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EXTRATO DE FLORES DE *Moringa oleifera*: ATIVIDADE LARVICIDA E EFEITO SOBRE TRIPSINA E ACETILCOLINESTERASE DE LARVAS DE *Aedes aegypti*

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RECIFE

AGOSTO, 2010

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Orientadora: Profa. Dra. Patrícia Maria Guedes Paiva

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"Extrato de flores de *Moringa oleifera*: atividade larvicida e efeito sobre tripsina e acetilcolinesterase de larvas de *Aedes aegypti*"

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“Cada um de nós compõe a sua história e cada ser em si carrega o dom de ser capaz, de ser feliz” (Almir Sater e Renato Teixeira)

RESUMO

Dengue é uma arbovirose transmitida pelo *Aedes aegypti* e o controle do mosquito é fundamental para reduzir a propagação da doença. As larvas de *A. aegypti* têm desenvolvido resistência a inseticidas organofosforados. O uso de compostos naturais que promovam mortalidade pode evitar a emergência de larvas resistentes, devido à rotatividade dos inseticidas. Este trabalho relata a atividade larvicida (CL_{50} de 0.925%, p/v) do extrato de flores de *Moringa oleifera* sobre o quarto instar larval (L4) de *A. aegypti.* Inibidor de tripsina de natureza protéica (MoFTI, 169,9 kDa, K_i: 0,38 nM), triterpeno (β-amirina), esteróide (β-sitosterol) e flavonóides (kaempferol e quercetina) foram detectados no extrato; lectina não foi detectada. Tripsina do extrato do intestino de L4 foi inibida por MoFTI (K_i: 0,6 nM); entretanto, a atividade de acetilcolinesterase (AChE) do extrato de L4 inteiras não foi alterada. Ensaio em condições *in vivo* mostrou que a atividade de tripsina do intestino de L4 tratadas com o extrato de flores de *M. oleifera* diminuiu ao longo do tempo (0 a 1440 min) e foi fortemente inibida (98,6 %) após 310 min de incubação; a atividade de AChE do extrato de L4 inteiras não foi afetada neste período. O estudo aponta o extrato de flores de *M. oleifera* como uma ferramenta biodegradável para o controle de larvas de *A. aegypti* e sugere que o mecanismo larvicida envolve a inibição da tripsina do intestino das L4 por MoFTI.

Palavras-chave: *Moringa oleifera*; *Aedes aegypti*; atividade larvicida; inibidor de tripsina; tripsina; acetilcolinesterase.

ABSTRACT

Dengue Fever is an arboviruses transmitted by *Aedes aegypti* and mosquito control is fundamental to reduce disease spreading. *A. aegypti* larvae have developed resistance to organophosporous insecticides and the use of natural compounds that promote mortality may avoid emergence of resistant larvae due to rotation of insecticides. This work reports the larvicidal activity (LC_{50} of 0.925% w/v) of *Moringa oleifera* flower extract on fourth larval instars (L4) of *A. aegypti*. Proteinaceous trypsin inhibitor from *M. oleifera* flower (MoFTI, 169.9 kDa, K_i: 0.38 nM), triterpene (β-amyrin), sterol (β-sitosterol) and flavonoids (kaempferol and quercetin) were detected in the extract; lectin was absent. Trypsin from L4 gut extract was inhibited by MoFTI (K_i: 0.6 nM), however acetylcholinesterase (AChE) activity from total L4 extract was not altered. *In vivo* assay showed that gut trypsin activity from L4 treated with *M. oleifera* flower extract decreased along the time (0 to 1440 min) and was strongly inhibited (98.6 %) after 310 min incubation; AChE activity from total L4 extract was not affected in this period. The study points out *M. oleifera* flower extract as a biodegradable tool for *A. aegypti* larvae control and suggests that larvicidal mechanism involves inhibition of L4 gut trypsin by MoFTI.

Keywords: *Moringa oleifera*; *Aedes aegypti*; larvicidal activity; trypsin inhibitor; gut trypsin; acethylcholisnesterase.

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AChE – Acetilcolinesterase

AH – Atividade hemaglutinante

BApNA - N-benzoil-DL-arginil-ρ-nitroanilida

Bti – *Bacillus turingiensis* var. *israelensis*

ClaveLL – Lectina do líquen *Cladonia verticillaris* (do inglês *Cladonia verticillaris Lichen Lectin*)

cMoL – Lectina coagulante de *Moringa oleifera* (do inglês *coagulant M. oleifera lectin*)

DDT – Dicloro difenil tricloro etano

DTNB – Ácido 5,5'-dithiobis-(2-nitrobenzóico)

L1, L2, L3 e L4 – Primeiro, segundo, terceiro e quarto ínstares larvais de *Aedes aegypti*

MoFTI – Inibidor de tripsina de flores de *Moringa oleifera* (do inglês *Moringa oleifera flower trypsin inhibitor*)

MuBL – Lectina de entrecasca de *Myracrodruon urundeuva* (do inglês *Myracrodruon urundeuva bark lectin*)

MuHL – Lectina de cerne de *Myracrodruon urundeuva* (do inglês *Myracrodruon urundeuva heartwood lectin*)

MuLL – Lectina de folha de *Myracrodruon urundeuva* (do inglês *Myracrodruon urundeuva leaf lectin*)

OMS – Organização Mundial de Saúde

PMSF – Fluoreto de Fenilmetilsulfonil

SDS-PAGE – Eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio (do inglês s*odium dodecyl sulphate polyacrylamide gel electrophoresis*)

TLC – Cromatografia em camada delgada (do inglês *thin layer chromatography*)

WSMoL – Lectina solúvel em água de *M. oleifera* (do inglês *water-soluble M. oleifera lectin*)

SUMÁRIO

1. INTRODUÇÃO

Cerca de 40% da população mundial encontra-se hoje em risco de dengue, doença infecciosa comum em regiões tropicais e subtropicais; o agente etiológico, o arbovírus DENV, existe em quatro sorotipos distintos (HUHTAMO *et al*., 2008; OMS, 2009). A dengue é transmitida principalmente através da picada de fêmeas do mosquito *Aedes aegypti* (SILVA *et al*., 2008); o controle de populações do inseto é de grande importância desde que não existe ainda vacina contra a doença (OMS, 2009).

O uso de inseticidas tem induzido a seleção de linhagens de mosquitos resistentes, o que tem impulsionado a busca por inseticidas naturais isentos de toxicidade ao meio ambiente (BRAGA e VALLE, 2007; SILVA *et al*., 2008). Atividade inseticida contra *A. aegypti* tem sido descrita para compostos de plantas como os metabólitos secundários e as proteínas (BROUSSALIS *et al*., 2010; OCHIENG *et al*., 2010; OLIVEIRA *et al*., 2010; PRASAD *et al*., 2010).

O ciclo de vida do mosquito *A. aegypti* envolve as fases de ovo, larva (quatro instares: L1, L2, L3 e L4), pupa e adulto; tripsina e enzimas com atividade semelhante estão presentes em todas as fases do ciclo do mosquito, embora com diferentes níveis de expressão de acordo com o estágio de desenvolvimento (BOROVSKY e MEOLA, 2004). A tripsina, devido à sua presença indiscriminada e envolvimento nos processos de digestão em insetos, é sugerida como interessante alvo de agentes inseticidas (HILDER *et al*., 1987; BROADWAY, 1995).

Os inibidores de tripsina são capazes de induzir a morte de insetos por inibir a atividade catalítica da enzima, prejudicando a digestão de alimentos de natureza protéica e, conseqüentemente, reduzindo a biodisponibilidade e absorção dos nutrientes (HILDER *et al*., 1987; BROADWAY, 1995; CARLINI e GROSSI-DE-SÁ, 2002; BHATTACHARYYA *et al*., 2007a, 2007b). Atividade inseticida de inibidores de tripsina tem sido descrita; larvas

alimentadas com ração contendo inibidores de proteases podem apresentar perda de peso, atraso no desenvolvimento e morte por inanição (CARLINI e GROSSI-DE-SÁ, 2002; BHATTACHARYYA *et al*., 2007a, 2007b).

Moringa oleifera, planta nativa da Índia, tem despertado interesse devido ao seu potencial na indústria e medicina (MAKKAR e BECKER, 1996; MCCONNACHIE *et al*., 1999; FOIDL *et al*., 2001; KARADI *et al*., 2006). As sementes de moringa apresentam moléculas com atividade coagulante e são capazes de promover clarificação em águas turvas. As flores apresentam atividade antioxidante, devido à presença de α e γ-tocoferol, e antimicrobiana, devido à presença do alcalóide pterigospermina (MAKKAR e BECKER, 1996; GUEVARA *et al*., 1999; SÁNCHEZ-MACHADO *et al*., 2006; ONG, 2008).

Coelho *et al*. (2009) relataram que extrato aquoso de sementes de *M. oleifera* atrasou o desenvolvimento de larvas de *A. aegypti*, bem como apresentou atividade larvicida contra L4. A busca por substâncias naturais e biodegradáveis com propriedades inseticidas justifica a investigação do efeito larvicida do extrato de flores de *M. oleifera* sobre *A. aegypti*.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Dengue

A dengue é uma doença infecciosa que ocorre em regiões tropicais e subtropicais (Figura 1); nas décadas recentes, a dengue tem se tornado um importante problema de saúde pública internacional desde que aproximadamente 2,5 bilhões de pessoas, ou seja, dois quintos da população mundial, vivem em áreas de risco de transmissão da doença. Cerca de 50 milhões de casos são registrados mundialmente a cada ano, o que caracteriza a dengue como uma pandemia (OMS, 2009).

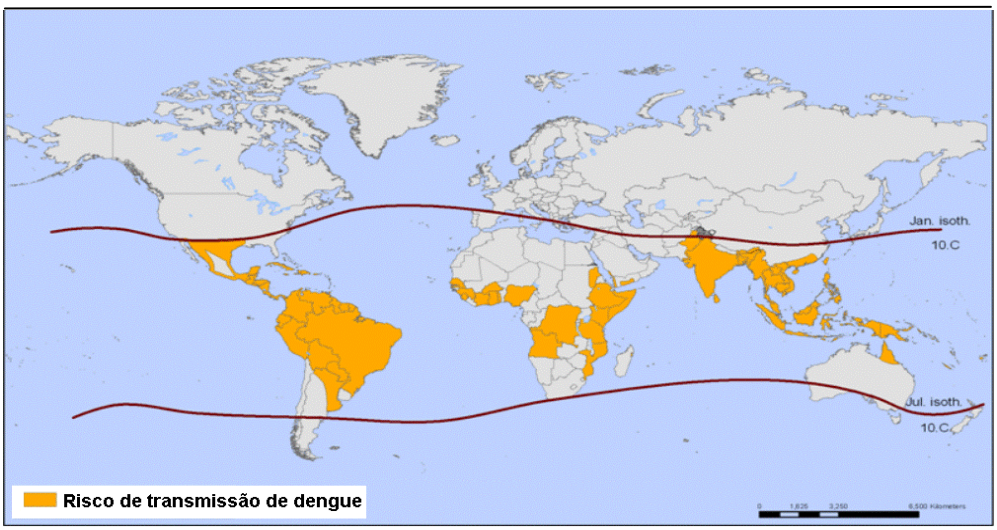


Figura 1. Países com risco de transmissão de dengue.

