UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
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TESE DE DOUTORADO

EFEITO DA LECTINA SOLÚVEL EM ÁGUA DE SEMENTES DE *Moringa oleifera* (WSMoL) SOBRE A OVIPOSIÇÃO DE *Aedes aegypti* E AVALIAÇÃO DA ATIVIDADE OVICIDA

NATALY DINIZ DE LIMA SANTOS

ORIENTADORA: Profa. Dra. Patrícia Maria Guedes Paiva
COORIENTADORA: Profa. Dra. Daniela Maria do Amaral Ferraz Navarro

RECIFE
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BANCA EXAMINADORA:

_____

Profa. Dra. Patrícia Maria Guedes Paiva (Orientadora)
(Universidade Federal de Pernambuco - UFPE)

_____

Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho (Titular Interno)
(Universidade Federal de Pernambuco - UFPE)

_____

Dr. Emmanuel Viana Pontual (Titular Externo)
(Universidade Federal de Pernambuco - UFPE)

_____

Profa. Dra. Michele Dalvina Correia da Silva (Titular Externo)
(Universidade Federal Rural do Semi-Árido - UFERSA)

_____

Prof. Dr. Elizeu Antunes dos Santos (Titular Externo)
(Universidade Federal do Rio Grande do Norte – UFRN)

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Dedico a minha mãe, ***Marly Diniz de Lima,***
pela dedicação e amor incondicional.
Essa conquista é nossa!

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A MUSA DAS LECTINAS

Poema de Noel Tavares
Dedicado à doutoranda Nataly Santos

Sob a sombra da Moringa
A fonte d'água respinga
Antes barrenta, hoje limpa
Como água de cacimba.
Sementes com proteínas
Se transformam em lectinas
E o forte agente ovicida
Não poupa a vida da pupa
Nem da larva, que agoniza,
E o *Aedes aegypti*,
Vira colônia de machos
Sem a prole feminina
Como o vetor desatina,
a dengue logo declina
Enquanto em sua oficina
A musa das lectinas
sua a bata e a camisa;
Deus ajuda a quem pesquisa.

Salve as lectinas!

RESUMO

O mosquito *Aedes aegypti* é o vetor de três arboviroses denominadas febre Chikungunya, febre amarela e dengue. Esta última é uma das doenças infecciosas mais importantes mudialmente e epidemias de dengue vêm ressurgindo em países tropicais e subtropicais. O controle do vetor é a melhor estratégia de combate à doença. Os inseticidas químicos atualmente utilizados são tóxicos para o meio ambiente e seu uso excessivo tem levado ao desenvolvimento de populações resistentes. Neste cenário, tem aumentado a busca por inseticidas naturais para controle de populações de *A. aegypti*. Lectinas, proteínas que reconhecem carboidratos, isoladas de plantas apresentam atividade inseticida. A lectina solúvel em água isolada de sementes de *Moringa oleifera* (WSMoL) promoveu mortalidade (CL_{50} de 0,197 mg/mL) contra larvas de *A. aegypti* no quarto estágio (L4) e extrato das sementes contendo WSMoL retardou o desenvolvimento larval. Uma vez conhecidos os efeitos deletérios de WSMoL sobre as larvas dessa espécie de mosquito, o presente trabalho investigou: 1) o efeito de WSMoL sobre a oviposição de fêmeas grávidas de *A. aegypti* em condições de laboratório e utilizando ovitrampas (armadilhas para captura de ovos) em ensaios de campo simulado; 2) a eficácia de WSMoL em aumentar a eficiência de armadilhas para captura de fêmeas (MosquiTRAP^{TM}); 3) a atividade ovicida de WSMoL sobre ovos de *A. aegypti* frescos e estocados; 4) o envolvimento de sensores olfatórios das fêmeas na resposta de oviposição frente a WSMoL; 5) a influência de diferentes doses de radiação gama nos efeitos da lectina sobre larvas, ovos e oviposição. A lectina foi isolada através de protocolo previamente estabelecido. Ensaio de oviposição em laboratório foi realizado em gaiolas (33 x 21 x 30 cm) contendo um par de recipientes de vidro, sendo um deles preenchido com 50 mL do extrato de sementes, fração proteica 0-60% ou WSMoL isolada (concentração final: 0,1 mg/mL de proteínas) e o outro com 50 mL de água destilada (controle negativo). Cada recipiente continha uma peça de papel de filtro como suporte para oviposição. As fêmeas grávidas (25) foram liberadas no interior das gaiolas e após 16 h o número de ovos foi determinado. A eclodibilidade dos ovos depositados pelas fêmeas foi também avaliada. Para isso, 50 ovos foram selecionados e imersos na mesma solução em que foram depositados. Após 144 h, o número de larvas foi registrado. A resposta de oviposição das fêmeas grávidas em condições de campo simulado foi avaliada em uma área experimental localizada no campus da Universidade Federal de Minas Gerais contendo grandes gaiolas (2,5 x 2,5 x 2 m) em seu interior. Em cada gaiola foram colocadas duas ovitrampas contendo palhetas de madeira, uma preenchida com WSMoL (0,1 mg/mL) e outra com água de torneira (controle negativo). Em seguida, 40 fêmeas grávidas previamente selecionadas em laboratório foram liberadas no centro da gaiola. Após 2 h, as ovitrampas foram retiradas e o número de ovos determinado. Ensaios utilizando par de ovitrampas contendo infusão de *Panicum maximum* (um eficiente estimulante de oviposição) e água destilada foram realizados como controle positivo. Amostra da lectina foi avaliada quanto à presença de compostos voláteis por cromatografia gasosa acoplada à espectrometria de massas (GC-MS) e ensaio de olfatometria utilizando olfatômetro horizontal de dupla escolha foi realizado para investigar o envolvimento de sensores olfatórios na resposta das fêmeas frente a WSMoL. MosquiTRAP™ (versão 1.0) foi utilizada para avaliar a eficácia de WSMoL como um atraente para captura de fêmeas de *A. aegypti* em condições de campo simulado. As armadilhas foram preenchidas com WSMoL (teste), infusão de *P. maximum* (controle positivo) ou água de torneira (controle negativo). Quarenta fêmeas grávidas foram liberadas no centro de cada gaiola contendo um par de armadilhas. O número de fêmeas capturadas foi avaliado após 180 min. A capacidade do extrato de sementes, fração 0-60% e WSMoL em impedir a eclosão de ovos estocados de *A. aegypti* foi também avaliada. Por fim, foi determinado o efeito da irradiação gama de WSMoL, nas doses de 10 mGy e 10 Gy, sobre as

atividades larvicida, ovicida e efeito na oviposição. As preparações brutas (extrato de sementes e fração proteica 0-60%) não afetaram a oviposição de *A. aegypti*. Diferentemente, WSMoL apresentou significante ($p<0,05$) efeito estimulante sobre a oviposição tanto em condições de laboratório (73 ± 2,1%) quanto em condições de campo simulado (65 ± 14%) utilizando ovitrampas. O efeito de WSMoL em condições de campo foi similar ao da infusão de *P. maximum* (67 ± 11 %). Nas ovitrampas contendo WSMoL, o número de ovos presentes na superfície do líquido foi maior que o de ovos depositados na palheta. A presença de compostos voláteis não foi detectada na solução de WSMoL e os resultados do ensaio de olfatometria revelaram que as fêmeas não foram atraídas por WSMoL através de resposta envolvendo sensores olfativos. A não-volatilidade de WSMoL, a presença da maioria dos ovos na superfície do líquido nas ovitrampas contendo WSMoL e os resultados do ensaio de olfatometria indicam que os mecanismos de percepção de WSMoL pelas fêmeas provavelmente envolvem sensores de contato (gustatórios). WSMoL não interferiu na eficiência da armadilha MosquiTRAP^{TM} para captura de fêmeas grávidas, provavelmente devido ao fato de que nesse tipo de armadilha, as fêmeas não podem entrar em contato com a solução. Extrato de sementes, fração proteica 0-60% e WSMoL reduziram a eclosão dos ovos depositados no ensaio de oviposição em condições de laboratório. Extrato, fração e WSMoL também apresentaram atividade ovicida contra ovos estocados, sendo os valores de CE_{50} (concentração efetiva de proteínas necessária para reduzir em 50% o número de ovos eclodidos) de 0,28, 0,18 e 0,1 mg/mL de proteínas, respectivamente. Análise por microscopia óptica revelou que WSMoL interferiu no desenvolvimento do embrião em ovos frescos, bem como causou a morte dos embriões presentes nos ovos estocados. A irradiação de WSMoL na dose de 10 mGy potencializou as atividades hemaglutinante, larvicida e ovicida da lectina, enquanto o efeito estimulante de oviposição foi abolido. Em conclusão, o presente trabalho relata, pela primeira vez, os efeitos ovicida e estimulante de oviposição de uma lectina e demonstra a atuação de uma molécula fixa (não volátil) como uma pista química utilizada pelas fêmeas grávidas de *A. aegypti* para seleção do sítio de oviposição. As atividades estimulante de oviposição, ovicida e larvicida de WSMoL fazem desta lectina uma molécula com excelentes características para controle de *A. aegypti*.

