *Culex quinquefasciatus*

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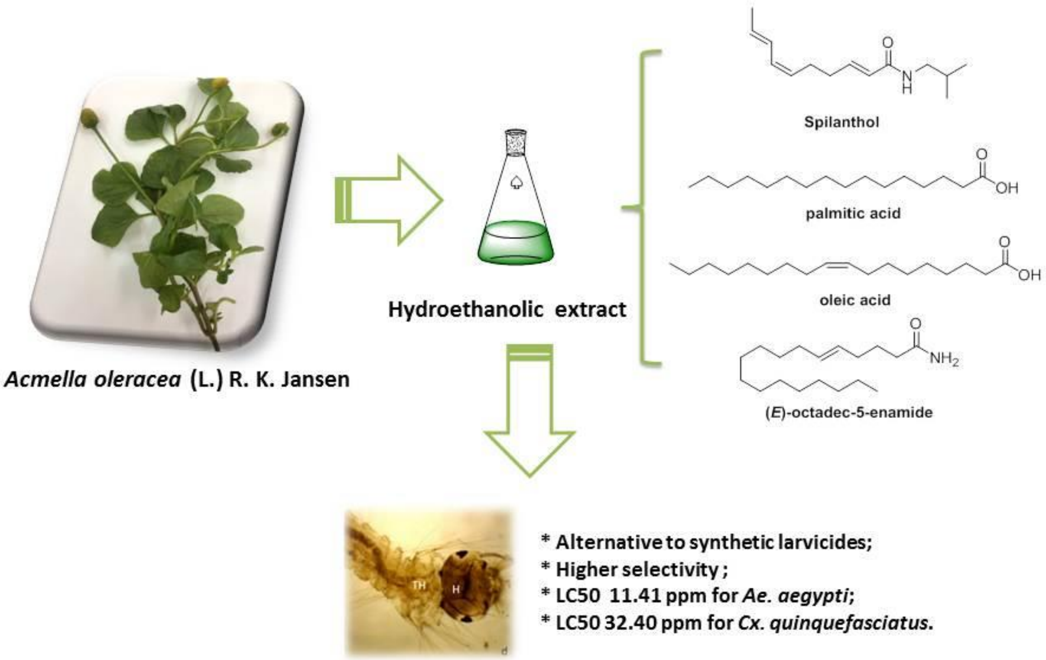
Highlights

- Larvicidal potential of hydroethanolic extract of leaves of *Acmella oleracea* against the *Aedes aegypti* and *Culex quinquefasciatus.*
- It was possible to determine the LC_{50} values of 11.41 ppm for *Ae. aegypti* and LC_{50} 32.40 ppm for *Cx. quinquefasciatus*.
- It the hydroethanolic extract from leaves of *Acmella oleracea* showed very low ecotoxicity.

Graphic Abstract



Abstract

Mosquitoes, such as *Aedes aegypti* and *Culex quinquefasciatus,* are important vectors of diseases, such as dengue fever, chikungunya fever, Zika virus, and filariasis, and these diseases are public health problems. The present study was carried out to evaluate the larvicidal activity of the hydroethanolic extract from leaves of *Acmella oleracea* leaves against 3rd instar larvae of the *Ae. aegypti* dengue vector and the *Cu. quinquefasciatus* filariasis vector. The hydroethanolic extract of caused significant mortality in *Ae. Aegypti* and *Cx. quinquefasciatus*. After 24 hours of exposure to the extract, it was possible to establish the LC_{50} values for the extract: 11.41 ppm for *Ae. aegypti* and LC_{50} 32.40 ppm for *Cx. quinquefasciatus*. It the hydroethanolic extract from leaves of *A. oleracea* showed very low ecotoxicity, for the strains of the fungus *Trichoderma sp.*, suggesting can be used without causing environmental damage. This is the first study that show the use of hydroethanolic extract from leaves of *A. oleracea* as an alternative to synthetic larvicides to eliminate larvae of *Ae. aegypti* and *Cx. quinquefasciatus* in an easy, cheap and safe way.