Fonte: OMS (2006)

O agente etiológico da dengue é um arbovírus pertencente ao gênero *Flavivirus* (família Flaviviridae) que ocorre em quatro sorotipos: DENV-1 a DENV-4 (HUHTAMO *et*

al., 2008). A dengue é transmitida pela picada de mosquitos do gênero *Aedes*, tendo sido descrita a ocorrência do vírus nas espécies *A. aegypti*, *A. albopictus* e *A. polynesiensis*, pertencentes ao subgênero *Stegomyia* (ROBHAIN e ROSEN, 1997). Devido à não existência de uma vacina contra a dengue, o controle de populações do mosquito vetor é fundamental para prevenir a transmissão da doença (OMS, 2009).

Segundo a Organização Mundial de Saúde (OMS), a dengue pode afetar crianças, adolescentes e adultos, raramente levando à morte (OMS, 2009). Os sintomas aparecem de 3 a 15 dias após a picada pelo inseto infectado e envolvem dor de cabeça, febre alta, forte dor no corpo e, em alguns casos, vômito. É frequente a ocorrência de manchas avermelhadas na pele (semelhantes ao sarampo ou rubéola) que aparecem 3 a 4 dias após o início da febre, acompanhadas de prurido; pessoas que desenvolvem a forma severa da doença (dengue hemorrágica) podem apresentar uma excessiva permeabilidade capilar responsável pelo sangramento de gengivas (gengivorragia), nariz (epistaxe), sangramento gastrointestinal, hematúria (sangue na urina) e aumento do fluxo menstrual (http://www.cives.ufrj.br/informacao/dengue/den-iv.html). As hemorragias variam de intensidade e podem desencadear choque por diminuição da pressão sanguínea seguido de morte (HUBERT e HALSTEAD, 2009).

2.2 O mosquito *Aedes aegypti*

A. aegypti (Diptera: Culicidae), cujo nome significa “o indesejável do Egito”, é originário da África, de onde migrou para as Américas e Ásia. O *A. aegypti* é o principal vetor da dengue e febre amarela e foi provavelmente introduzido no Brasil na metade do século XIX, através de navios negreiros (SILVA *et al*., 2008).

Medindo menos de 1 cm, o *A. aegypti* possui coloração preta com listras brancas no corpo e nas pernas. Seu ciclo de vida compreende os estágios de ovo, larva, pupa e adulto (Figura 2). A fase larval de *A. aegypti* compreende quatro instares denominados L1, L2, L3 e L4 (SILVA *et al*., 2008).

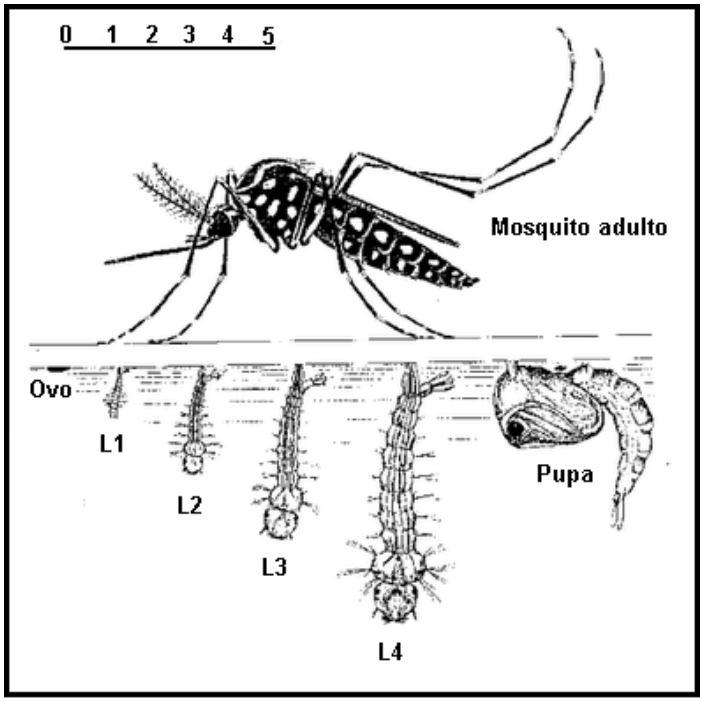


Figura 2. Ciclo biológico do mosquito *A. aegypti*.

(Fonte: Secretaria de Saúde e Defesa Civil do Estado do Rio de Janeiro, 2007).

Estudos desenvolvidos com populações de *A. aegypti* oriundas de quatro regiões bioclimáticas da Paraíba (municípios de Remígio, Boqueirão, Brejo dos Santos e Itaporanga) mostraram a duração média de cada fase do ciclo (BEZERRA e CASTRO JR., 2008). Com pequena variação, os quatro dias iniciais correspondem ao período de desenvolvimento embrionário (ovo) após os quais ocorre a eclosão. O primeiro instar larval dura de 1,4 a 2 dias passando para L2. A muda que origina o terceiro instar ocorre entre 1 e 2 dias após a emergência de L2. A fase de L3 dura 1 dia ao fim do qual aparece o último instar (L4) que

pode durar de 2 a 3 dias até se desenvolver em pupa. Esta ultima pode permanecer cerca de 2 dias após os quais o vetor da dengue atinge a fase reprodutiva, o mosquito adulto; os autores verificaram que os adultos podem viver de 42 a 44 dias (machos) ou até 46 dias (fêmeas).

A espécie *A. aegypti* alimenta-se de seiva vegetal, contudo, após o acasalamento, as fêmeas desenvolvem o hábito hematofágico desde que existem proteínas no sangue que são extremamente necessárias para o desenvolvimento dos ovos. A picada do mosquito ocorre durante as primeiras horas do dia e as últimas da tarde e não causa dor e nem coceira (SILVA *et al*., 2008).

As enzimas digestivas mais importantes presentes no intestino de *A. aegypti* em todas as fases de seu ciclo biológico são a tripsina e a quimotripsina, ambas muito expressas nas fases de larva e pupa (YANG *et al*., 2003). Segundo Venancio *et al*. (2009), existem pelo menos 66 genes em *A. aegypti* que codificam tripsinas: 12 em larvas, 15 em adultos e 39 em ambos; as tripsinas expressas apenas em larvas ou em adultos diferem entre si e, segundo os autores, isso resulta provavelmente da emergência do hábito hematofágico após a maturidade sexual.

2.3 Controle do *A. aegypti*

Inseticidas químicos têm sido bastante utilizados em programas de controle de doenças transmitidas por vetores; no entanto sabe-se que possuem alta toxicidade ao ambiente (BRAGA e VALLE, 2007). Os organoclorados e piretróides mantêm abertos os canais de sódio das membranas de neurônios e os organofosforados e carbamatos atuam como inibidores da acetilcolinesterase (AChE), enzima que catalisa a hidrólise da acetilcolina nas sinapses colinérgicas após a propagação do impulso nervoso. A fosforilação da AChE pelo organofosforado leva à inibição irreversível da enzima e promove a morte do inseto por

paralisia devido a contrações musculares intermináveis. O combate às larvas de *A. aegypti* tem sido feito principalmente pela utilização do organofosforado temefós; a genotoxicidade e mutagenicidade do temefós, em concentrações similares àquelas utilizadas no combate ao *A. aegypti*, foram descritas (AIUB *et al*., 2002).

O controle biológico dos insetos tem sido realizado pela utilização de invertebrados aquáticos ou larvas de peixes que se alimentam de insetos nas fases larvais e de pupa ou ainda utilizando fungos ou bactérias patogênicas como o *Bacillus thuringiensis* (Bti). No Brasil, o Programa Nacional de Controle da Dengue já utiliza o Bti (Fundação Nacional de Saúde, 2002). Dois mecanismos envolvidos no controle pelo Bti são sugeridos: A) a digestão de endotoxinas produzidas durante a esporulação da bactéria libera no intestino do inseto derivados com ação larvicida e B) interação entre glicoconjugados da membrana peritrófica do intestino dos insetos e endotoxinas que possuem domínios com atividade de lectina (proteína ligadora de carboidratos) prejudica os processos de digestão e absorção, levando as larvas à morte (GILL *et al*., 1992; BURTON *et al*., 1999). Araújo *et al*. (2007) avaliaram a atividade larvicida do Bti contra *A. aegypti*; tabletes contendo 15% (p/p) de esporos e cristais de endotoxinas causaram 100 % de mortalidade de L1 e L4.

2.4 Resistência a inseticidas

Populações de insetos consistem em uma mistura de indivíduos com susceptibilidade variável de acordo com suas características genéticas; uma mudança na resposta de uma população ao tratamento com um inseticida ou o aumento da concentração letal necessária para matar 50% de insetos (CL_{50}) podem ser devidos à redução na proporção de insetos susceptíveis (DAVISON, 1992).

De acordo com a OMS, resistência é a habilidade de uma população de insetos em tolerar uma dose de inseticida que, em condições normais, causaria sua morte. O dicloro difenil tricloro etano (DDT), um dos primeiros xenobióticos utilizados no controle de populações de insetos, mostrou uma grande eficiência que decaiu rapidamente devido ao surgimento de linhagens resistentes. Os mecanismos que conferem resistência aos insetos incluem diminuição da taxa de penetração do xenobiótico pela cutícula, detoxificação metabólica aumentada e diminuição da sensibilidade do sítio-alvo (BRAGA e VALLE, 2007).

A ocorrência de populações de *A. aegypti* resistentes ao temefós, único larvicida empregado no controle do mosquito até o ano 2000, tem sido comprovada; o desenvolvimento de resistência pelas larvas tem sido atribuído a alterações da enzima AChE, bem como a uma alta atividade das enzimas glutationa S-transferases e α- e β-esterases, responsáveis pelo metabolismo dos xenobióticos e conseqüente detoxificação do organismo (LIMA *et al*., 2003; BRAGA e VALLE, 2007; MELO-SANTOS *et al*., 2010).

2.5 Inseticidas naturais

A busca por inseticidas naturais e biodegradáveis tem como objetivos minimizar os danos ambientais, evitar o surgimento de larvas resistentes devido à rotatividade dos compostos utilizados no combate ao mosquito e identificar substâncias ativas contra linhagens resistentes (BRAGA e VALLE, 2007; SILVA *et al*., 2008). As plantas produzem fitotoxinas em resposta a ataques de fitopatógenos e herbívoros como uma estratégia de defesa; uma estratégia economicamente vantajosa e ecologicamente viável é a utilização de inseticidas extraídos de plantas, que podem ser produtos do seu metabolismo primário ou secundário (BROUSSALIS *et al*., 2010; OCHIENG *et al*., 2010; OLIVEIRA *et al*., 2010; PRASAD *et al*., 2010; SHI *et al*., 2010).