Palavras-chave: *Moringa oleifera*; mosquito da dengue; atividade ovicida; estimulante de oviposição; olfatometria; ovitrampa; radiação gama.

ABSTRACT

The mosquito *Aedes aegypti* is the vector of three arboviruses named Chikungunya fever, yellow fever and dengue. This last is one of the most important infectious diseases worldwide and dengue epidemics are re-emerging in tropical and subtropical countries. The vector control is the best strategy to combat the disease. The chemical insectides currently used are toxic to environment and their excessive use has led to developing of resistant populations. In this scenario, the search for natural insecticides for *A. aegypti* control has increased. Lectins, carbohydrate-binding proteins, isolated from plants show insecticidal activity. The water-soluble lectin isolated from *Moringa oleifera* seeds (WSMoL) promoted mortality (LC_{50} of 0.197 mg/mL) of *A. aegypti* fourth-stage larvae (L4) and seed extract containing WSMoL delayed larval development. Once known the deleterious effects of WSMoL on larvae of this mosquito species, the present work investigated: 1) the effect of WSMoL on oviposition by *A. aegypti* gravid females under laboratory conditions and using ovitraps (traps to capture eggs) in assays under semi-field conditions; 2) the efficacy of WSMoL in enhance the efficiency of traps used to capture females (MosquiTRAP^{TM}); 3) the ovicidal activity of WSMoL on fresh and stored *A. aegypti* eggs; 4) the involvement of female olfactory sensilla in the oviposition response toward WSMoL; 5) the influence of different doses of gamma radiation on the effects of lectin on larvae, eggs and oviposition. Oviposition assay in laboratory was performed in cages (33 x 21 x 30 cm) containing a pair of glass vessels; one filled with 50 mL of seed extract, 0-60% protein fraction or isolated WSMoL (final concentration: 0.1 mg/mL of protein) and the other with 50 mL of distilled water (negative control). Each vessel contained a piece of filter paper as support for oviposition. The gravid females (25) were released inside the cages and after 16 h the number of eggs was determined. The hatchability of the eggs laid by females was also evaluated. For this, 50 eggs were selected and imersed in the same solution at which they were laid. After 144 h, the number of larvae was recorded. The oviposition response by gravid females under semi-field conditions was evaluated in an experimental area located at the campus of the *Universidade Federal de Minas Gerais* containing wide cages (2.5 x 2.5 x 2 m) inside. In each cage, two ovitraps containing a wood paddle were placed and one was filled with WSMoL (0.1 mg/mL) and another with tap water (control). Next, 40 gravid females previously selected in laboratory were released in the center of the cage. After 2 h, the ovitraps were removed and the number of eggs was determined. Assays using a pair of ovitraps containing *Panicum maximum* infusion (an effective oviposition-stimulant) and tap water were performed as positive control. Lectin sample was evaluated for presence of volatile compounds by gas chromatography coupled to mass spectrometry (GC-MS) and olfactometry assay using double-choice horizontal olfactometer was performed aiming to investigate the involvement of olfactory sensilla in the response of females toward WSMoL. The MosquiTRAP™ (Version 1.0) was used to evaluate the efficacy of WSMoL as an attractant for capturing *A. aegypti* females under semi-field conditions. The traps were filled with WSMoL (test), *P. maximum* infusion (positive control) or tap water (negative control). Forty gravid *A. aegypti* females were released in the center of each cage containing a pair of traps. The number of captured females was evaluated in the cages after 180 min. The ability of seed extract, 0-60% fraction and WSMoL to impair the hatching of stored *A. aegypti* eggs was also evaluated. Finally it was determined the effect of gamma irradiation of WSMoL, at doses of 10 mGy and 10 Gy, on the larvicidal and ovicidal activities as well as effect on oviposition. The crude preparations (seed extract and 0-60% fraction) did not affect the *A. aegypti* oviposition. Differently, WSMoL showed a significant ($p<0.05$) oviposition-stimulant effect both under laboratory conditions (73 ± 2.1 %) and in ovitraps at field-simulated conditions (65 ± 14 %). The effect at field conditions was similar to that of *P. maximum* infusion (67 ± 11 %). In ovitraps containing WSMoL, the number of

eggs found at liquid suface was higher than that of eggs laid on the paddles. The presence of volatile compounds was not detected in WSMoL solution and the results from olfatometry assay revealed that the females were not attracted by WSMoL through response involving olfactory sensilla. The non-volatilty of WSMoL, the presence of eggs majority at the liquid surface in WSMoL ovitraps and the results from olfatometry assay indicated that the mechanisms of WSMoL perception by females probably involve contact (gustatory) sensilla. WSMoL did not interfere in the efficiency of MosquiTRAP^{TM} in capture gravid females, probably due to the fact that in this type of trap the females cannot have contact with the solution. Seed extract, 0-60% protein fraction and WSMoL reduced the hatching of eggs laid in the oviposition assay under laboratory conditions. Extract, fraction and WSMoL also showed ovicidal activity against stored eggs, being the EC_{50} (effective protein concentration required to reduce the number of hatched eggs in 50%) values of 0.28, 0.18 and 0.1 mg/mL, respectively. Analysis by optical miscroscopy revealed that WSMoL interfered on embryo developmente in fresh eggs as well as caused the death of the embryos present in stored eggs. The irradiation of WSMoL at 10 mGy dose potencialized the hemagglutinating, larvicidal and ovicidal activities of the lectin while the oviposition-stimulant effect was abolished. In conclusion, the present work reports for the first time the ovicidal and oviposition-stimulant effects of a lectin and demonstrate the acting of a fix (non-volatile) molecule as a chemical cue used by *A. aegypti* gravid females for selection of the oviposition site. The oviposition-stimulant, ovicidal and larvicidal activities of WSMoL make this lectin a molecule with excellent characteristics for *A. aegypti* control.

Keywords: *Moringa oleifera*; dengue mosquito; ovicidal activity; oviposition-stimulant; olfactometry; ovitrap; gamma radiation.

LISTA DE FIGURAS

Figure 10. Mortality of *Aedes aegypti* L4 in incubation with lectins from *Myracrodruon urundeuva* heartwood (A) and bark (B) as well as *Moringa oleifera* seeds (C). Lethal lectin concentration required to kill 50% (LC_{50}) of larvae in 24h was determined by probit analysis with a reliability interval of 95%.

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SUMÁRIO

1. INTRODUÇÃO

Insetos vetores, especialmente os mosquitos, são responsáveis pela disseminação de sérias doenças humanas em regiões tropicais. Interesse particular é dado à espécie *Aedes aegypti* (Culicidae), por ser o principal vetor de muitas arboviroses (viroses transmitidas por artrópodes, principalmente insetos ou ácaros) como a febre amarela, febre Chikugunya e a dengue (CONSOLI & OLIVEIRA, 1998; CALJON *et al.*, 2013).