Keywords

Acmella oleracea; Jambu; Amazon biodiversity; Larvicidal activity;

1. Introduction

Mosquito-borne diseases are still of significant concern, considering the high mortality and morbidity of these diseases among underdeveloped and developed countries (Carvalho and Moreira, 2017). In Brazil, 925 people died due to diseases transmitted by *Aedes aegypti*, such as dengue, chikunguya and, more recently, Zika virus, in 2016 (Brazil, 2017); *Aedes aegypti* is the main vector of these diseases.

Culex quinquefasciatus is the main vector of *Wuchereria bancrofit*, an agent responsible for lymphatic filariasis, which is an important cause of acute and chronic morbidity (WHO, 1997; Brazil, 2011) and has recently been reported to have the potential for expressive transmission of the Zika virus through *Cx. pipiens quinquefasciatus* (Guo et al., 2016). The control of *Cx. quinquefasciatus* is based on breeding prevention measures and the elimination of breeding places through improvements in basic sanitation or the use of larvicides (WHO, 1997).

It is therefore clear that the best way to prevent these diseases is to control their vectors through the use insecticides and larvicides; however, the uncontrolled and rampant use of these products, directly or indirectly, generates resistant populations of mosquitoes and many of these substances used are considered harmful to the environment (Belinato and Valle, 2015, Nkya et al., 2013).

The search for alternatives to synthetic insecticides stimulates the development of new technologies. In the Amazon, due to its rich biodiversity, oils, extracts, or active constituents from certain plants are being exploited for their uses as bioactive products (Guissoni et al., 2013).

Among several species, *Acmella oleracea* (L.) R. K. Jansen, popularly known as jambu, stands out. It belongs to the Asteraceae family, a small herbaceous plant with creeping and branching stems (Cardoso and Garcia; 1997, Barbosa et al., 2016). Jambu leaves and stalks are used in local cuisine in the Amazon (Cardoso and Garcia, 1997).

Jambu is rich in bioactive isobutylamides; the majority compounds this species is the alkaloid (2*E*,6*Z*,8*E*)-*N*-Isobutyl-2,6,8-decatrienamide, known as spilanthol (Gilbert and Favoreto, 2010). The presence of this substance and its derivatives gives the plant potential in the pharmaceutical, food, and health industries, with its best-studied property being its anaesthetic activity (Pandey and Agrawal, 2009). Interestingly, it also has insecticidal activity via spilanthol against *Periplaneta americana* L. (Kadir et al., 1989), *Plutella xylostella* (Sharma et al., 2012), and *Tuta absoluta* (Moreno et al., 2012).

Thus, aiming to deepen the studies on natural products from the Amazonian biodiversity, the objective of this papes was to evaluate the larvicidal activity of the *A. oleraceae* hidroethanolic extract (of the leaves), popularly known as jambú, against *Ae. Aegypti* and *Cx. quinquefasciatus*.

2. Material and Methods

2.1 Plant and larvae

The leaves of the jambu were collected in March 2017 in the Fazendinha District (S º02'30.40/W 5106'37.5), Macapá-AP, Brazil. The species was identified by botanic Rosangela Sarquis of Federal University of Amapá and deposited in the Herbarium IAN of Embrapa Amazônia Oriental under numbering: 196011.

For the larvicidal test, we used third instar larvae of *Ae. aegypti* Rockefeller and *Cx. quinquefasciatus* Macapá strain from the Arthropoda Laboratory of the Federal University of Amapá. The assay was conducted under controlled conditions, with temperatures between 25 ± 2 °C, relative humidity of 75 ± 5%, and a photoperiod of 12 hours.

Figure 1. Image of the leaves of *A. oleracea* collected for later drying and production of the extract.