2.5.1 Metabólitos secundários

Os metabólitos secundários presentes em vegetais superiores não estão envolvidos de modo direto no crescimento e desenvolvimento da planta, mas atuam na defesa contra fitopatógenos (herbívoros e microrganismos), como atraentes de polinizadores e dispersores de sementes (TAIZ e ZEIGER, 2004). Os metabólitos secundários podem ser nitrogenados (alcalóides, aminoácidos não protéicos, aminas, alcamidas, glicosídeos cianogênicos e glicosinolatos) e não-nitrogenados (monoterpenos, diterpenos, triterpenos, tetraterpenos, sesquiterpenos, saponinas, flavonóides, esteróides, cumarinas) e são precursores de diversos derivados produzidos de acordo com as necessidades do tecido ou órgão da planta e o estágio de desenvolvimento (WINK, 2003). Quando produzidos na forma de precursores inativos, os metabólitos secundários se tornam ativos em caso de ferimento, infecções ou quando ingeridos por herbívoros.

Esses compostos promovem respostas celulares por diferentes mecanismos. Os alcalóides podem atuar como agonistas ou antagonistas de neurotransmissores e neuroreceptores ou acarretar distúrbios na replicação e transcrição. Taninos e outros fenóis formam pontes de hidrogênio e ligações iônicas com proteínas induzindo modificações conformacionais que podem levar à perda de função das proteínas. Os compostos lipofílicos, tais como terpenos e saponinas, interagem com membranas formando poros e induzindo distúrbios na permeabilidade celular (WINK, 2003).

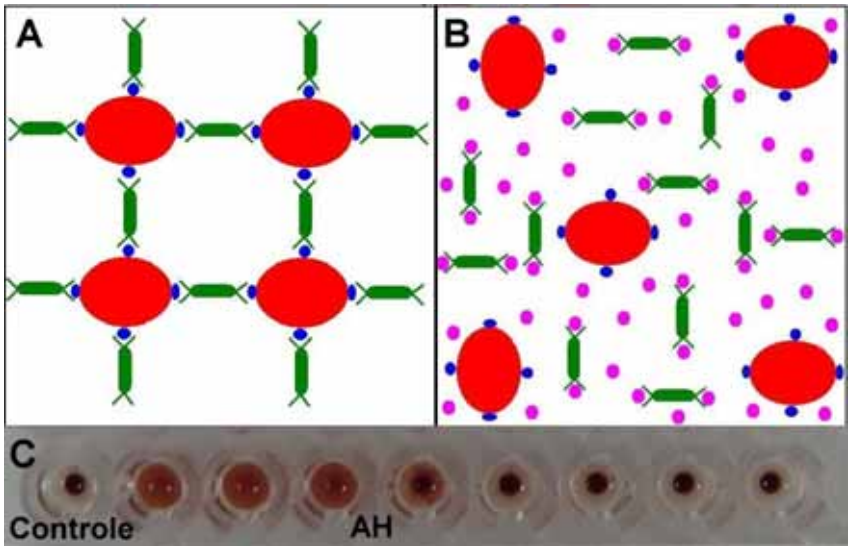
Metabólitos secundários com atividade inseticida têm sido descritos. Compostos fenólicos derivados do ácido elágico presentes em galhos e casca de *Laguncularia racemosa* quando incorporados a uma dieta artificial oferecida a larvas de *Spodoptera littoralis*, após seis dias de exposição, inibiram em até 90% o crescimento das larvas (SCHI *et al*., 2010). Extratos orgânicos de *Hybanthus parviflorus*, contendo β-sitosterol, foram ativos contra larvas

de *Ceratitis capitata* diminuindo a formação de pupa e emergência de adultos ou induzindo mortalidade das larvas (BROUSSALIS *et al*., 2010).

Atividade larvicida contra *A. aegypti* tem sido descrita para extratos de plantas contendo óleos essenciais (PITASAWAT *et al*., 2007; AUTRAN *et al*., 2009) e outros metabólitos secundários como limonóides (WANDSCHEER *et al*., 2004), quinonas (IOSET *et al*., 2000) e saponinas (CHAPAGAIN *et al*., 2008). O esteróide β-sitosterol extraído de *Abutilon indicum* apresentou atividade larvicida com CL_{50} de 11,49 ppm e os flavonóides quercetina (35,7 μg/mL) e kaempferol (30,65 μg/mL) isolados de folhas de *Gardenia ternifolia* promoveram mortalidade de L2 (RAHUMAN *et al*., 2008; OCHIENG *et al*., 2010).

2.5.2 Lectinas

Lectinas, proteínas ou glicoproteínas de origem não imune, interagem com carboidratos através de no mínimo dois sítios de ligação e, por isso, aglutinam células e precipitam polissacarídeos, glicoproteínas ou glicolipídeos, sem ocasionar modificações em suas estruturas (GOLDSTEIN *et al*., 1980). A palavra lectina, proveniente do latim (*Lectus*, selecionar/escolher), reflete a especificidade com que ocorre a ligação reversível entre uma lectina e o seu carboidrato específico (KENNEDY *et al*., 1995; MATSUI *et al*., 2001). A presença de lectinas em determinada amostra é verificada através do ensaio de atividade hemaglutinante (AH) realizado em suspensão de eritrócitos e confirmado pela inibição da AH quando em presença de carboidratos (Figura 3).



Pontual, E.V. (2008)

Figura 3. Atividade Hemaglutinante de lectina. A) Esquema da malha formada pela ligação da lectina aos carboidratos da superfície dos eritrócitos. B) Inibição da AH por carboidrato e C) Aspecto do ensaio de AH em microplaca. lectina – ; eritrócito – e carboidrato – .Controle: NaCl 0,15 M.

Atividade inseticida de lectinas contra espécies de pragas de importância econômica tem sido descrita; ClaveLL, a lectina extraída do líquen *Cladonia verticillaris* e as lectinas ligadoras de quitina extraídas do cerne (MuHL), da casca (MuBL) e da folha (MuLL) de *Myracrodruon urundeuva* (Aroeira do Sertão) possuem atividade termiticida contra soldados e operários de *Nasutitermes corniger* (SÁ *et al*., 2008; SILVA *et al*., 2009; NAPOLEÃO *et al*., 2010). Sugere-se que o efeito deletério ocorre devido à interação entre domínios ligadores de carboidratos das lectinas e unidades de N-acetil-glicosamina da matriz peritrófica dos insetos (SÁ *et al*., 2009). As lectinas que são resistentes a proteases do trato digestivo de homens e outros animais podem se ligar a carboidratos da superfície de células da mucosa intestinal e interferir nos processos de digestão e absorção dos alimentos reduzindo a eficiência do aproveitamento dos nutrientes (VASCONCELOS e OLIVEIRA, 2004;

KANSAL *et al*., 2006). Napoleão *et al*. (2010) descreveram a resistência de MuHL, MuBL e MuLL à digestão pela tripsina do intestino de *N. corniger*; adicionalmente os autores sugeriram que a atividade termiticida pode ter sido decorrente da atividade bacteriostática e bactericida sobre simbiontes do intestino das térmitas.

O controle de populações de *A. aegypti* utilizando preparações contendo lectinas também pode representar uma alternativa econômica e viável; MuHL e MuBL possuem atividade larvicida contra L4 de *A. aegypti* com CL_{50} de 0,04 e 0,125 mg/mL, respectivamente. Atividade larvicida (CL_{50} de 0,197 mg/mL), bem como atraso no desenvolvimento larval de *A. aegypti*, foram descritos para WSMoL, a lectina solúvel em água extraída de sementes de *M. oleifera*; as larvas apresentaram ausência da camada epitelial que delimita o lúmen do intestino bem como aumento do lúmen e hipertrofia de segmentos (COELHO *et al*., 2009).

2.5.3 Inibidores de proteases

A interação entre inibidores de proteases e enzimas proteolíticas pode resultar em modificações conformacionais na molécula da protease decorrentes da formação de complexos estáveis inativos ou com baixa atividade (LIAO *et al*., 2007).

Compostos fenólicos do tipo flavonóides podem inibir tripsina pela formação de duas ou mais pontes de hidrogênio e interações eletrostáticas com a região S^1 da molécula da enzima; quercetina, miricetina, morina e kaempferol inibem tripsina sendo as concentrações que inibem 50% da atividade máxima (CI_{50}) iguais a 0,010, 0,015, 0,027 e 0,06 mM, respectivamente (MALIAR *et al*., 2004).

Inibidores de proteases de natureza protéica são classificados em inibidores de serino, treonino, cisteíno, aspártico e metaloproteases, de acordo com o resíduo nucleofílico presente

no sítio ativo da enzima sobre a qual ocorre inibição (FEAR *et al*., 2007). Os inibidores de serinoproteases interagem de maneira estável com o sítio ativo das enzimas impedindo sua ligação com o substrato e, conseqüentemente, sua atividade hidrolítica (BODE e HUBER, 2000).

Devido à especificidade e versatilidades dos inibidores de proteases, as ciências médica e farmacêutica têm explorado seu potencial aplicativo como agentes antifúngicos, antiprotozoários, antivirais e terapêuticos no tratamento de doenças, entre as quais estão o câncer e a *diabetes mellitus* (KOBLINSKI *et al*., 2000; FEAR *et al*., 2007).

Os inibidores de proteases são comumente expressos em tecidos vegetais e sua biossíntese pode ser regulada em resposta ao ataque de pragas e herbívoros (BHATTACHARYYA *et al*., 2007a). A atividade inseticida de inibidores de tripsina ocorre devido à diminuição na biodisponibilidade de aminoácidos e pobre absorção de nutrientes que levam o inseto à morte por inanição (CARLINI e GROSSI-DE-SÁ, 2002; MACEDO *et al*., 2002; BHATTACHARYYA *et al*., 2007a, 2007b; OLIVEIRA *et al*., 2007; RAMOS *et al*., 2009).

O controle de pragas de interesse econômico através do bloqueio da digestão pela utilização de inibidores de tripsina (serinoprotease) tem despertado considerável interesse devido à ocorrência generalizada dessa enzima no intestino de insetos (HILDER *et al*., 1987; BROADWAY, 1995). Larvas de *Anagasta kuehniella*, praga que se alimenta de produtos em estoque, alimentadas com inibidor de tripsina de sementes de *Adenanthera pavonina* (1% p/p) apresentaram atividade de tripsina diminuída, alteração no ciclo biológico (aumento do período larval e de pupa) e redução de emergência e sobrevivência de adultos (MACEDO *et al*., 2010). Os inibidores de tripsina de sementes de *Cajanus cajan* e *Vigna mungo* também inibiram a tripsina do intestino de *Achaea janata*, *Helicoverpa armigera* e *Spodoptera litura* e foram resistentes à digestão por proteinases intestinais das larvas; alimentação artificial

contendo os inibidores acarretou em diminuição do peso e da taxa de sobrevivência das larvas seguindo uma curva dose-resposta (PRASAD *et al*., 2010).

Inibidores de tripsina de *Glycine max* e *Archidendron ellipticum* causaram inibição do crescimento e diminuição do peso corporal de larvas de *S. litura* (BHATTACHARYYA *et al*., 2007a). Kansal e*t al*. (2008) verificaram que o inibidor de tripsina isolado de sementes de *Vigna radiata* causou morte e atraso no crescimento de larvas de *Helicoverpa armigera*. Tratamento do besouro *Anthonomus grandis* (Bicudo-do-algodoeiro) com o inibidor de tripsina isolado de *Glycine max* resultou em larvas com peso e tamanho reduzidos, indução de mortalidade em adultos e deformidades em todos os estágios de desenvolvimento; em adultos, as deformidades observadas incluíram ausência de tórax e asas (FRANCO *et al*., 2004).