A dengue é a arbovirose de maior incidência no mundo, sendo endêmica em todos os continentes, exceto na Europa. Atualmente, é uma das doenças infecciosas mais freqüentes encontradas nos países em desenvolvimento, e as taxas de soroprevalência estão acima de 50% em países endêmicos (TAUIL, 2002). É uma infecção reemergente causada por diferentes sorotipos virais e que se manifesta de diferentes formas clínicas, algumas graves e letais, como a febre de dengue hemorrágica (MICIELI & CAMPOS, 2003). A distribuição e a abundância dessa doença são fortemente influenciadas pela urbanização descontrolada, sistemas de distribuição de água e gestão de resíduos inadequados, promovendo altas densidades e dispersão dos mosquitos vetores entre diferentes áreas geográficas (WEAVER & VASILAKIS, 2009). Como não há a diponibilidade de vacinas, alguns agentes terapêuticos têm sido desenvolvidos e administrados em voluntários em estágios pré- clínicos e clínicos da doença (BENTSI-ENCHILL *et al*., 2013). Dessa forma, o controle do vetor é a única solução disponível para redução da transmissão desse vírus (LEE *et al*., 2013).

Com o surgimento de formas resistentes do mosquito aos inseticidas convencionais utilizados, tem crescido a procura por substâncias naturais que sejam efetivas no combate ao mosquito adulto, ovos e/ou larvas de *A. aegypti*, bem como interfiram no comportamento de oviposição e hematofágico das fêmeas, e que sejam isentas de toxicidade para o meio ambiente. As plantas possuem compostos ou substâncias que podem afetar a biologia, o

desenvolvimento e a reprodução dos insetos. A síntese dessas substâncias pode estar relacionada a uma função auto-ecológica de defesa. As funções dos produtos naturais das plantas podem ser múltiplas, envolvendo elementos do metabolismo primário e secundário. Entre as proteínas de defesa, estão incluídas enzimas (tais como as quitinases), as lectinas e os inibidores de enzimas digestivas, dentre outras (CARLINI & GROSSI-DE-SÁ, 2002; WINK, 2003). O estudo de novas moléculas derivadas de fontes vegetais, com diferentes atividades biocidas, pode auxiliar no combate aos mosquitos vetores.

As lectinas são proteínas que se ligam a carboidratos e apresentam alto potencial biotecnológico, incluindo ação tóxica sobre insetos. Sementes de *Moringa oleifera* são popularmente usadas para o tratamento de água barrenta e contêm lectinas com atividade coagulante (SANTOS *et al.*, 2009; FERREIRA *et al.*, 2011). A alta solubilidade em água da lectina WSMoL (do inglês *water soluble Moringa oleifera lectin*) estimulou a avaliação do efeito da mesma sobre o ciclo biológico do *A. aegypti*. WSMoL apresentou atividade larvicida sobre larvas no quarto estágio e extrato das sementes contendo WSMoL atrasou o desenvolvimento das larvas (COELHO *et al.*, 2009).

Uma vez conhecidos os efeitos deletérios de WSMoL sobre as larvas dessa espécie de mosquito, o presente trabalho investigou, em síntese: 1) o efeito de WSMoL sobre a oviposição de fêmeas grávidas de *A. aegypti* em condições de laboratório e utilizando ovitrampas (armadilhas para captura de ovos) em ensaios de campo simulado; 2) a atividade ovicida de WSMoL sobre ovos de *A. aegypti* frescos e estocados; 3) o envolvimento de sensores olfatórios das fêmeas na resposta de oviposição frente a WSMoL; 4) a eficácia de WSMoL em aumentar a eficiência de armadilhas para captura de fêmeas (MosquiTRAP^{TM}); 5) o efeito de diferentes doses de radiação gama nas atividades larvicida, ovicida e estimulante de oviposição de WSMoL.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Arboviroses transmitidas pelo mosquito *Aedes aegypti*

Os mosquitos constituem um grupo altamente diversificado e relevante na classe dos insetos, pois transmitem diferentes patógenos causadores de doenças à saúde humana, em áreas tropicais e subtropicais (CALJON *et al*., 2013). As arboviroses são doenças infecciosas virais transmitidas por vetores artrópodes a hospedeiros vertebrados, em áreas periurbanas ou urbanas, devido à introdução e elevada densidade populacional dos vetores infectados nessas áreas (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2012).

O mosquito *Aedes aegypti* (Linnaeus, 1762) é vetor de três das principais arboviroses que despertam interesse aos órgãos públicos de Saúde atualmente: a febre Chikungunya, a febre amarela e a dengue. Em razão da importância epidemiológica e do grau de participação de *A. aegypti*, torna-se necessário o incentivo e a utilização de estratégias de manejo populacional deste inseto, visando diminuir a incidência da doença, bem como os índices de óbitos registrados (GUBLER, 2011).

2.1.1. Febre Chikungunya

A febre Chikungunya é uma doença reemergente e em constante expansão, a qual foi descrita pela primeira vez na Índia em 1824 e cuja primeira epidemia ocorreu na Tânzania em 1953. Após esse período, já foram relatadas epidemias em várias áreas da África e da Ásia (Figura 1), onde a doença é considerada endêmica, embora os ciclos de transmissão nesses continentes sejam consideravelmente diferentes. O termo “chikungunya”, proveniente do idioma Makonde (falado em algumas regiões de Moçambique) significa “aqueles que se

dobram" e se refere à forte dor que a doença causa nas articulações (KUCHARZ & CEBULA-BYRSKA, 2012).

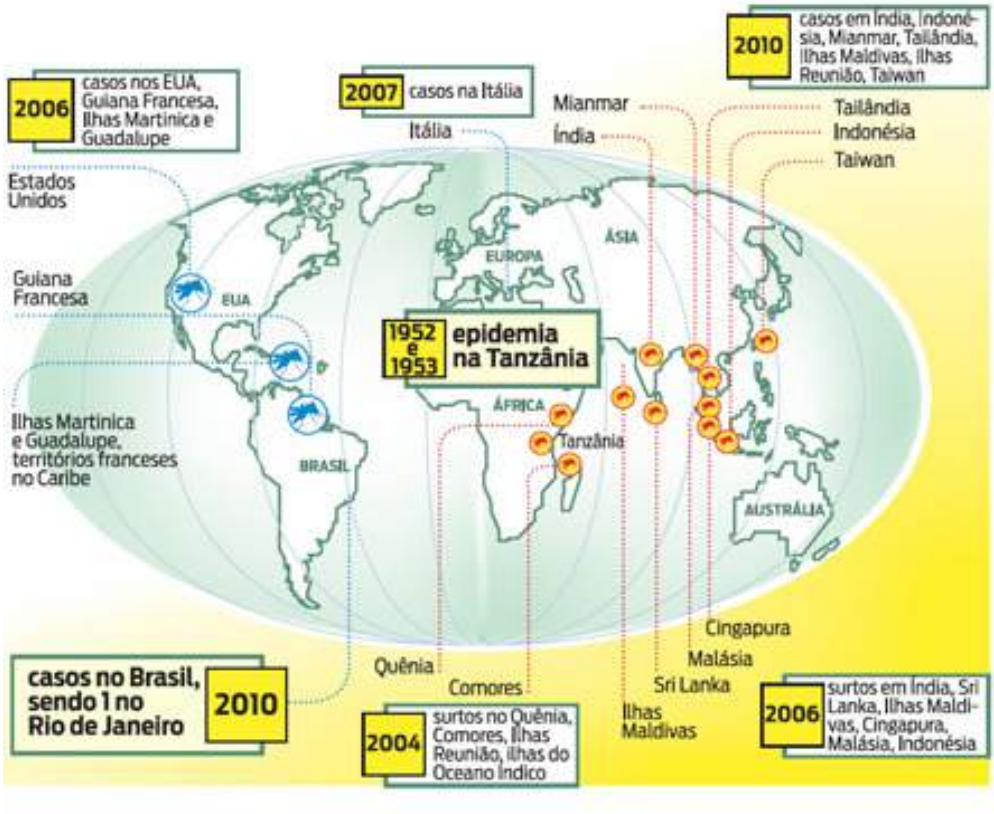

Figura 1. Distribuição global da febre Chikungunya.

Adaptada de: http://alagoasreal.blogspot.com.br/2010/12/expectativa-por-mais-casos-da-nova.html

Esta doença é causada pelo vírus de RNA (fita simples de polaridade positiva) Chikungunya (CHIKV), o qual pertence à família Togaviridae, gênero *Alphavirus*. Na Ásia, os homens são os hospedeiros primários do CHIKV, sendo o *A. aegypti* descrito como vetor primário em epidemias urbanas (BIRENDRA *et al*, 2012). É uma doença de baixa letalidade, mas com manifestações clínicas e sintomáticas que deixam os pacientes debilitados principalmente pela presença, na fase aguda, de febre alta e de dores intensas nas articulações, avançando até a artrite crônica, com alterações neurológicas e hematológicas (BIRENDRA *et al*, 2012). No momento, nenhuma vacina tem sido descrita para o tratamento dessa doença.