2.2 Preparation of the hydroethanolic extract of *Acmella oleracea*

The leaves of *A. oleracea* were dried at room temperature for 10 days, triturated, and stored. Subsequently, 74 g of crushed leaves were weighed and placed under maceration for 10 days using ethyl alcohol (70%) (1.5 L) as the solvent, and then the solution was filtered and excess solvent was subjected to rotary evaporation under reduced pressure and thereafter lyophilized.

2.3 Gas chromatography-mass spectrometry (GC-MS)

We evaluated the samples using a gas chromatograph (GCMS-QP 2010) equipped with an auto-sampler injection systems (AOC-20i, Shimadzu). The following settings were used: electron impact detection (Shimadzu MS2010 Plus), electronic impact of 70 eV, and fragments detected from 50-400 Da. Separations were performed on a fused silica capillary column (RTX-5MS with i.d. = 0.25 mm, length = 30 m, and film thickness = 0.25 µm) in a stream of helium (1.03 mL/min). The sample was solubilized in dichloromethane (2 µg/mL) and 1.0 µL of the solution was subjected to the following experimental conditions: injector temperature, 210 °C; detector temperature, 250 °C; carrier gas, helium; flow rate, 3.0 mL/min; and split injection with a split ratio of 1/10. The column temperature was programmed from 80 °C, with an increase of 6 °C/min, to 250 °C, ending with a 5-min isothermal step at this temperature; the total analysis time was 35.33 min.

2.4 Larvicidal activity

The extract were dissolved in dimethylsulphoxide (DMSO5) at different concentrations (2.5, 5, 7.5, 10 and 15 ppm) for *Ae. aegypti* and at (5, 10, 20, 30 and 40 ppm) for *Cx. quinquefasciatus*. Five replicates were carried out with ten larvae each. Negative controls contained distilled water containing the same amount of DMSO (1%) present in the respective test sample. The larval mortality rate was determined after 24 h of incubation. Larvae were considered dead when they did not respond to stimuli or did not rise to the solution surface, in contrast to those observed in the control. The bioassay experiments were conducted according to the WHO standard (2005).

2.5 Morphological study of larvae

After treatment, larvae were fixed in formalin (10%) and the external morphology was analysed under light microscopy (Output DC 6V/20W) and photographed with a digital camera (MDCE-SC USB 2.0) with scopelmage 9.0 software.

2.6 Isolation of the filamentous fungus *Trichoderma ssp*

The fungus used in this study was obtained from the Brazil nut (*Bertholletia excelsa*). To obtain the isolates, the malt extract medium (2%) was treated with the antibiotic chloramphenicol. Isolates from Brazil nut urchins were obtained by surface scraping of the structures of the microorganisms. After the isolation procedure, the petri dishes containing the isolate were transferred to a BOD greenhouse incubator with an adjusted photoperiod of 12 hours and temperature of 31 ± 1 °C. After 7 days of incubation under the conditions described above, the colony and morphological structure (conidiophores and conidia) of the isolate were evaluated for the identification of *Trichoderma* at the genus level, based on the morphological keys of the sections and species developed by Gams and Bisset (1998).

2.7 Activity of *Trichoderma ssp*

The fungi were grown in solid BDA medium, where 20 g of agar and 20 g of dextrose were weighed and added to distilled water and potato broth. The pH of the medium was corrected to 7.0 with the aid of NaOH (0.1 M) and HCl (0.1 M). After autoclaving, 32.40 ppm the solution of the jambu extract was impregnated in the culture medium; DMSO (1%) was used as a control. The test was performed in triplicate. Growth was observed at 24, 48, 72, and 96 hours. Inhibition of mycelial growth (PIC) was calculated by Equation: (%) PIC = RGC – RGT/RGC × 100, where RGC = radial growth of control (cm) and RGT = radial growth of treatment (cm) (Vale et al. 2011).