2.6 *Moringa oleifera*

M. oleifera (Figura 4), lírio branco ou quiabo de quina, pertence à família das Moringaceae. É nativa da Índia e amplamente cultivada nos trópicos desde que sobrevive por longo período em solos pobres e com baixo teor de umidade (MCCONNACHIE *et al*., 1999). A moringa desperta grande interesse devido às suas propriedades medicinais e utilização como planta forrageira, bem como por ser uma fonte promissora de óleos e biogás (MAKKAR e BECKER, 1996; FOIDL *et al*., 2001; KARADI et al., 2006).

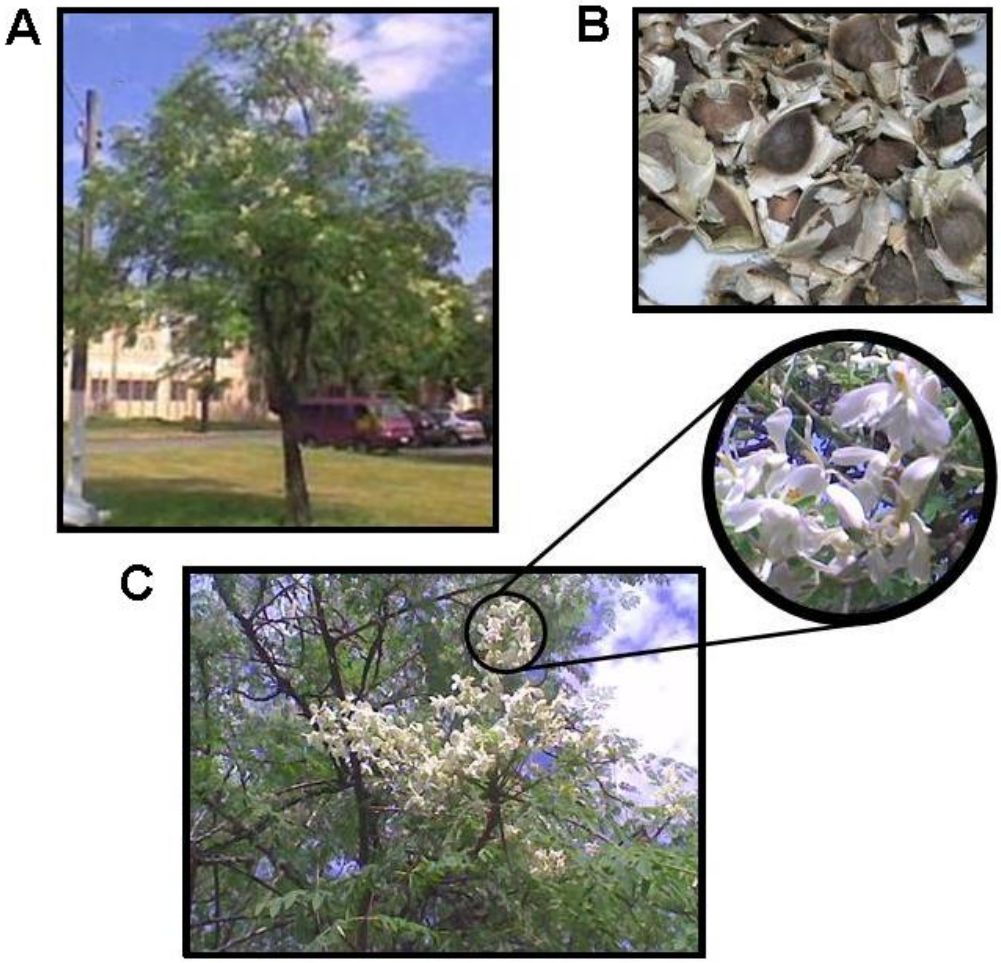


Figura 4. Aspectos de *M. oleifera*: (A) árvore, (B) sementes e (C) inflorescência.

Fotos: Pontual, E.V., 2008 (A e C) e www.mfrural.com.br (B)

Sementes de *M. oleifera* possuem propriedades coagulantes devido à presença de proteínas e de um polieletrólito orgânico de 3 kDa hábeis em clarificar águas turvas (OKUDA, *et al*., 2001; GHEBREMICHAEL *et al*., 2005; SANTOS *et al*., 2009); sua utilização no tratamento de água para consumo humano em países em desenvolvimento tem sido descrita. Dentre as proteínas envolvidas no mecanismo de coagulação promovido pelas sementes de *M. oleifera* foi descrita a lectina cMoL (SANTOS *et al*., 2009).

As flores de *M. oleifera* cruas ou após cozimento brando são utilizadas como alimento constituindo uma rica fonte de íons cálcio e potássio e dos antioxidantes naturais α- e γ-

tocoferol (RAMACHANDRAN *et al*., 1980; MAKKAR e BECKER, 1996; GUEVARA *et al*., 1999; FOIDL *et al*., 2001; SÁNCHEZ-MACHADO *et al*., 2006). As flores de moringa são utilizadas com fins medicinais como colagogo, diurético, hipoglicemiante e tônico (KHARE, 2007; PARROTTA, 2009). Pterigospermina, um alcalóide com atividade antifúngica e antibacteriana, é encontrado nas flores (LIZZY *et al*., 1968; ONG, 2008).

3. OBJETIVOS

3.1 Objetivo geral

Investigar extrato aquoso de flores de *M. oleifera* quanto à presença de compostos inseticidas (metabólitos secundários, lectinas, inibidor de tripsina e inibidor de AChE) e atividade larvicida contra *A. aegypti*. Avaliar as atividades de tripsina e AChE de L4 tratadas com o extrato de flores em condições *in vivo*.

3.2 Objetivos específicos

- Determinar a taxa de mortalidade de L1 e L4 após incubação com o extrato de flores de *M. oleifera*.
- Investigar o extrato de flores de *M. oleifera* quanto à presença de metabólitos secundários.
- Determinar o perfil eletroforético, em gel de poliacrilamida, de proteínas do extrato de flores de *M. oleifera* sob condições desnaturantes (sulfato sódico de dodecila) e redutoras (β-mercaptoetanol).
- Avaliar a presença de lectinas no extrato de flores de *M. oleifera*, através da determinação de atividade hemaglutinante.
- Investigar o extrato de flores de *M. oleifera* quanto à presença de inibidores de tripsina e de AChE utilizando enzimas comerciais e substratos sintéticos.
- Determinar a atividade de tripsina e AChE em extratos de L4.
- Determinar o efeito de quercetina comercial sobre tripsina de L4.
- Avaliar o efeito do extrato de flores de *M. oleifera* sobre tripsina e AChE de L4.
- Determinar as atividades de tripsina e AChE em L4 tratadas com o extrato de flores.

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5. ARTIGO

EFFECT OF LARVICIDAL EXTRACT FROM *Moringa oleifera* FLOWERS ON GUT TRYPSIN AND ACETHYLCHOLINESTERASE ACTIVITY FROM *Aedes aegypti* LARVAE

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Effect of larvicidal extract from *Moringa oleifera* flowers on gut trypsin and acethylcholinesterase activity from *Aedes aegypti* larvae

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Abstract

Dengue Fever is an arboviruses transmitted by *Aedes aegypti* and mosquito control is fundamental to reduce disease spreading. *A. aegypti* larvae have developed resistance to organophosporous insecticides and the use of natural compounds that promote mortality may avoid emergence of resistant larvae due to rotation of insecticides. This work reports the larvicidal activity (LC_{50} of 0.925% w/v) of *Moringa oleifera* flower extract on fourth larval instars (L4) of *A. aegypti*. Proteinaceous trypsin inhibitor from *M. oleifera* flower (MoFTI, 169.9 kDa, K_i: 0.38 nM), triterpene (β-amyrin), sterol (β-sitosterol) and flavonoids (kaempferol and quercetin) were detected in the extract; lectin was absent. Trypsin from L4 gut extract was inhibited by MoFTI (K_i: 0.6 nM), however acetylcholinesterase (AChE) activity from total L4 extract was not altered. *In vivo* assay showed that gut trypsin activity from L4 treated with *M. oleifera* flower extract decreased along the time (0 to 1440 min) and was strongly inhibited (98.6 %) after 310 min incubation; AChE activity from total L4 extract was not affected in this period. The study points out *M. oleifera* flower extract as a biodegradable tool for *A. aegypti* larvae control and suggests that larvicidal mechanism involves inhibition of L4 gut trypsin by MoFTI.

Keywords: *Moringa oleifera*; *Aedes aegypti*; larvicidal activity; trypsin inhibitor; gut trypsin; acethylcholisnesterase.

1. Introduction

Dengue Fever is a mosquito-borne infection that has become a major international public health concern, mainly in tropical and sub-tropical regions. Today approximately 2.5 billion people (two-fifths of the world population) are in risk to be infected and 50 million cases of dengue fever are recorded every year worldwide, characterizing a pandemia (World Health Organization, 2009). The Dengue Fever is caused by an arbovirus and the main vector is the predominantly urban mosquito *Aedes aegypti* (Culicidae). Mosquito control is fundamental to reduce the disease spreading since there is no vaccine for Dengue Fever.

A. aegypti development occurs through stages of egg, larvae (four instars: L1, L2, L3 and L4), pupa and adult. *A. aegypti* larvae have been controlled in Public Health Programs mainly using the organophosphorous temephos which target is the serine hydrolase acetylcholinesterase (AChE); this enzyme, after propagation of nervous impulse, hydrolyses the acetylcholine from cholinergic synapses. Phosphorylation of AChE by organophosphorous leads to irreversible inhibition of enzyme and promotes insect death by paralysis due to interminable muscular contractions. Resistance of *A. aegypti* larvae to insecticides has been attributed to insensitive AChE as well as higher activity of glutathione S-transferases and α- and β-esterases (Braga and Valle, 2007; Melo-Santos et al., 2010). Genotoxicity and mutagenicity of temephos was detected by single cell gel electrophoresis (comet assay), SOS/umu, and Ames/*Salmonella* assays in concentrations similar to those routinely used to combat *A. aegypti* (Aiub et al., 2002).

A strategy used in Brazil as part of the National Program of Dengue Control (Fundação Nacional de Saúde, 2002) is the biological control with *Bacillus thuringiensis* serovar *israelensis* (Bti). The endotoxin Cry1AC, produced during Bti sporulation, is digested by enzymes of larvae midgut releasing larvicidal toxins; tablet containing spore and crystals (15%, w/w) of *B. thuringiensis* was able to cause 100 % mortality of larvae and was

suggested for use in programs to control dengue vector (Araújo et al., 2007). Cry1AC has a *N*-acetylgalactosamine-specific lectin domain that binds glycoconjugates at insect midgut (Gill et al., 1992; Burton et al., 1999).

The use of natural and biodegradable insecticides to minimize environmental damage, can promote mortality of resistant larvae and avoid emergence of resistant larvae due to rotation of compounds used in *A. aegypti* control. Lectins, hemagglutinating proteins with carbohydrate-binding property, from *Myracrodruon urundeuva* bark and heartwood were able to kill *A. aegypti* L4 and it was suggested that binding of lectins to peritrophic membrane of larvae was involved in the larvicidal activity (Sá et al., 2009). Larvicidal activity against *A. aegypti* was reported for plant extracts containing secondary metabolites such as essential oils, limonoids, quinones and saponins (Ioset et al., 2000; Wandscheer et al., 2004; Pitasawat et al., 2007; Chapagain et al., 2008; Autran et al., 2009).