Muitos casos de febre Chikungunya foram relatados em diferentes regiões de Madagascar e, nos períodos epidêmicos, a espécie *Aedes albopictus* também foi identificada como vetor (RAHARIMALALA *et al.*, 2012). No Brasil, a entrada do vírus da febre Chikungunya está relacionada ao aumento da densidade de *A. aegypti*, como também a introdução do vírus através do intenso fluxo de viajantes internacionais. Já foram registrados três casos dessa doença nos estados do Rio de Janeiro e São Paulo, mas não houve a disseminação do vírus pelo país. Tanto o *A. aegypti* quanto o *A. albopictus*, que estão presentes nas Américas, podem transmitir o vírus CHIKV nas diferentes regiões do continente (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2010).

2.1.2 Febre Amarela

A febre amarela é uma doença viral infecciosa aguda, de curta duração, mas de gravidade variável, causada por um arbovírus da família Flaviviridae, gênero *Flavivirus*. É encontrada em países da África, das Américas Central e do Sul e pode apresentar-se sob duas modalidades: urbana e silvestre. A espécie *A. aegypti* transmite a febre amarela urbana, enquanto os mosquitos *Haemagogus* e *Sabethes* transmitem a febre amarela silvestre (TOMORI, 2002).

No Brasil, a febre amarela foi erradicada e apesar de não serem relatados surtos urbanos nas Américas desde 1954, a presença do mosquito mantém o risco de epidemia. Na África, é comum a ocorrência de surtos urbanos e rurais, podendo causar milhares de mortes por ano. Diferentemente da febre Chikungunya, há vacina disponível para a prevenção da febre amarela e o certificado de vacinação contra esta doença, é um dos principais requisitos de entrada em muitos países (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2011).

2.1.3 Dengue

A dengue destaca-se entre as enfermidades reemergentes e é considerada um dos maiores problemas de Saúde Pública no Brasil e no mundo, principalmente pelos inúmeros índices de casos registrados anualmente, como também pela elevada letalidade (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2012). Trata-se, caracteristicamente, de uma enfermidade presente nos centros urbanos de regiões tropicais e subtropicais, uma vez que as condições ambientais e características sócio-econômicas dessas regiões favorecem a proliferação e o desenvolvimento do vetor (GUBLER, 2002).

Aproximadamente 3,6 bilhões de pessoas vivem em áreas de risco e 50 a 100 milhões de infecções ocorrem anualmente. Nos primeiros meses de 2013, já foram registrados 204.650 casos de dengue no Brasil, um aumento de 190% dos casos notificados comparados aos dados de 2012 (70.489). Contudo, houve uma redução de 44% nos casos graves e de 20% nos óbitos (MINISTÉRIO DA SAÚDE, 2013).

É uma doença de grande complexidade, devido às interações entre humanos, mosquitos e vários sorotipos virais, como também pelas estratégias de sobrevivência do mosquito vetor (MEDEIROS *et al*., 2011). É causada por um vírus do gênero *Flavivirus* que é encontrado em quatro sorotipos antigenicamente diferentes, denominados DENV-1, DENV-2, DENV-3, DENV-4. A infecção por um desses sorotipos virais confere proteção permanente para o mesmo sorotipo e imunidade parcial e temporária para os outros três. No momento, os 4 sorotipos circulam concomitantemente no Brasil (Figura 2) e são responsáveis pela alta incidência da doença nas Américas (WILDER-SMITH *et al.*, 2010).

O vírus da dengue é de RNA, fita simples de polaridade positiva, e possui genoma de aproximadamente 11 kb organizado de uma forma que confere uma proficiente propriedade replicativa (PERERA *et al.*, 2008). Os diferentes sorotipos virais podem manifestar-se

clinicamente, sob duas formas principais: a dengue clássica ou febre de dengue (sintomática ou assintomática) e as formas graves e letais, como a febre hemorrágica de dengue, às vezes com síndrome de choque de dengue (ARAÚJO *et al*., 2009).

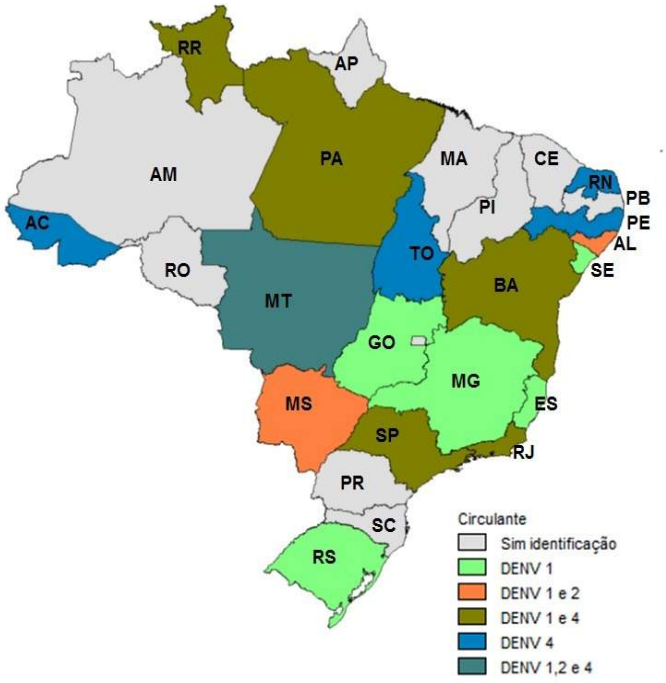

Figura 2. Representação dos sorotipos virais da dengue (DENV) em circulação no Brasil, em 2012. (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2012).

O vírus da dengue é transmitido pelas fêmeas dos mosquitos do gênero *Aedes*. O principal vetor nas Américas é o mosquito *A. aegypti* (vetor primário), embora o *A. albopictus* também desempenhe um importante papel na Ásia e atue como vetor secundário. No Brasil, as duas espécies estão presentes em alta densidade, e assim podem servir como vetores da doença (JOHNSON *et al*., 2002). O homem e outros primatas são os hospedeiros vertebrados, o que caracteriza o hábito antropofilíco do vetor (GUBLER, 2002).

A transmissão da doença é iniciada quando a fêmea é contaminada ao realizar um repasto sanguíneo em um indivíduo infectado que esteja na fase virêmica da doença. Após um

período de 10 a 14 dias, a fêmea se torna capaz de transmitir o vírus por toda sua vida através de suas picadas (SIM *et al.*, 2012). Embora a transmissão vertical do vírus seja relatada, os mosquitos adquirem principalmente o DENV pela alimentação sanguínea de humanos infectados (ANGEL, 2008). Primeiramente, o vírus infecta o epitélio intestinal dos mosquitos e se replica para, em seguida, espalhar-se através da hemolinfa e se replicar em outros órgãos, tais como corpo gorduroso e traquéia, finalmente infectando as glândulas salivares aproximadamente 10-14 dias após o repasto sanguíneo (SALAZAR *et al.*, 2007). Na saliva, o DENV pode ser inoculado em hospedeiros humanos quando o mosquito se alimenta, provocando assim, a disseminação da doença (RIBEIRO & ARCA, 2009).

Desde o início dos anos 70, a Organização Mundial da Saúde (OMS) está envolvida, de maneira bastante ativa, no desenvolvimento e na promoção de estratégias de tratamento e controle da doença, que é endêmica em mais de 100 países da Ásia, África e América (WILDER-SMITH & GUBLER, 2008). Diferentemente da febre amarela, não há vacina contra o vírus da dengue, apesar de muitas pesquisas serem realizadas visando o desenvolvimento de vacinas e anti-virais que contribuam para o entendimento da patogênese viral, seguida de intervenção farmacêutica. Logo, o controle do vetor é uma estratégia essencial de controle da doença (HERRERO *et al*., 2013).