2.8 Statistical analysis

Lethal concentrations (LC_{50} and LC_{90}) were determined after 24 h of incubation and calculated using Probit analysis with StatGraphic Centurium XV software, version 15.2.11. If the control mortality of the treated groups was between 5 – 20 %, the analysis was corrected according to the WHO (2005) formula: mortality (%) = X − Y/X × 100, where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

3 Results

3.1 Gas chromatography-mass spectrometry (GC-MS)

The presence of four major substances was observed by gas chromatography (Figure 2). Among the compounds found in the extract, the majority was spilanthol (**1**), followed by linoleic acid (**3**), and palmitic acid (**2**), respectively in the forms of ethyl ester and octadecanamide (**4**).

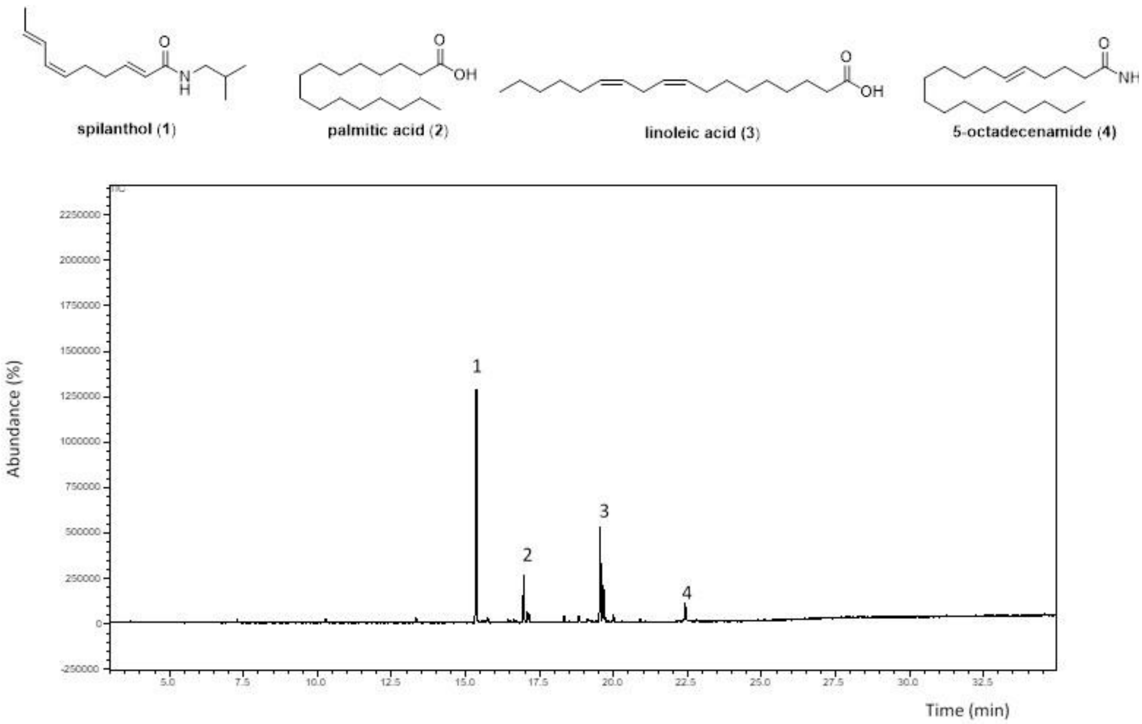


Figure 2. Major compounds of the hydroethanolic extract from leaves of the *A. oleracea.*

3.2 Larvicidal activity of *Acmella oleracea* extract

The larvicidal bioassays for *Ae. aegypti* showed 58% mortality in 24 hours at a concentration of 15 ppm and 18% at the lowest concentration (2.5 ppm) (**Figure 3**). This demonstrates a promising result, considering that the concentrations are low.