Larval stages in *A. aegypti* development correspond to phagoperiod and the digestive process is critical and highly active in larvae (Yang et al., 1971; Ho et al., 1992). Kunz (1978) reported that larvae of *A. aegypti* possess at least 12 serine proteinases with molecular mass of 20 to 25 kDa, some with trypsin-like and others with chymotrypsin-like activity. Borovsky and Meola (2004) reported that in *A. aegypti* the amount of trypsin synthesized in the larval gut is higher than chymotrypsin and that trypsin biosynthesis increases during larval development. Analysis of *A. aegypti* genome revealed that 51 genes that encode trypsin are expressed in larval stage (Borovsky and Meola, 2004; Venancio et al., 2009).

Damage to digestion process can be a strategy to control insect population. Borovsk and Meola (2004) showed that inhibition of trypsin biosynthesis in *A. aegypti* larvae reared with a decapeptide named trypsin-modulating oostatic factor resulted in larval mortality. Plant trypsin inhibitors are able to reduce larval survival rate by decreasing in the essential amino acids bioavailability and poor nutrient absorption (Carlini and Grossi-de-Sá, 2002; Macedo et

al., 2002; Macedo et al., 2003; Bhattacharyya et al., 2007a, 2007b; Oliveira et al., 2007; Ramos et al., 2009).

Moringa oleifera (Moringaceae family) is a tree widely cultivated throughout the tropics and subtropics due to its medicinal properties, used as a forage plant and a source of biogas and oil with industrial applications (Foidl et al., 2001). The seeds contain coagulant proteins and organic polyelectrolyte of 3 kDa able to remove water turbidity and have been used in developing countries to water treatment for human consumption (Okuda et al., 2001; Ghebremichael et al., 2005; Santos et al., 2009). The water-soluble *M. oleifera* lectin (WSMoL) isolated from seeds promoted *A. aegypti* L4 mortality (LC_{50} of 0.197 mg/ml); morphological changes including hypertrophy of the segments and absence of epithelial layer that delimits the larval gut were observed (Coelho et al., 2009). The flowers contain antioxidants (α and γ-tocopherol) and pterigospermin, alkaloid with fungicidal and bactericidal activities (Lizzy et al., 1968; Foidl et al., 2001; Sánchez-Machado et al., 2006; Ong, 2008). The flowers are eaten raw or after lightly blanched and have medicinal use as cholagogue diuretic, hypoglycemic and tonic (Khare, 2007; Parrotta, 2009).

This work determined the effect of *M. oleifera* flower extract on survival of *A. aegypti* L1 and L4, the first and latest larval instars; the flower extract was evaluated for secondary metabolites, lectin as well as trypsin and AChE inhibitors. The effect of *M. oleifera* flower extract on gut trypsin and AChE from L4 was investigated; trypsin and AChE activities from L4 treated with *M. oleifera* flower extract at *in vivo* conditions were also determined.

2. Materials and Methods

2.1 Plant material

M. oleifera Lam. (Division Magnoliophyta, Class Magnoliopsida, Subclass Dilleniidae, Order Capparidales, Family Moringaceae) has the vernacular names “moringa” in Portuguese, “árbol del ben” in Spanish and horseradish tree in English. Flowers were collected in Recife City, State of Pernambuco, Northeastern Brazil. A voucher specimen is deposited under number 73345 at the herbarium “Dárdano de Andrade Lima” (Empresa Pernambucana de Pesquisa Agropecuária, IPA, Recife, Brazil).

2.2 M. oleifera flower extract

M. oleifera fresh flowers (50 g) were added to distilled water (100 ml) and after homogenisation in a blender (10 min at 27°C), followed by filtration through gauze and centrifugation (9,000 *g,* 15 min, 4 ºC), the extract (clear supernatant) was obtained. The extract was dried by lyophilization, ressuspended in a concentration of 4.5 % (dry weight/volume) in distilled water and evaluated for protein concentration according to Lowry et al. (1951) using serum albumin (31.25-500 µg/ml) as standard.

2.3 A. aegypti larvae

A. aegypti eggs were hatched in distilled water at a temperature in the range 25-27 ºC. Cat food (Whiskas®) was offered to larvae; L1 and L4 were separated and used in the larvicidal assays. For identification of larval stage, color of the anterior region (head) and length of larvae (L1: 0.11 cm ± 0.02; L2: 0.39 cm ± 0.02; L3: 0.47 cm ± 0.04; L4: 0.63 cm ± 0.10) were observed (Coelho et al., 2009).

2.4 Larvicidal activity from M. oleifera extract

Larvicidal activity was performed according to an adaptation of the World Health Organization (1981) method described by Navarro et al. (2003); bioassay with L1 (25) used

tissue culture plate due to reduced dimensions of larvae while L4 (25) were placed into glass beaker. Larvae were incubated with *M. oleifera* flower extract at 0.669-1.115 % (w/v), corresponding to 1.336-2.227 mg/ml in protein concentration, and distilled water (negative control); the final volume of each assay was 2 (L1) and 20 mL (L4). Mortality rate (%) was determined after 24 h of incubation at 27 ±2 °C and 12–12 (light–dark) photoperiod using a stereomicroscope (Leica MZ6). Three independent experiments were run in quadruplicate.

2.5 Investigation of M. oleifera extract for secondary metabolites

Phytochemical evaluation of *M. oleifera* flower extract (15 µl) was performed by thin layer chromatography (TLC) on silica sheet (Merck, Germany). The presence of alkaloids (mobile phase: 100:11:11:26 [v/v] EtOAc/HCOOH/AcOH/H_2O; revealer: Dragendorff's reagent), terpenoids and steroids (mobile phase: 100:0.5:0.5:0.5 [v/v] EtOAc/HCOOH/AcOH/H_2O; revealer: Liebermann-Burchard's reagent), saponins (mobile phase: 100:11:11:26 [v/v] EtOAc/HCOOH/AcOH/H_2O; revealer: anisaldehyde), iridoids (mobile phase: 100:11:11:26 [v/v] EtOAc/HCOOH/AcOH/H_2O; revealer: vanillin-sulphuric acid), coumarins (mobile phase: 50:50:50 [v/v] Et_2O/toluene/10% AcOH; detection: UV 365 nm), cinnamic derivatives, phenylpropanoglucosides, flavonoids and phenolic acids (mobile phase: 100:11:11:26 [v/v] EtOAc/HCOOH/AcOH/H_2O; revealer: Neu's reagent), condensed proanthocyanidins and leucoanthocyanidins (mobile phase: 100:11:11:26 [v/v] EtOAc/HCOOH/AcOH/H_2O; revealer: vanillin-chloridric acid), and hydrolysable tannins (mobile phase: 40:50:10 [v/v] n-BuOH/Me_2CO/phosphate buffer pH 5.0; revealer: 1% iron alum) were investigated (Stiasny, 1912; Wallenfels, 1950; Neu, 1956; Roberts et al., 1956; Markhan, 1982; Wagner and Bladt, 1996; Harborne, 1998).

2.6 Polyacrylamide gel electrophoresis (PAGE) of M. oleifera extract

M. oleifera flower extract (100 µg of protein) was evaluated by PAGE (7-18%, w/v, gradient gel) under denaturing conditions containing sodium dodecyl sulphate (SDS-PAGE) according to Laemmli (1970) in presence or absence of the reducing agent β-mercaptoethanol. Polypeptides and molecular mass markers (myosin, 198.8 kDa; β-galactosidase, 115.7 kDa; bovine serum albumin, 96.7 kDa; ovalbumin, 53.5 kDa; carbonic anhydrase, 37.1 kDa; soybean trypsin inhibitor, 29.1 kDa; lysozyme, 19.5 kDa from Bio-Rad, USA) were stained with 0.02% (v/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid.

2.7 Hemagglutinating assay for lectin detection

Hemagglutinating activity was carried out in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho (1992) using rabbit erythrocyte suspension (2.5%, v/v) treated with glutaraldehyde (Bing et al., 1967). One hemagglutination unit was defined as the reciprocal of the highest dilution of sample that promotes full agglutination of erythrocytes (Chumkhunthod et al., 2006).

2.8 Investigation of trypsin inhibitor activity from M. oleifera flower extract

2.8.1. Inhibition of bovine trypsin by M. oleifera flower extract

Trypsin inhibitor activity was assayed using 0.1 mg/ml bovine trypsin (Sigma-Aldrich, USA) in 0.1 M Tris-HCl pH 8.0 containing 0.02 M $CaCl_2$. Bovine trypsin (5 µl) was incubated (5 min, 37 ºC) with *M. oleifera* flower extract (50 µl, 135-244 µg of protein) in Tris-HCl pH 8.0 (140 µl). Following, the synthetic substrate N-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA) dissolved in dimethyl sulfoxide was added (5 µl) and the mixture was incubated (30 min, 37 ºC). The substrate hydrolysis was followed by measurement of

absorbance at 405 nm (A405nm) and the inhibitory activity was determined by remaining hydrolytic activity towards BApNA.

A Dixon plot analysis was employed to determine the constant of inhibition (K_i) for bovine trypsin (Segel, 1975). Enzyme inhibition was carried out at two different BApNA concentrations ([BApNA]; 4 mM and 8 mM). Samples were prepared to achieve inhibitor concentrations (nM) of 0.794-1.44 nM. The initial slope *v* was determined for each inhibitor concentration. Dixon plots were generated using the reciprocal velocity (1/*v*) versus inhibitor concentration. Intersection of the two regression lines for each [BApNA] yielded the K_i.

2.8.2. Reverse zymography

M. oleifera flower extract was submitted to SDS-PAGE on 7-18% (w/v) gradient gel containing 0.1% (w/v) casein and the electrophoresis was performed at 4 ºC. After running, the gel was removed and placed in 2.5% Triton X-100 with continuous stirring for 45 min at 25 ºC to remove SDS. The gel was washed with distilled water three times and then incubated with the development buffer (10 mM Tris–HCl pH 7.6, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% Brij-35, and 450 µg/100 ml trypsin) at 37 ºC for 9 h to digest out the background substrate. After proteolysis by incubation with trypsin, the gel was stained with 0.02% (v/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid. The undigested bands corresponded to the proteins with inhibitory activity (Le and Katunuma, 2004).

2.9 Investigation of AChE inhibitor activity from M. oleifera flower extract

AChE inhibitor activity was assayed using 1 µg/ml electric eel (*Electrophorus electricus*) type VI-S acetylcholinesterase (Sigma-Aldrich, USA) in Tris-HCl 0.5 M, pH 8.0. Enzyme activity was determined using 0.062 M acetylthiocholine (Sigma-Aldrich, USA) as substrate and 0.25 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) as colour-developing

agent. The enzyme (20 µl) was incubated with the substrate (20 µl) and DTNB (200 µl) during 3 min at 25 ºC. The thiocholine generation in the presence of DTNB was determined by measurement of the increase of absorbance at 405 nm resulting from the formation of thiolate dianion of DTNB. The effect of *M. oleifera* flower extract (0.1-20,000 µg/ml of protein) on AChE was determined by previous incubation (60 min, 25 ºC) of the extract (10 µl) with the enzyme (10 µl; 1.0 µg/ml). Assays were performed in quadruplicate. The organophosphorous (0.1-1,000 µg/ml) dichlovors (Sigma-Aldrich, USA) and temephos (Sigma-Aldrich, USA) were used as positive controls.