2.2 Biologia do vetor *Aedes aegypti*

O *A. aegypti* pertence ao Filo Arthropoda, Classe Insecta/Hexapoda, Ordem Diptera, Família Culicidae. É uma espécie cosmopolita, com ampla ocorrência em regiões tropicais e subtropicais, mas é originário do norte da África (FORATTINI & BRITO, 2003). Possui hábito doméstico e como parte do seu comportamento sinantrópico, o mosquito se reproduz principalmente nas paredes de recipientes domésticos com água armazenada e a fêmea precisa

de grandes quantidades de sangue (hematofagia) para realizar a oviposição (CONSOLI & OLIVEIRA, 1998).

É essencialmente um mosquito urbano, pois ocorre em maior abundância em cidades, vilas e povoados. Entretanto, no Brasil, México e Colômbia, já foi localizado em zonas rurais, provavelmente transportado de áreas urbanas em vasos domésticos onde se encontravam ovos e larvas (CONSOLI & OLIVEIRA, 1998; BRAKS *et al*., 2004). Esse vetor é conhecido nas Américas por seu alto grau de antropofilia e normalmente está associado à presença humana, pois nas habitações humanas, ou próximo a elas, as fêmeas do mosquito podem encontrar abrigo e hospedeiros para o repasto sanguíneo (FORATTINI, 2002). Os dípteros possuem um complexo sistema de glândulas salivares, e é este sistema que permite que vírus, protozoários e outras formas de vida se utilizem deles como veículos de transmissão até os hospedeiros vertebrados (REITER, 2001).

A alta plasticidade da espécie demonstra a capacidade de adaptação a diferentes situações ambientais, tendo sido encontrados adultos em altitudes elevadas e larvas em águas poluídas. Diferentes criadouros artificiais têm sido descritos para esta espécie, como pneus, vasos de plantas, garrafas entre outros reservatórios plásticos. Essas condições favorecem o aumento da densidade populacional do vetor (TAUIL, 2002; CHAVES *et al.*, 2013).

Os mosquitos se desenvolvem através de metamorfose completa (holometabolia), e o ciclo de vida do *A. aegypti* compreende quatro fases: ovo, larva (quatro estágios denominados L1, L2, L3 e L4), pupa e adulto (Figura 3), com alimentação e hábitos distintos (LOZOVEI, 2001). Em condições favoráveis de temperatura, umidade e disponibilidade de alimento, o tempo transcorrido entre o estágio de ovo a adulto varia em média de 10 a 13 dias (FORATTINI, 2002).

Os estágios imaturos e dependentes de água são representados pelos ovos, larvas e pupas. Na forma adulta, o inseto se apresenta na forma alada. Os adultos possuem o corpo

coberto por escamas escuras e prateadas e possuem hábitos diurnos com pico da atividade hematofágica durante os períodos matutino (entre 6 e 7h) e vespertino (entre 17 e 19h) (CÔNSOLI & OLIVEIRA, 1998; FORATTINI, 2002).

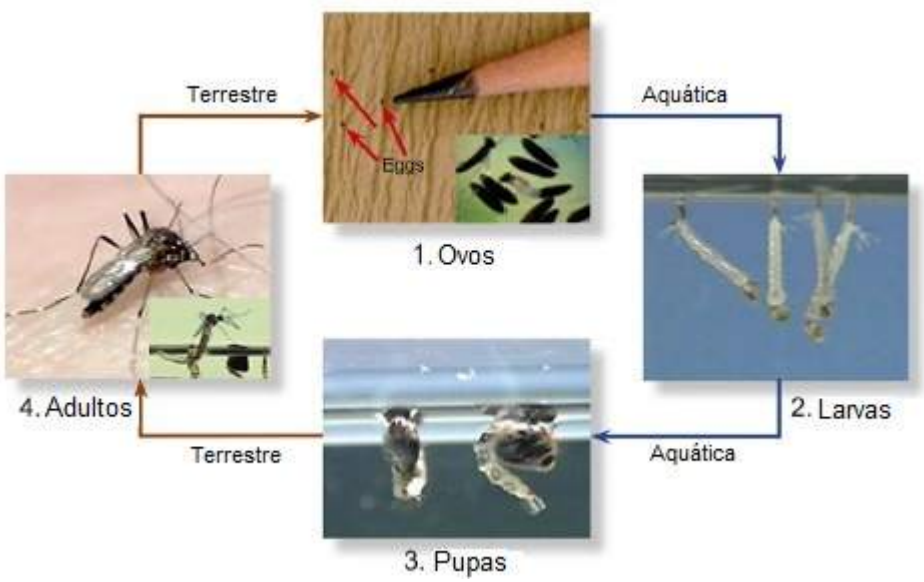

Figura 3. Ciclo biológico de *Aedes aegypti*

(URDANETA-MARQUEZ & FAILLOUX, 2011).

Os ovos medem, aproximadamente, 1 mm de comprimento, apresentando um contorno alongado e fusiforme. São depositados, individualmente, nas paredes internas dos depósitos que servem como criadouros, próximos à superfície da água. No momento da postura os ovos são brancos (Figura 4A), mas, rapidamente, adquirem a cor negra brilhante (Figura 4B). Os ovos são bastante resistentes à dessecação por muitos anos e essa capacidade é um sério obstáculo para o combate ao vetor e um importante fator de adaptação a ambientes adversos. Esta condição permite que os ovos sejam transportados a grandes distâncias (dispersão passiva), em recipientes secos, tornando-se assim o principal meio de dispersão do inseto (CONSOLI & OLIVEIRA, 1998; MINISTÉRIO DA SAÚDE, 2001; FORATTINI, 2002).

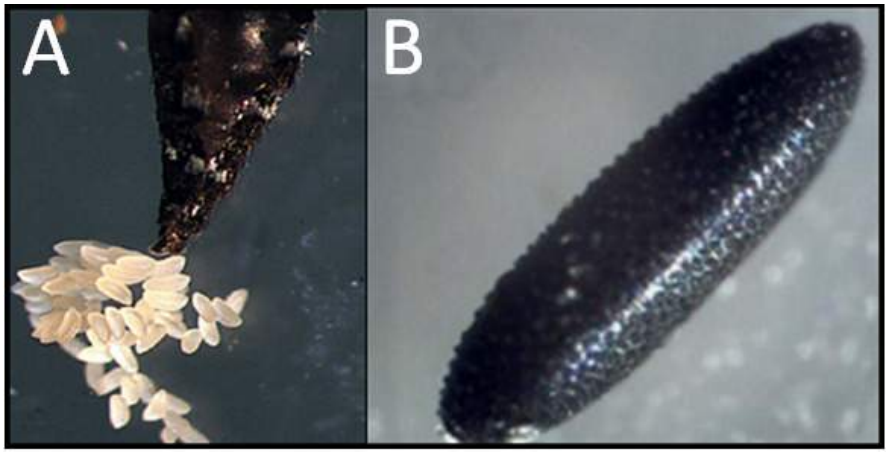

Figura 4. Ovos de *Aedes aegypti*. Ovos no momento da oviposição (A). Estrutura fusiforme do ovo (B).

Fonte: http://www.ioc.fiocruz.br

Após contato com água, a maioria dos ovos eclode rapidamente dando origem a larvas no primeiro estágio (L1). As larvas do *A. aegypti* são formadas por cabeça, tórax e abdômen (Figura 5A), sendo a fase larvária o período de maior alimentação e crescimento. O desenvolvimento larvário pode apresentar variações no tempo de duração, pois é dependente de fatores ambientais como umidade relativa do ar, temperatura, luz, pH da água do criadouro, disponibilidade de alimento e densidade das larvas no criadouro. Em condições ótimas, o período entre a eclosão e a pupação pode não exceder 5 dias (CONSOLI & OLIVEIRA, 1998; MINISTÉRIO DA SAÚDE, 2001).

Após a fase larvária, surgem as pupas (Figura 5B), que não se alimentam. É nesta fase que ocorre a metamorfose do estágio larval para o adulto. As pupas se mantêm a maior parte do tempo na superfície da água, flutuando, o que facilita a emergência do inseto adulto. O estado pupal dura, geralmente, de 2 a 3 dias. O corpo da pupa é dividido em cefalotórax (cabeça e tórax unidos) e abdômen. Lateralmente, possui uma aparência em forma de uma vírgula. A pupa tem um par de tubos respiratórios ou trompetas, as quais atravessam a água e permitem a respiração (MINISTÉRIO DA SAÚDE, 2001).