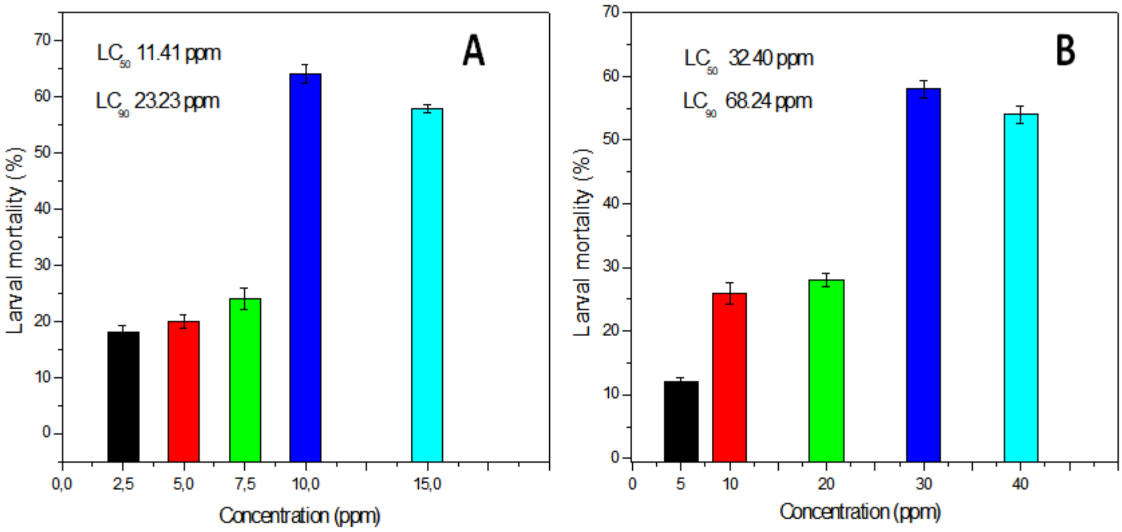


Figure 3. Larvicidal activity of *Acmella oleracea* hydroethanolic extract in *Aedes aegypti* (**A**) and *Culex quinquefasciatus* (**B**) larvae at 24 h.

In the tests for *Cx. quinquefasciatus*, no mortality was observed for the concentration of 15 ppm, in this way the concentration was increased to 40 ppm where it was possible to observe the mortality 54% after the period of 24 hours in exposure to the extract and of 12% for the (5ppm) in the same period.

The LC_{50} and LC_{90} values for *Ae. aegypti* in 24 hours were 11.41 ppm and 23.23 ppm, respectively (Table 1), with a *p* value <0.05 (0.0124). The LC_{50} and LC_{90} values for *Cx. quinquefasciatus* were 32.40 ppm and 68.24 ppm, respectively, with a *p* value <0.05 (0.0148), thus demonstrating that the higher the concentration, the greater the mortality and efficiency of the extract of *A. oleracea* for larval control.

Table 1. Larvicidal activity (LC_{50} and LC_{90}) for *Aedes aegypti* and *Culex quinquefasciatus* in 24.

A. oleracea	***Aedes aegypti***	***Culex quinquefasciatus***
	24 h	24 h
LC_{50}*	11.41 ppm	32.40 ppm
C.I	7.98±26.27 ppm	22 ± 84.67 ppm
LC_{90}*	23.23 ppm	68.24 ppm
C.I	16.11±86.44 ppm	46.15 ± 284 ppm

* LC50 and LC90 in ppm. C.I. = confidence interval

3.3 Analysis of larval morphology after 24h

In the obtained optical microscope images, the larvae of *Ae. aegypti* (**Figure 3a-c**) have normal morphological segments (head (H), thorax (TH), and abdomen (AB) and no changes were observed in their cuticle, respiratory tract (S), or anal papillae. However, the larvae treated with 15 ppm of extract showed, after 24 hours (Figure 4d-f), a discoloration that started from the thorax and continued to the end of the abdomen.