2.10 Trypsin activity from L4 gut extract

Groups of 50 live L4 were immobilized by placing them at 4 ºC for 10 min. The gut of each larva was removed using a needle (8 mm length; 0.3 mm caliber) and immediately homogenized in tissue grinder with 1 ml of 0.1 M Tris-HCl pH 8.0 containing 0.15 M NaCl. The homogenate was centrifuged (9,000 *g*, 4 °C, 15 min) and the supernatant (L4 gut extract) was evaluated for protein concentration and trypsin activity. L4 gut extract (20 µl, 28 µg of protein) was incubated (30 min, 37 ºC) with 8 mM BApNA (5 µl) in Tris buffer (175 µl). The enzyme activity was followed by measurement of absorbance at 405 nm (A405nm). One unit of trypsin activity was defined as the amount of enzyme that hydrolyzes 1 µg of BApNA per minute. Control of substrate hydrolysis was performed by incubation (30 min, 37 °C) of bovine trypsin (0.5 µg) with 8 mM BApNA (5 µl).

Zymography for proteases was carried out according to the method described by Garcia-Carreño et al. (1993). Sample of L4 gut extract (20 µg of protein) was submitted to SDS-PAGE using a 12.5% (w/v) gel at 4 ºC. After electrophoresis, the gel was immersed in 2.5% Triton X-100 in 0.1 M Tris-HCl pH 8.0 to remove SDS and incubated (30 min, 4 °C) with 3% casein (w/v) in 0.1 M Tris-HCl pH 8.0. The temperature was then raised to 37 ºC and

kept for 90 min to allow the digestion of casein by the active polypeptides. Finally, the gel stained for protein with 0.02% (v/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid was washed with destaining solution (40% methanol, 10% acetic acid, and 50% distilled water). Light bands against the dark background indicated proteolytic activity.

2.11 Acetylcholinesterase activity from total L4 extract

Groups of 50 L4 were immobilized by placing them at 4 ºC for 10 min and following were homogenized in tissue grinder with 1 ml of 0.1 M Tris-HCl pH 8.0 containing 0.15 M NaCl. The homogenate was centrifuged (9,000 *g*, 4 °C, 15 min) and the supernatant (L4 extract) was evaluated for protein concentration and AChE activity. Total L4 extract (10 µl, 37 µg of protein) was incubated with 0.062 M acetylthiocholine (20 µl) and 0.25 mM DTNB (200 µl) during 3 min at 25 ºC and the increase of the absorbance at 405 nm was monitored. One unit of AChE activity was defined as the amount of enzyme capable of converting 1 µmol of substrate per minute.

Zymography for AChE was performed according to Mohamed et al. (2007). Total L4 extract (100 µg of protein) was submitted to SDS-PAGE on 6-10% (w/v) gradient gel. After electrophoresis, the gel was washed three times with 50 mM phosphate buffer pH 7.5. The gel was incubated (16 h, 27 °C) in substrate buffer (50 mg of acetylthiocholine iodide dissolved in 65 ml of 100 mM sodium phosphate buffer pH 7.5, 5 mL of 100 mM sodium citrate, 10 ml of 30 mM copper sulfate, 10 mL H_2O and 10 mL of 5 mM potassium ferricyanide). After visualization of AChE bands, the gel was incubated in 10% (v/v) acetic acid. AChE activity appeared as polypeptide bands of brown color.

2.12 Effect of quercetin on trypsin activity from L4 gut extract

L4 gut extract (20 µl, 28 µg of protein) was incubated (5 min, 37 ºC) with 0.01-0.1 mM quercetin (Merck, Germany) in 0.1 M Tris-HCl, pH 8.0. Following, 8 mM BApNA (5 µl) was added and after incubation (30 min, 37 ºC) trypsin activity was recorded as described in 2.10. Assay was performed in triplicate.

2.13 Effect of M. oleifera flower extract on trypsin activity from L4 gut extract

L4 gut extract (20 µl, 28 µg of protein) was incubated (5 min, 37 ºC) with *M. oleifera* flower extract (135-480 µg of protein corresponding to inhibitor concentration of 0.794-2.23 nM) in 0.1 M Tris-HCl, pH 8.0. Following, 8 mM BApNA (5 µl) was added and after incubation (30 min, 37 ºC) trypsin activity was recorded as described in 2.10. Assay was performed in triplicate. Inhibition curve was plotted using the GraFit 3.0 software and the K_i for L4 gut trypsin was determined using the Dixon plot analysis as described in 2.8.1 item using different BApNA concentrations (4 mM and 8 mM).

Zymography of the L4 gut extract (20 µg of protein) after incubation (30 min, 27 ºC) with *M. oleifera* flower extract (210 µg of protein) was performed on SDS-PAGE 12.5% (w/v) gel as described in item 2.10. L4 gut extract (20 µg) was also incubated (30 min, 27 °C) with the serine protease inhibitor PMSF (1 mM; 5 µl) which was used as positive control in zymography.

2.14 Effect of M. oleifera flower extract on AChE activity from total L4 extract

M. oleifera flower extract (0.1-20,000 µg/ml of protein) was incubated (60 min, 25 ºC) with total L4 extract (26 µl, 37 µg of protein). Following, AChE activity was determined as described in 2.11 and a curve was plotted using the GraFit 3.0 software. Assays were performed in quadruplicate. The organophosphorous dichlovors and temephos at concentrations of 0.1-1,000 µg/ml were used as positive controls. The K_i were calculated

using the formula $K_i = IC_{50}/(1+([acetylthiocoline]/K_m))$, where IC_{50} is the concentration of inhibitor required to produce 50 % inhibition of the enzymatic reaction and K_m is the Michaelis-Menten constant (Cheng and Prusoff, 1973).

Zymography of total L4 extract (100 µg of protein) after incubation (60 min, 27 ºC) with *M. oleifera* flower extract (500 µg of protein) was performed on SDS-PAGE 6-10% (w/v) gel as described in item 2.11.

2.15 Trypsin and AChE activities from L4 treated with M. oleifera flower extract

To assess trypsin and AChE activities from live larvae treated with *M. oleifera* flower extract, larvicidal bioassay was performed as described in 2.9. L4 were incubated with the extract (0.925%, w/v) during different times (20, 40, 60, 120, 215, 310 and 1440 min). After each time of incubation live larvae were used to prepare L4 gut and total L4 extracts that were evaluated for trypsin and AChE activities as described in 2.10 and 2.11, respectively. Trypsin and AChE activities from dead larvae found after 1440 min incubation and live larvae from control (distilled water) were also determined. Three independent assays were performed in triplicate.

2.16 Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA) and data were expressed as a mean of replicates ± SD. Significant differences between treatment groups were analysed by Student´s t-test (significance at $p<0.05$) using Origin 6.0 program. The lethal concentrations required to kill 16% (LC_{16}), 50% (LC_{50}) and 84% (LC_{84}) of larvae in 24 h were calculated by probit analysis with a reliability interval of 95% using the computer software StatPlus® 2006 (AnalystSoft, Canada).

3. Results

Bioassay using L1 revealed that survival rate (92%) determined after incubation of larvae with *M. oleifera* extract at all tested concentrations was the same as that detected in the negative control. *M. oleifera* flower extract was toxic on *A. aegypti* L4 (Figure 1) and the LC_{16}, LC_{50}, and LC_{84} values calculated by probit analysis were 0.675, 0.925 and 1.159 %; these values correspond in protein concentrations to 1.384, 1.851, and 2.319 mg/ml, respectively. L4 was the selected stage to next assays due to the deleterious effect of *M. oleifera* extract on this larval instar.

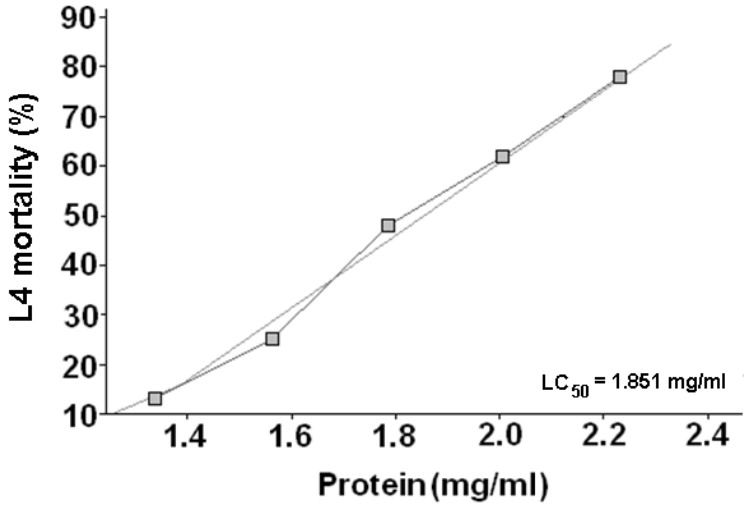

Figure 1. Mortality of *A. aegypti* L4 in incubation with *M. oleifera* flower extract. Lethal protein concentration required to kill 50% (LC_{50}) of larvae in 24 h was determined by probit analysis with a reliability interval of 95%.

M. oleifera flower extract was evaluated for presence of secondary metabolites by TLC. Fluorescent spots with yellow and green colors were observed when Neu's reagent was used indicating the presence of the flavonoids kaempferol and quercetin in the extract. The presence of β-amyrin (triterpene) and β-sitosterol was detected after reaction of the extract with acetic anhydride in the presence of concentrated sulfuric acid (Liebermann-Burchard's reagent).

M. oleifera flower extract containing 8.91 mg/ml of protein showed a single polypeptide band of 169.9 kDa on SDS-PAGE (Figure 2A); the same electrophoretic profile was detected in presence of the reducing agent β-mercaptoethanol. The extract did not present hemagglutinating activity on rabbit erythrocytes revealing that the conditions used for protein extraction did not solubilize lectin.