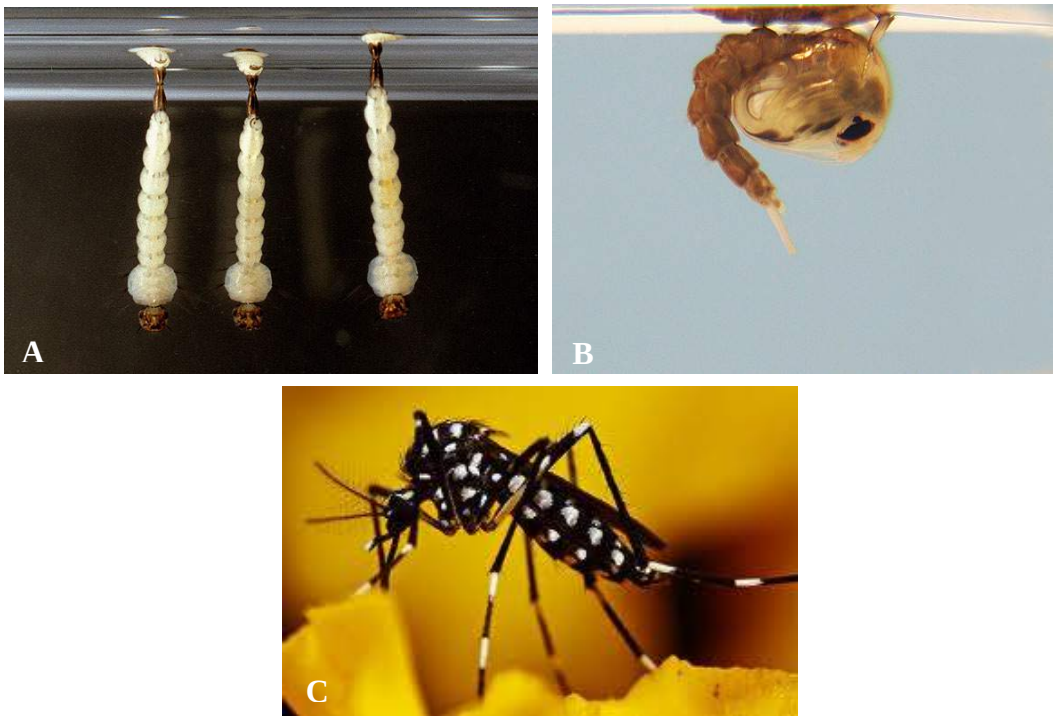

Figura 5. Larvas (A), pupas (B) e adulto (C) de *A. aegypti*.

Fonte: (A) www.sabbatini.com (B) www.abc.net.au/science/news/health (C) www.infoescola.com

Em seguida ocorre a emergência do adulto (Figura 5C), que representa a fase reprodutiva do inseto. Como ocorre em outros insetos alados, o adulto representa um importante forma de dispersão. Entretanto, com o *A. aegypti* é provável que haja mais transporte passivo de ovos e larvas em recipientes do que dispersão ativa pelo inseto adulto. O adulto apresenta uma coloração escura, com faixas brancas nas bases dos segmentos tarsais e um desenho em forma de lira no mesonoto (CONSOLI & OLIVEIRA, 1998; SERVICE, 1996). Dentro de 24 h após emergirem, podem acasalar. O acasalamento geralmente se dá durante o vôo, mas, ocasionalmente, pode ser realizado sobre uma superfície vertical ou horizontal. Uma única inseminação é suficiente para fecundar todos os ovos que a fêmea venha a produzir durante sua vida (SERVICE, 1996). Como parte do seu comportamento sinantrópico, o mosquito se reproduz principalmente nas paredes de recipientes domésticos com água armazenada e a fêmea precisa de grandes quantidades de sangue para a oviposição.

A fecundação se dá durante a postura e o desenvolvimento do embrião se completa em 48 horas, em condições favoráveis de umidade e temperatura. Após o término do desenvolvimento embrionário, os ovos, como mencionado anteriormente, são capazes de resistir a longos períodos de dessecação, que podem prolongar-se por mais de um ano (TELANG *et al*., 2013).

Os machos e as fêmeas alimentam-se de carboidratos extraídos dos vegetais. O macho distingue-se essencialmente da fêmea por possuir antenas plumosas e palpos mais longos. O repasto sanguíneo das fêmeas fornece proteínas para o desenvolvimento dos ovos e ocorre quase sempre durante o dia, nas primeiras horas da manhã e ao anoitecer. Em geral, a fêmea faz uma postura após cada repasto sanguíneo e o intervalo entre a alimentação sanguínea e a postura é, em regra, de três dias, em condições de temperatura satisfatórias. Frequentemente, a fêmea se alimenta mais de uma vez entre duas sucessivas posturas. Quando a fêmea de *A. aegypti* está infectada pelo vírus do dengue ou da febre amarela, pode haver transmissão transovariana resultando no nascimento de larvas portadoras do vírus (OPAS, 1987). Apesar de não realizarem alimentação sanguínea, o papel dos machos de *Aedes* na manutenção da circulação viral é de grande importância devido às formas de transmissão vertical e venérea (TU *et al*, 1998; KOW *et al*, 2001).

2.2.1. Comportamento de oviposição de *A. aegypti*

A localização e a seleção de um possível criadouro para a oviposição envolvem respostas visuais, olfativas e táteis. A aceitação ou rejeição dos sítios de oviposição por fêmeas grávidas pode estar relacionada a fatores químicos, os quais os insetos podem detectar através de estruturas cuticulares, sensilas e neurônios quimiossensoriais presentes nas antenas, aparelhos bucais, margens das asas e patas (Figura 6A e 6B). As sensilas olfativas detectam

substâncias voláteis do ar, enquanto os sensores gustatórios respondem a químicos de baixa volatilidade (BOHBOT & VOGT, 2005).

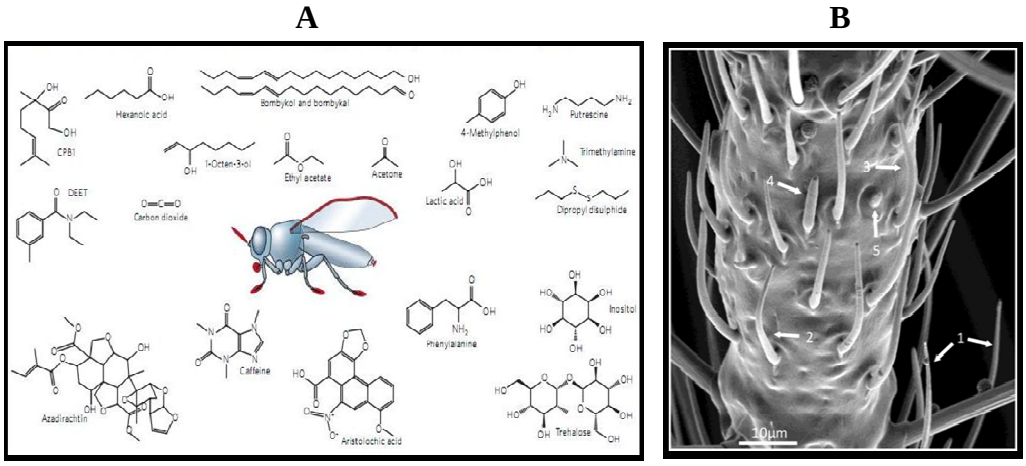

Figura 6. (A) Representação esquemática (regiões vermelhas) da localização das sensilas nos insetos. **(B)** Tipos morfológicos de sensilas de *A. aegypti*.

Fontes: (A) adaptado de Naters & Carlson (2006). (B) Stanczyk *et al.* (2010)

Além das pistas visuais ou olfativas, a seleção dos sítios de oviposição pelas fêmeas de *A. aegypti* também é influenciada por fatores químicos e físicos da água. A cor, densidade óptica da água, o tipo de substrato, temperatura, bem como pistas olfativas químicas provenientes de larvas de mosquitos já presentes no local são algumas condições relevantes na oviposição. As larvas de *A. aegypti* são conhecidas por liberarem substâncias no ambiente aquático, as quais podem atuar na água atraindo fêmeas grávidas co-específicas. A determinação do comportamento de oviposição por fêmeas de *A. aegypti* em criadouros também pode estar relacionada a fatores exógenos como chuva, umidade relativa do ar, temperatura e velocidade do vento. A presença de bactérias é também descrita com atividade atraente para as fêmeas. (BENTLEY & DAY, 1989; TILAK *et al*., 2005).