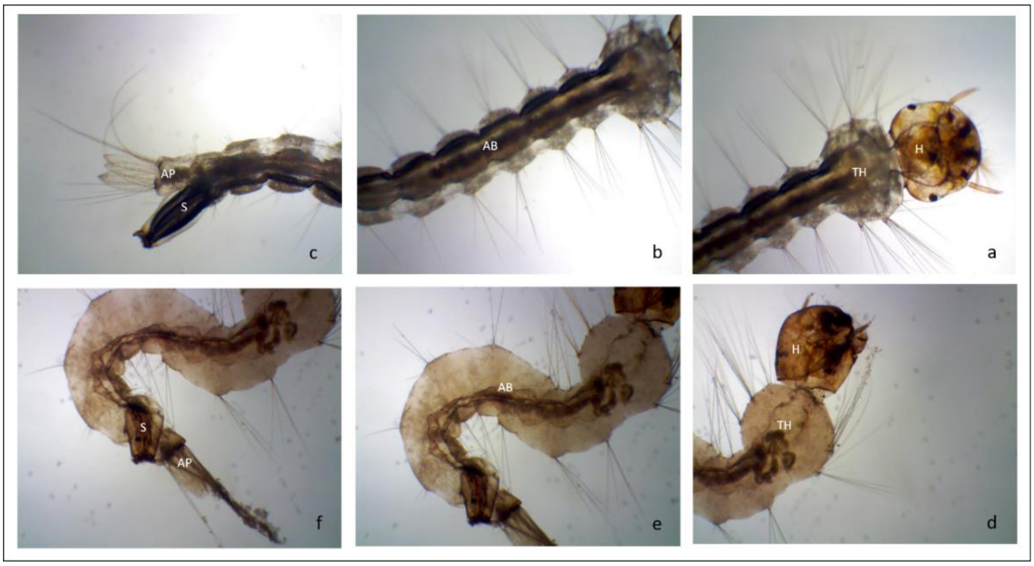


Figure 4. Optical microscope images of *Aedes aegypti* larvae. Control (a-c) not showing changes, head (H), thorax (TH), abdomen (AB), respiratory siphon (S) and papilla anal (AP). Treatment with extract at 15 ppm after 24h exposure (d-f).

When observing the larvae of *Cx. quinquefasciatus* (**Figure 5a-f**), it is possible to verify that both the larvae in the control and in the treatment did not present alterations in the external structure, making it possible to observe the division of the segments, the presence of the bristles, and the siphon and papilla without apparent changes.

Figure 5. Optical microscope images of *Culex quinquefasciatus* larvae. It was submitted to *Acmella oleracea* extract at 40 ppm (d-f), head (H), thorax (TH), abdomen (AB), respiratory siphon (S) and anal papilla (AP). Control (a-c) with no changes.

3.4 Activity Fungus *Trichoderma* ssp

The study showed low values for %PIC 1.78. This result indicat that of the hydroethanolic extract from leaves of *A. oleracea* no induced significant toxic responses on test-microorganisms, fungus *Trichoderma* ssp, up until of 96 h for concentration of 32.40 ppm.

4 Discussion

The compound (2*E*,6*Z*,8*E*)-*N*-Isobutyl-2,6,8-decatrienamide (spilanthol) was identified as the major substance present in the hydroethanolic extract of jambú leaves. The identification of espilantol by electron impact (70 eV) showed the appearance of two characteristic fragmentation signals, resulting from hemolytic C-C bond cleavage, at m/z = 81 (100%) and m/z = 141 (72%) (Hiserodt et al., 2004).

In this study, the hydroethanolic extract of *A. oleracea* showed an LC_{50} of 11.41 ppm after 24 hours for *Ae. aegypti*, unlike the results described by Simas et al. (2013), who studied the crude ethanolic extract of the leaves of *A. oleracea* and observed an LC_{50} value of 251 ppm overall and an LC_{50} value of 145 ppm in the hexane partition. Notably, the chemical composition of the metabolites can be influenced by the development site, seasonality, age, temperature, water stress, ultraviolet radiation, mechanical factors, and pathogen attack of the product (Gobbo-Neto and Lopes, 2007).