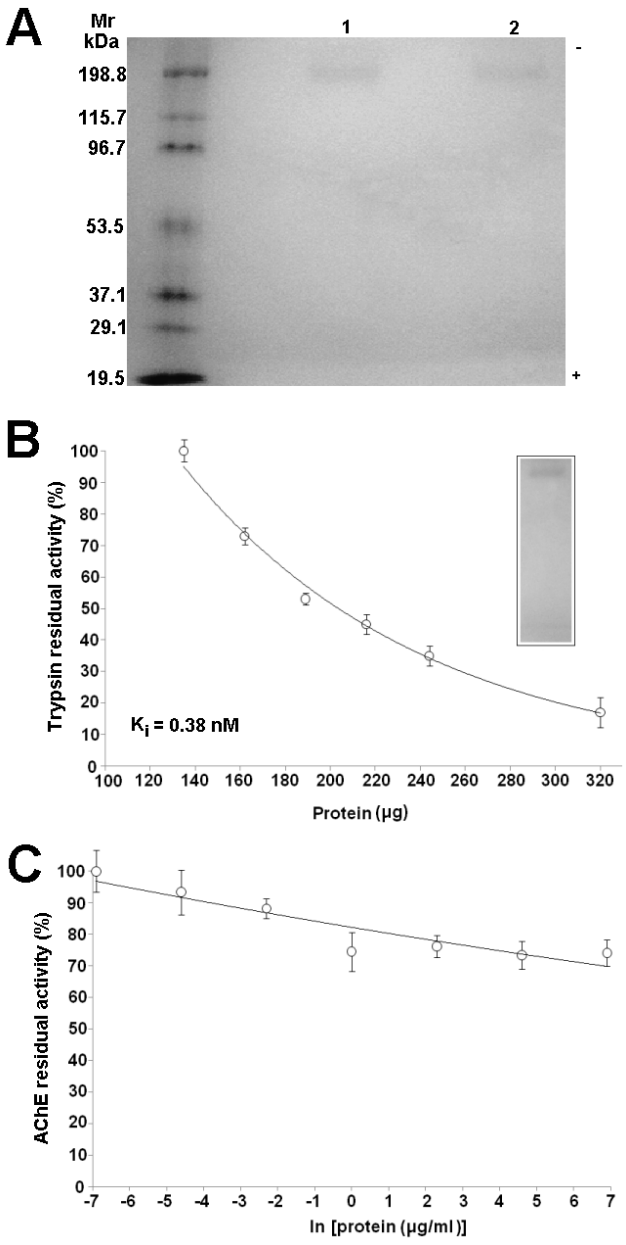

Figure 2. Characterization of *M. oleifera* flower extract. (A) SDS-PAGE of molecular mass standards and extract peptides (100 µg of protein) in absence (1) and presence (2) of β-mercaptoethanol stained with Coomassie Brilliant Blue. (B) Bovine trypsin inhibitor activity and reverse zymography (inset). (C) Electric eel AChE activity at presence of *M. oleifera* flower extract.

The extract was able to inhibit bovine trypsin (Table 1) with K_i of 0.38 nM (Figure 2B); the *M. oleifera* flower trypsin inhibitor was called MoFTI. The reverse zymography shows that the polypeptide band of 169.9 kDa detected on SDS-PAGE was stained by Coomassie Blue (Figure 2B, inset) revealing that it was not digested by bovine trypsin. This result agrees with the presence of MoFTI in the extract. The activity of electric eel AChE was slightly inhibited by extract (Figure 2C) whereas the enzyme was inhibited by positive controls dichlovors and temephos (Table 2).

Table 1. Bovine trypsin and L4 gut trypsin-like activities in presence of *M. oleifera* flower extract and quercetin.

Sample	Enzyme activity*
Bovine trypsin (control)	0.205 ± 0.019 [a]
Bovine trypsin + *M. oleifera* flower extract	0.091 ± 0.011 [b]
Bovine trypsin + quercetin	0.112 ± 0.021 [c]
L4 gut extract (control)	0.319 ± 0.006 [d]
L4 gut extract + *M. oleifera* flower extract	0.220 ± 0.010 [e]
L4 gut extract + quercetin	0.325 ± 0.068 [d]

Concentrations of bovine trypsin and quercetin were 0.1 mg/ml and 0.1 mM, respectively. Protein in *M. oleifera* flower extract and L4 gut extract were 200 and 28 µg, respectively. *Absorbance at 405 nm. Enzyme activity corresponds to the increase of absorbance due to BAPNA hydrolysis and reduction in absorbance reveals enzyme inhibition. Different letters indicate significant differences between treatments.

Table 2. Electric eel AChE and L4 AChE activities in presence of *M. oleifera* flower extract and organophosphorous insecticides.

Sample	Enzyme activity*
Electric eel AChE (control)	0.0270 ± 0.0009 [a]
Electric eel AChE + *M. oleifera* flower extract	0.0235 ± 0.0015 [a]
Electric eel AChE + dichlorvos	0
Electric eel AChE + temephos	0
Total L4 extract (control)	0.0094 ± 0.0005 [b]
Total L4 extract + *M. oleifera* flower extract	0.0090 ± 0.0003 [b]
Total L4 extract + dichlorvos	0.0048 ± 0.0001 [c]
Total L4 extract + temephos	0.0056 ± 0.0001 [d]

Concentrations of electric eel AChE, dichlovors and temephos were 1 µg/ml, 100 µg/ml and 1000 µg/ml, respectively. Protein amount in *M. oleifera* flower extract and Total L4 extract were 200 and 37 µg, respectively. *Absorbance at 405 nm. AChE activity corresponds to the increase of absorbance resulting from the formation of the thiolate dianion of DTNB and reduction in absorbance reveals enzyme inhibition. Different letters indicate significant differences between treatments.

L4 gut extract (28 μg of protein) showed trypsin activity (234 mU/ml). This activity was not inhibited by quercetin but reduction of activity was detected after incubation of L4 gut extract with MoFTI (Table 1); K_i of 0.6 nM was determined (Figure 3A). Zymography of L4 gut extract showed multiple polypeptide bands (Figure 3B1) and protease activities were abolished when L4 gut extract was incubated with PMSF indicating the presence of serine protease in larval preparation (Figure 3B2). Zymography of L4 gut extract incubated with *M. oleifera* flower extract shows the absence of three polypeptide bands in comparison with untreated L4 gut extract revealing that activities of three enzymes were inhibited by MoFTI (Figure 3B3).

Total L4 extract (75 μg) contains AChE (1.59 mU/ml) activity since promoted acetylthiocholine hydrolysis revealed by DTNB reaction; this activity was not inhibited by *M. oleifera* flower extract (Figure 3C). Zymography also revealed that AChE activity from total L4 extract (Figure 3D1) was not inhibited by *M. oleifera* flower extract (Figure 3D2). Dichlovors and temephos inhibit L4 AChE with K_i of 8.64 x 10^{-10} M and 2.19 x 10^{-8} M, respectively (Table 2; Figure 3E).

Treatment of L4 with *M. oleifera* extract at LC_{50} concentration resulted in inhibition of trypsin activity from L4 gut extract. Exponential correlation ($y = 564.69e^{-0.0143x}$; R^2: 0.9798, y= incubation time in min and x= L4 gut trypsin activity in mU/ml) was detected between increasing period of incubation and loss of enzyme activity (Figure 4A). The enzyme activity was almost completely inhibited (98.6 %) after 310 min incubation in comparison with control (0 min). Gut extracts of live and dead larvae incubated with *M. oleifera* flower extract by 1440 min were not able to promote BApNA hydrolysis. Incubation of *A. aegypti* L4 with *M. oleifera* flower extract did not result in significant reduction of AChE from total L4 extract (Figure 4A). Trypsin and AChE activities from untreated L4 remained actives during all time of experiment (Figure 4B).

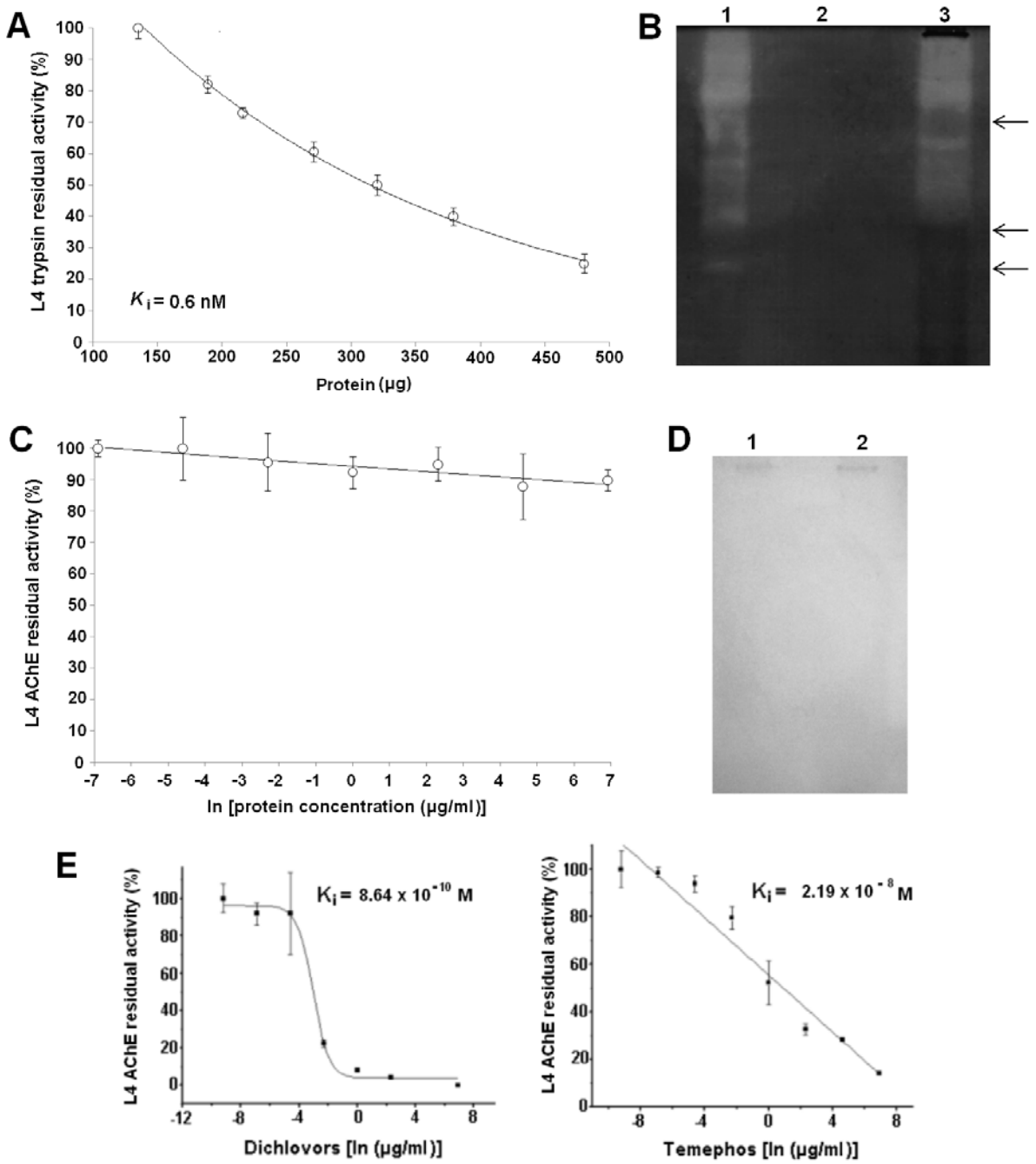

Fig. 3. Effect of *M. oleifera* flower extract on gut trypsin and AChE activities from *A. aegypti* L4. (A) Inhibition of trypsin activity from L4 gut extract by *M. oleifera* flower extract. (B) Zymography for proteases of L4 gut extract (1) and L4 gut extract incubated with PMSF (2) and *M. oleifera* flower extract (3). (C) Effect of *M. oleifera* flower extract on AChE activity from total L4 extract. (D) Zymography for AChE from total L4 extract (1) and total L4 extract after incubation with *M. oleifera* flower extract (2). (E) AChE activity from total L4 extract at presence of positive controls dichlorvos and temephos. The points are the mean of four experiments ± SD. Arrows indicate the polypeptide bands absent in L4 gut extract incubated with *M. oleifera* flower extract in comparison with untreated L4 gut extract.