Fêmeas de *A. aegypti* apresentam preferência por depositarem seus ovos nas bordas de recipientes que tendem a armazenar água de chuva ou de torneira. O tempo requerido entre a

alimentação sanguínea e a deposição de ovos relaciona-se com fatores que dependem de controle endógeno no inseto, como a assimilação de aminoácidos essenciais que favorecem a maturação dos ovos. Estes fatores somados às condições ambientais permitem ao inseto estabelecer um ritmo circadiano, culminando na oviposição (KLOWDEN & BLACKMER, 1987).

A oviposição ocorre mais frequentemente no período diurno com um pico matutino (6 às 8h) e outro vespertino (16 às 18h). As fêmeas grávidas são atraídas por recipientes escuros ou sombreados (CLEMENTS, 1992; COBERT & CHADEE, 1992; GOMES *et al.* 2006). O número médio de ovos por postura é 120, o qual depende da quantidade de sangue ingerido durante o repasto sanguíneo (FORATTINI, 2002). A eclosão das larvas ocorre cerca de dois dias após a oviposição, caso as condições ambientais sejam favoráveis, e tem como principal estímulo o contato do ovo com a água do criadouro (FORATTINI, 2002).

A escolha do sítio de oviposição por fêmeas grávidas é o principal fator responsável pela distribuição dos mosquitos e a subsequente dispersão desses vetores em diferentes áreas geográficas. O monitoramento da densidade de *A. aegypti* e sua distribuição é crítica na previsão de epidemias de dengue ou seu risco em áreas ativas de *Aedes* (HUDSON, 1956).

A Ecologia Química é a ciência que permite o estudo dos infoquímicos – substâncias que, dentro de um contexto natural, transmitem informações numa interação entre indivíduos, produzindo uma resposta comportamental ou fisiológica, a qual pode ser vantajosa ou desvantajosa – procurando identificar e sintetizar substâncias que possam levar informações entre os organismos, como também em estratégias de controle de populações de mosquitos vetores (LAZZARI, *et al*. 2004). Estudos dos aspectos de comunicação entre insetos favorecem a busca e o desenvolvimento de novas substâncias químicas a serem utilizadas em programas de manejo integrado de pragas. Em insetos, os odores são muito importantes na localização de presas, na defesa e agressividade, na seleção de plantas, na escolha de locais de

oviposição, na corte e acasalamento, na organização das atividades sociais e em diversos outros tipos de comportamento (VILELA & DELLA LUCIA, 2001). Os infoquímicos são classificados em feromônios e aleloquímicos.

Os feromônios agem na comunicação intra-específica e são divididos segundo sua função como feromônio de alarme, sexual, de atração e agregação entre outros (SPIEGEL *et al*. 2011); os aleloquímicos agem na comunicação interespecífica e podem se originar tanto de plantas, como insetos, alimentos ou outras fontes. Odores sintéticos, feromônios sintéticos e óleos essenciais têm sido estudados quanto ao efeito sobre a oviposição de *A. aegypti* (SEENIVASAGAN & VIJAYARAGHAVAN, 2010; SRITABUTRA *et al*., 2011).

2.3. Controle de populações de mosquitos *A. aegypti*

Epidemias de dengue são frequentes, devido à ampla circulação dos quatro sorotipos virais no continente e ausência de vacinas que confiram imunidade permanente às variações genéticas virais. Logo, o controle vetorial torna-se a principal medida de combate à doença. Na tentativa de manter a incidência das enfermidades transmitidas por insetos sob controle, são destinadas, continuamente, quantias significativas de recursos para programas contra os vetores de doenças (BALY *et al*., 2007).

As altas densidades dos mosquitos estão relacionadas ao comportamento sinantrópico e ao hábito antropofílico dessa espécie. Além disso, outros fatores intimamente relacionados à biologia do vetor, somados a problemas típicos dos centros urbanos, como pobreza, lixo e alta densidade populacional, contribuem para a ocorrência de surtos. A água é um elemento indispensável à sobrevivência das populações, mas quando se torna escassa, a única forma de obtê-la é através do armazenamento em depósitos domésticos, que servem como criadouros do vetor (CLARO *et al*., 2004; BRAGA & VALLE, 2007).

O controle específico para o mosquito *A. aegypti* pode ser direcionado aos estágios imaturos aquáticos, para os adultos, ou para ambos simultaneamente (SERVICE, 1996). O método mais eficaz para controlar e prevenir a infestação de mosquitos ocorre através do uso de larvicidas, mas compostos ovicidas também são extensivamente estudados.

O controle químico, um dos métodos mais antigos a ser utilizado, consiste na aplicação de substâncias químicas como larvicidas e ovicidas, tais como óleos, repelentes, organosfosfatos, organofosforados e piretróides, entre outros (LUNA *et al*., 2004; CHUNG *et al*., 2009). O uso de larvicidas químicos constitui a principal medida adotada pelos Programas de Saúde Pública. Entretanto, em diferentes partes do mundo e no Brasil, tem sido registrada a resistência de populações de *A. aegypti* a esses inseticidas convencionais. Diante dessa problemática, têm sido investigadas novas formas de controle alternativo de vetores de doenças, a partir do uso de inseticidas biológicos, químicos naturais e reguladores de crescimento. Essas novas estratégias de controle permitem uma rotatividade dos inseticidas em programas de controle de vetores (VIEGAS JÚNIOR, 2003; CAVALCANTE *et al*., 2006).

Em estratégias para os estágios imaturos, pode-se utilizar o controle biológico, através de organismos predadores, patógenos e parasitas naturais, capazes de parasitar ou predar os mosquitos em várias fases evolutivas. Algumas linhagens de bactérias entomopatogênicas, do gênero *Bacillus*, produzem toxinas proteicas com um alto grau de especificidade a insetos vetores que, quando ingeridas, provocam mortalidade das larvas. As duas espécies mais utilizadas como larvicidas são o *Bacillus sphaericus* (Bs) e *Bacillus thuringiensis* serovar *israelensis* (Bti). O Bti é utilizado principalmente para controle de espécies do gênero *Aedes* (ARAÚJO *et al*., 2007). Esta especificidade e não-toxicidade para vertebrados têm levado ao seu sucesso comercial. No entanto, os insetos podem desenvolver resistência a este valioso recurso de controle biológico (JAYARAMAN *et al*., 2005; OCAMPO *et al*., 2011).

O controle genético também pode ser utilizado através da produção de machos híbridos e estéreis, ou pelo uso de citoplasmas incompatíveis, translocações, introdução de genes letais ou genes que tornem os mosquitos refratários como vetores, produzindo, por meio da divisão meiótica, um número excessivo de machos (ESTEVA & YANG, 2005; LEE *et al*., 2013).

O controle físico, mais conhecido como controle ambiental ou mecânico consiste na substituição, drenagem ou redução dos locais de reprodução dos insetos. A participação da população é fundamental na localização e eliminação dos criadouros, principalmente intradomiciliares e peridomiciliares, que funcionam como os principais focos para proliferação desse inseto (CONSOLI & OLIVEIRA, 1998; HEMME *et al*., 2009).

O controle direcionado aos estágios adultos também ocorre através de materiais de proteção pessoal, como o uso de janelas, portas, ventiladores e telas contra os mosquitos, ou ainda, pela aplicação de vapores de óleos, aerossóis, névoas e neblinas ou aplicações em pequenas quantidades de inseticidas concentrados como Malathion e outros piretróides (SERVICE, 1996; LUCIA *et al*., 2009).

2.3.1. Controle alternativo: inseticidas naturais

Tradicionalmente, produtos derivados de plantas ou fitoinseticidas têm sido utilizados por comunidades humanas em muitas partes do mundo contra vetores. Os fitoinseticidas podem agir como larvicidas, ovicidas, inibidores do crescimento, desreguladores do desenvolvimento, repelentes, atraentes e estimulantes de oviposição (COELHO *et al*., 2009; KABIR *et al*., 2013).