The hydroethanolic extract of the leaves of *A. oleracea* collected in the district of Fazendinha - Macapá Brazil, was effective for killing the larvae of *Ae. aegypti*, required only a low dose to be effective, was easy to prepare, and was cheap. It is noteworthy that the presence of the 2*E*-type unsaturated bonds present in alkanoamines are associated with insect toxicity (Jacobson, 1954).

In contrast, other studies reported insecticidal activity from *Clausena anisata* extract with an LC_{50} value of 59.65 ppm in 24 hours (Mukandiwa et al., 2015), *Xanthium strumarium* seed extract with an LD_{50} of 531.07 ppm against *Ae. caspius* and 502.32 ppm for *Cx. pipiens* larvae (Mekhlafi et al., 2017), and methanolic extract from the leaves of *Crataeva magna* with LC_{50} values of 121.69, 132.09, and 147.27 ppm for *Anophelles stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*, respectively, at 24 hours of exposure (Veni et al., 2016).

The insecticidal activity of spilanthol isolated from the *Spilanthes acmella* extract for *Periplaneta americana* L. showed high activity against the adults of this species, with an LD_{50} value of 2.46 ppm; electrophysiological experiments suggested that spilanthol interferes in the nervous system (Kadir et al., 1989). A study of the *Spilanthes acmella* flower head extract also showed activity against the 2nd instar larvae of *Plutella xylostella*, presenting LC_{50} values of 1.49, 5.14, and 5.04 ppm for espilanthol, the hexanic extract, and the methanolic extract, respectively (Sharma et al., 2012).

In the study by Pandey et al. (2007), the hexanic extract of the flowers of *Spilanthes Acmella* L. var. Oleraceae Clarck showed variable mortality for the larvae of three species of three species of vectors: *Anopheles stephensi*, *An. culicifacies*, and *Cx. quinquefasciatus*, with LC_{50} values of 4.57 ppm, 87 ppm, and 3.11 ppm, respectively.

In the tests carried out in this work, the LC_{50} value for *Cx. quinquefasciatus* was 32.40 ppm; however, the solvent used and the part of the plant used to determine this activity were different in both works, and therefore the larvicidal actions of the extracts are more difficult to compare. It is also worth mentioning that ethanol was used as the solvent in this work and not hexane; ethanol is less toxic to the environment than hexane, relatively easy to access, less volatile, and, consequently, safer to handle.

Soonwera and Phasomkusolsil (2016), when studying the effect of the oils of *Cymbopogon citratus* and *Syzygium aromaticum* on the morphology of *Ae. aegypti* and *Anopheles dirus*, observed morphological alterations in the larvae in comparison to the control, where they presented deformations in the neck and stretching, as well as loss of siphon; in this work, it was also possible to observe changes in *Ae. aegypti*, where there was a loss in the clarity of the segmentation when compared to the control and a discoloration in the cuticle, suggesting that substances present in the extract may interact with chitin. However, Valotto et al. (2010), did not identify external morphological alterations in the larvae but instead observed the expulsion of the peritrophic matrix to the external environment, containing all of the food, as a means of eliminating the larvicidal substance.

Morphological studies in the larvae of *Cx. quinquefasciatus* also demonstrated alterations caused by a nanoemulsion of *Pterodon emarginatus* in the abdomen, thorax, and anal papillae (Oliveira et al., 2017); however, this effect was not observed in our results, suggesting that the mortality of *Cx. quinquefasciatus* is not related to external damage of the integument. Studies have shown that secondary metabolites with insecticidal effects may act in different ways, such as inhibiting feeding, regulating growth, or acting on the neuroendocrine system and interfering with tegument exchange and/or metamorphosis (Menezes, 2005; Maciel et al., 2010).