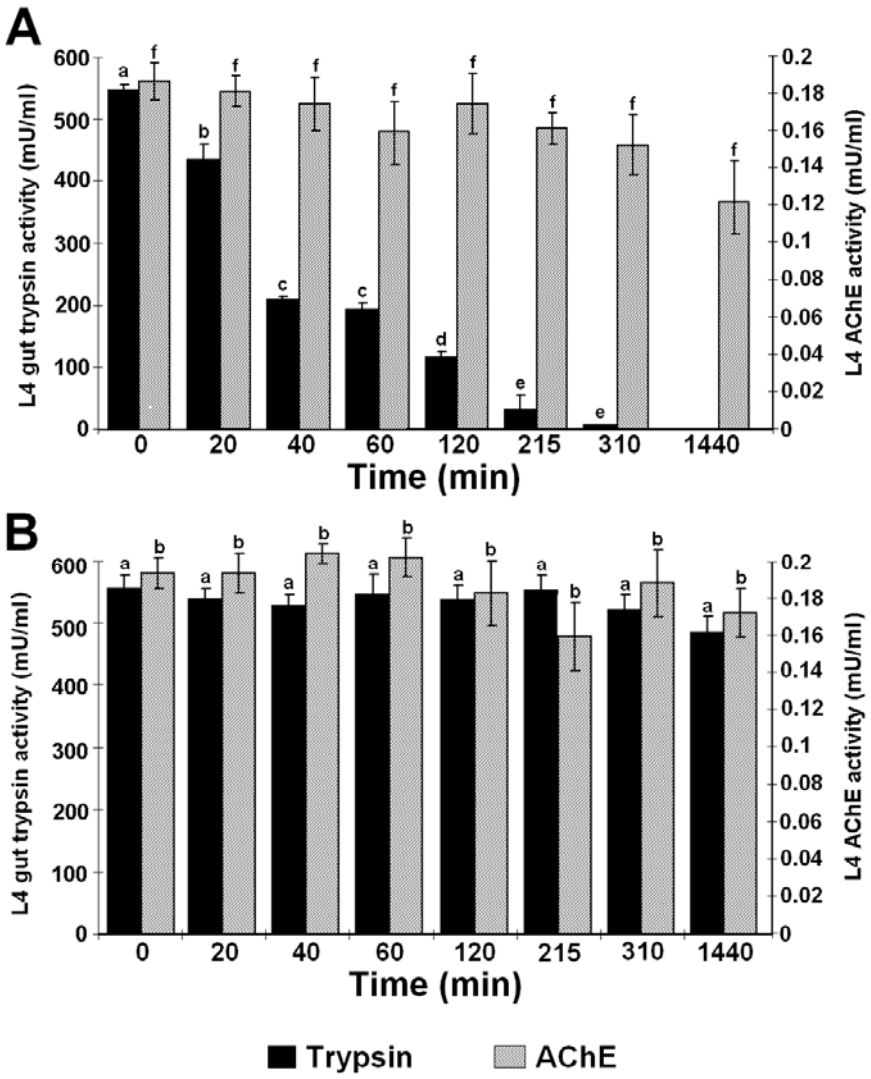

Fig. 4. Effect of *M. oleifera* flower extract on trypsin and AChE activities from live *A. aegypti* L4. Enzyme activities from (A) L4 treated with *M. oleifera* flower extract at LC_{50} (1.851 mg/ml) and (B) L4 from control (distilled water) measured after different incubation times. The bars are represented as the mean of three experiments ± SD. Data were analyzed with a Student´s t-test (Origin 6.0 program) to determine significant differences ($p<0.05$) among treatments. The different letters indicate significant differences between treatments.

4. Discussion

The search for alternative insecticides to control of *A. aegypti* population is of great importance in dengue endemic countries. In this work an aqueous extract of *M. oleifera* flowers was investigated for larvicidal activity on *A. aegypti* L1 and L4. To search for larvicidal agents in the flower extract, assays were conducted to determine the presence of secondary metabolites, lectin as well as trypsin and AChE inhibitors. The activities of trypsin

and AChE from L4 treated with the extract were also evaluated in an attempt to explain larvicidal mechanism.

Larvicidal activity of *M. oleifera* flower extract was only detected on L4. Unlike *M. oleifera* flower extract, those from *Albizzia amara* and *Ocimum basilicum* leaves were toxic on L1 and L4. *M. oleifera* flower extract was more efficient in promoting L4 mortality (LC_{50} of 0.925 %) than *A. amara* and *O. basilucum* extracts which LC_{50} were 7.515% and 5.124%, respectively (Murugan et al., 2007). Ferreira et al. (2009) also demonstrated that a water *M. oleifera* seed extract was larvicidal on *A. aegypti* L3 (LC_{50} of 1,260 μg/ml) and it was suggested the involvement of protein in the larvicidal activity.

M. oleifera flower extract contains the secondary metabolites β-amyrin, β-sitosterol, kaempferol and quercetin which were already described as larvicidal agents. A mixture of β-amyrin and 12-oleanene 3β, 21β-diol was highly effective against *Culex quinquefasciatus* larvae (Nikkon et al, 2010) and β-sitosterol from *Abutilon indicum* was a potential new mosquito larvicidal compound with LC_{50} of 11.49, 3.58 and 26.67 ppm against *A. aegypti*, *A. stephensi* and *C. quinquefasciatus*, respectively (Rahuman et al, 2008). Quercetin (35.7 μg/ml) and kaempferol (30.65 μg/ml) isolated from *Gardenia ternifolia* leaves showed larvicidal effect against *A. aegypti* L2 (Ochieng et al., 2010). Flavonoids bind to trypsin S^1 region through hydrogen bonds and electrostatic interactions; porcine trypsin was inhibited by quercetin, myricetin, morin and kaempferol with inhibitory concentrations (IC) of 0.010 mM, 0.015 mM, 0.027 mM and 0.06 mM, respectively (Maliar et al., 2004).

Santos et al. (2009) detected lectin in saline extract of *M. oleifera* flowers but the *M. oleifera* flower extract here evaluated was prepared with water and did not show hemagglutinating activity; this result indicates that solubilization of flower lectin depends on favorable electrostatic interactions between the charged residues of the lectin and salt ions present in extraction solution similar to other plant lectins (Sá et al., 2009; Napoleão et al.,

2010). The presence of lectin in the *M. oleifera* flower extract was investigated since larvicidal activity on *A. aegypti* has been reported for plant lectins (Coelho et al., 2009; Sá et al., 2009). The absence of lectin indicates that larvicidal activity of *M. oleifera* flower extract did not involve the action of this group of insecticidal proteins.

M. oleifera flower extract contains trypsin inhibitor activity. The presence of a single polypeptide band in SDS-PAGE reveals that one polypeptide was water extracted and reverse zymography showed that this polypeptide corresponds to a trypsin inhibitor (MoFTI). SDS-PAGE also revealed that MoFTI did not contain disulfide bridges in its structure since the same electrophoretic pattern was detected in presence or absence of reducing agent able to break this interaction force. MoFTI may consist in a flower defensive strategy against pathogens as well as may act in the control of protease activity, similarly to other protease inhibitors (García-Carreño, 1996).

The larvicidal activity of *M. oleifera* extract on L4 stimulated the evaluation of L4 enzymes. Trypsin activity from L4 gut was inhibited by *M. oleifera* flower extract. Zymography revealed that activities of three proteases were inhibited and this result is in accordance to the presence of several trypsin-like enzymes in *A. aegypti* larvae as described by Kunz (1978) and Venancio et al. (2009). The sensibility of L4 gut trypsin to commercial quercetin was tested aiming to investigate if the larvicidal activity of *M. oleifera* flower extract was due to trypsin inhibitory activity of this constituent. The enzyme activity was not altered and this result rules out inhibition of *A aegypti* trypsin by the flavonoid as larvicidal mechanism. Larvicidal activity of *M. oleifera* extract may be due to inhibition of gut trypsin by MoFTI. Damage to digestion process by insecticides inhibitors of trypsin and trypsin-like enzymes found in insect guts has been associated to insect mortality and can be a strategy for control of insect population (Carlini and Grossi-de-Sá, 2002). It has been reported that effects of trypsin inhibitor on larvae include reduction in body weight, decreasing in the survival rate

as well as delay and disruption of development (Macedo et al., 2002; Macedo et al., 2003; Bhattacharyya et al., 2007a, 2007b; Oliveira et al., 2007; Ramos et al., 2009). The absence of larvicidal activity of *M. oleifera* flower extract on L1 may be explained by low expression of trypsin in this larval stage. The increasing of trypsin biosynthesis in *A. aegypti* midgut during larval development was reported by Borovsky and Meola (2004) that determined trypsin activities (ng/gut) of 2.4, 17.4, 58.6 and 60 in L1, L2, L3 and L4, respectively.

L4 extract contains AChE activity, enzyme target of inorganic insecticides (Braga and Valle, 2007). Flavonoids are able to reversibly inhibit human butyryl- and acetyl-cholinesterases (Khan et al., 2009; Katalinić et al., 2010) and presence of quercetin and kaempferol in *M oleifera* flower extract stimulated us to investigate if AChE activity from L4 would be sensitive to *M. oleifera* extract. L4 AChE was not inhibited by extract and this result is in according with the absence of electric eel AChE inhibitor in the flower extract demonstrated here. The detected L4 AChE inhibition by organophosphorous of recognized action on *A. aegypti* larvae assegurates that assay conditions were proper for detection of enzyme activity. The data shows that larvicidal activity of *M. oleifera* flower extract was not due to AChE inhibition by flavonoids.

L4 were treated with *M. oleifera* flower extract aiming to determine if the effect on L4 gut trypsin and L4 AChE activities will be also detected by assay at *in vivo* conditions. Increase in incubation period was accompanied by progressive reduction of L4 gut trypsin activity and no alteration in AChE activity. Similarly to *M. oleifera* flower extract, a trypsin inhibitor from *Archidendron ellipticum* seeds promoted a decrease of trypsin-like activity on *Spodoptera litura* larvae reared on diet containing the inhibitor in comparison to larvae fed with control diet, demonstrating the influence of trypsin inhibitor on larval gut physiology (Bhattacharyya et al., 2007a). The results from *in vivo* assay corroborates with the hypothesis

that larvicidal mechanism of flower extract involves the inhibition of gut trypsin and no interference on L4 AChE activity.

5. Conclusions

This study points out *M. oleifera* flower aqueous extract as a new biodegradable tool for control of *A. aegypti* larvae. The extract contains MoFTI, a protein with trypsin inhibitor activity; *in vivo* assay indicates that the larvicidal mechanism involves gut trypsin inhibition by MoFTI.

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6. CONCLUSÃO

Os resultados deste trabalho apontam o extrato aquoso de flores de *M. oleifera* como uma nova ferramenta biodegradável para uso no controle de populações de larvas de *A aegypti*. O extrato contem MoFTI, uma proteína com atividade inibidora de tripsina; ensaio em condições *in vivo* indica que o mecanismo larvicida do extrato envolve a inibição da tripsina do intestino das larvas por MoFTI.