Endophytic fungi that live in association with plants without inducing any visible symptoms of pathogenicity (Dastogeer et al., 2017) may associate with the roots and produce interesting metabolites with applications in agriculture, industry, and pharmaceuticals (Souza et al., 2004).

The study indicate too that hydroethanolic extract from leaves of jambu is an optimal source of nitrogen or carbon for culture of fungus *Trichoderma* ssp about boratory incubation condition (pH 7 and 31 ± 1 °C), however field studies would be required to confirm whether the degradation of the jambu extract would be the same, enhanced or reduced in the real situation.

The non-toxicity of the hydroethanolic extract from leaves of jambu for the fungus *Trichoderma ssp*. Is promising since it is an endophytic and cosmopolitan fungus, can be present in the soil, and presents as a bioprotector, promoting growth and relieving the biotic and abiotic stress of plants (Mastouri et al., 2010). *Trichoderma harzianum* are also aids in the growth of plants in saline environments, which have higher water content and better photosynthetic performance (Yasmeen e Siddiqui, 2017). Therefore, it is suggested that the hydroethanolic extract from sheets of *A. oleracea* has no toxicity and can be used without damage to the environment.

The hydroethanolic extract of *A. oleracea*, against the larvae of the *Ae. aegypti* and *Cx. quinquefasciatus*, showed a significant result when compared to the adopted literature, mainly because it is a compound of the chemical derivatives and not only of an isolated product.

We must emphasize, however, that the chemical specificity of the species used is directly influenced by the seasonality, leading to the biochemical changes of the main metabolites, both constituent and extractable, as well as the extraction method.

5. Conclusions

It is concluded that the hydroethanolic extract from leaves of jambu was more toxic to *Aedes aegypti* larvae (LC_{50} 11.41 ppm); consequently, higher selectivity was suggested in the studied concentrations when compared to the effects on *Culex quinquefasciatus* larvae (LC_{50} 32.40 ppm). This is the first study that show the use of hydroethanolic extract from leaves of *A. oleracea* as an alternative to synthetic larvicides to eliminate larvae of *Ae. aegypti* and *Cx. quinquefasciatus* in an easy, cheap and safe way.

It was observed that the hydroethanolic extract from leaves of jambu has no toxicity and can be used without causing environmental damage.

Conflict of interest statement

We declare that we have no conflict of interest.

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4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Este trabaho abriu perspectivas quanto ao uso de *Acmella oleracea* para atividade biológica larvicida para espécie de *Aedes aegypti* cepa rockefeller e *Culex quinquefasciatus.* O extrato hidroetanólico 70% foi mais tóxico para *Ae. aegypti* com CL_{50} 11,41 ppm, do que para *Cx. quinquefasciatus* que apresentou CL_{50} 32,40. A composição química do extrato apresentou quatro compostos marjoritários ácido palmistico, ácido linoléico, 5-octadecanamida e espilantol, onde estes podem estar relacionada a atividade larvicida apresentada pelo extrato.

O mesmo não apresentou toxicidade para fungos *Trichoderma ssp,* sugerindo assim que o extrato hidroetanólico de Jambu pode ser utilizado como um meio de controle para estes vetores sem danos ao meio ambiente.

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Anexo 1 - Normas de publicação dos respectivos periódicos

INTRODUCTION

Official Journal of the South African Association of Botanists (http://www.sabotany.com) The South African Journal of Botany publishes original papers that deal with the classification, biodiversity, morphology, physiology, molecular biology, ecology, biotechnology, ethnobotany and other botanically related aspects of plants.

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Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Any species or infraspecific taxon studied is to be referenced against appropriate literature used to identify the material concerned. Give full scientific name(s) of plant(s) used, as well as cultivar (cv.) or variety (var.) where applicable. All growth conditions should be properly described. Sufficient detail of the techniques used should be provided to allow easy repetition.

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Results should be clear and concise. Do not include material appropriate to the Discussion.

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If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

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Acknowledgements

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