Os inseticidas naturais muitas vezes não têm qualquer efeito sobre as populações não-alvo e são biodegradáveis, além de serem localmente disponíveis em muitas partes do mundo,

inclusive as mais afetadas por doenças transmitidas por mosquitos. Uma estratégia viável para a redução das populações de insetos é o uso de extratos de plantas, associado a outros métodos de controle, uma vez que sistemas auto-sustentáveis de produção requerem metodologias menos agressivas que, preferencialmente, sejam parte do agroecossistema e, assim, mais duradouras (CAVALCANTE *et al*., 2006; GERIS *et al*., 2012).

Extrato hexânico de *Myroxylon balsamum* (óleo vermelho) foi eficiente contra o terceiro estágio larval de *A. aegypti* (SIMAS *et al*., 2004). Extratos metanólicos de folhas, cascas, alburno e cerne da criptoméria (*Cryptomeria japonica*) foram também analisados contra o quarto estágio larval (L_4) de *A. aegypti* e *A. albopictus*. Os resultados dos testes larvicidas demonstraram que a fração n-hexano do extrato metanólico do alburno teve um excelente efeito inibitório, provocando 100% de mortalidade das larvas em 24 h, numa concentração de 400 µg/mL, diferentemente dos extratos que foram obtidos das outras partes da planta (CHENG *et al*., 2008). Extratos brutos etanólicos de cascas do tingui (*Magonia pubescens*) mostraram atividade larvicida para *A. aegypti* e *A. albopictus* (SILVA *et al*., 2004). Estudos recentes têm apontado o tingui como uma fonte natural de agente larvicida por causa da presença de atividades larvicidas em extratos e frações ricas em saponinas de vários tecidos dessa planta, tais como frutos, raízes, cascas e folhas (WIESMAN & CHAPAGAIN, 2003; CHAPAGAIN, 2006). Extratos etanólicos obtidos de frutos e folhas de *Melia azedarach* têm mostrado efeitos larvicida e deterrente contra *A. aegypti* (WANDSCHEER *et al*., 2004; CORIA *et al*., 2008).

Óleos essenciais também têm sido bastante utilizados como larvicidas, devido a sua natureza lipofílica, que interfere diretamente nas funções do metabolismo básico, bioquímico, fisiológico e comportamental dos insetos (NISHIMURA, 2001). Por exemplo, os óleos essenciais de *Thymus vulgaris*, *Satureja hortensis* e *Thymus satureioide* mostraram ser eficazes sobre a mortalidade larval de *Culex quinquefasciatus*, com valores de CL_{50} menores

que 50 g/ml (PAVELA, 2009); óleo essencial extraído de flores de *Dendropanax morbifera*, com efeito tóxico sobre quarto estágio larval de *A. aegypti*, apresentou CL_{50} de 62,32 ppm (CHUNG *et al*., 2009) enquanto que o óleo derivado da inflorescência de *Piper marginatum* exibiu uma potente ação larvicida em pequenas concentrações sobre estágio L_4 de *A. aegypti* num valor de CL_{50} de 20 ppm (AUTRAN *et al*., 2009).

Lectinas – proteínas que se ligam a carboidratos – isoladas de entrecasca, cerne e folha de *Myracrodruon urundeuva* apresentaram também atividade larvicida sobre larvas de *A. aegypti* no quarto estágio (L4) (SÁ *et al*., 2009; NAPOLEÃO *et al*., 2012). A lectina isolada de sementes de *M. oleifera* (WSMoL), a qual apresenta elevada solubilidade em água, também foi larvicida contra larvas L4 de *A. aegypti* (COELHO *et al*., 2009).

Diversos compostos de origem vegetal têm sido investigados quanto à atividade ovicida sobre *A. aegypti*. Tem sido demonstrado que extratos orgânicos e óleos essenciais foram capazes de impedir a eclosão dos ovos (GOVINDARAJAN, 2011; GOVINDARAJAN & KARUPPANNAN, 2011; WARIKOO *et al.*, 2011). Extratos de sementes e folhas de *Delonix elata* e óleo essencial de *Cananga odorata* apresentaram atividade ovicida contra *A. aegypti* (MARIMUTHU *et al.*, 2012; PHASOMKUSOLSIL *et al.*, 2012).

As plantas podem ser também fontes de substâncias repelentes de mosquitos. Repelentes de oviposição são bastante estudados visando eliminar potenciais sítios de oviposição bem como afastar fêmeas grávidas de hospedeiros humanos. Óleos essenciais repelem as fêmeas principalmente devido à presença de monoterpenos e sesquiterpenos em sua composição (AUTRAN *et al.*, 2009; NERIO *et al.*, 2010).

Compostos que promovam aumento da taxa de oviposição de *A. aegypti* também têm sido procurados, desde que sua propriedade atrativa pode ser utilizada em armadilhas para captura de ovos (ovitrampas) juntamente com agentes larvicidas. Metabólitos secundários produzidos pelo fungo *Trichoderma viride* e infusões obtidas após fermentação de materiais

orgânicos, tais como feno de capim, folhas, bambu senescente e rações de animais, mostraram agir como estimulantes de oviposição para fêmeas grávidas dos gênerois *Aedes* e *Culex* (LAMPMAN & NOVAK, 1996; RITCHIE, 2001; PONNUSAMY *et al*., 2010; SANTOS *et al*., 2010).

2.3.2 Armadilhas para monitoramento e controle de *A. aegypti*

As armadilhas para controle e monitoramento de insetos consistem, geralmente, de combinações de atrativos aos quais os insetos respondem. Diferentes espécies não são igualmente atraídas pelos mesmos estímulos (LEHTONEN & PAHLONEN, 2004). Os atrativos podem ser de natureza química (como os feromônios sintéticos ou naturais e os aleloquímicos), física (como a transparência da água, luminosidade e temperatura) ou biológica, como a utilização de iscas animais e humanas (BARBOSA *et al*., 2007). Segundo Vargas (2002), tais armadilhas podem ser utilizadas para capturar ovos (ovitrampas), larvas (larvitrampas) e adultos (armadilhas adesivas, luminosas e iscas).

Diferentes tipos de armadilhas são utilizados em ensaios para estudar o comportamento de insetos, monitoramento de densidade e controle populacional. Iscas humanas já foram utilizadas e consideradas como o melhor método de captura de mosquitos. Porém, quando um sujeito vivo é utilizado como isca, há o iminente risco de contrair uma doença. Sendo assim, armadilhas com atraentes específicos proporcionam um acompanhamento mais seguro e consistente (FÁVARO *et al*., 2008; GAMA *et al*., 2007).

As ovitrampas são descritas usualmente como recipientes de cor escura, que podem ser preeenchidas por água ou outro líquido e possuem, no seu interior, uma palheta de madeira que serve como suporte de postura dos ovos (Figura 7A). São as armadilhas mais utilizadas pela Vigilância Sanitária em atividades de monitoramento de populações do vetor, e

substâncias atrativas para as fêmeas grávidas ou inseticidas reguladores de crescimento podem ser adicionadas a esses recipientes. Também podem ser utilizadas substâncias repelentes em reservatórios de água armazenados para prevenir a reprodução dos mosquitos em áreas domésticas; entretanto é necessária a implementação do uso por toda a comunidade para prevenção de doenças transmitidas por *Aedes* (LENHART *et al*., 2005; GAMA *et al*., 2007).

Figura 7. Armadilhas de monitoramento: (**A**) ovitrampa e (**B**) Mosquitrap

Fonte: (A) Foto: Santos, N.D.L; (B) Resende *et al*., 2010.

A MosquiTRAP^{TM} (Versão 1.0, Ecovec Ltda.) é uma armadilha adesiva descrita por Eiras (2002), a qual foi desenvolvida baseando-se no comportamento de fêmeas grávidas de *A. aegypti* quando elas exploram os sítios de reprodução. Ela consiste de um cilindro plástico preto de 1 L preenchido com água, um atraente de oviposição e um cartão adesivo. Essa armadilha pode explorar tanto estímulos visuais (cor preta) quanto olfativos (compostos voláteis) para atrair as fêmeas grávidas. Quando as fêmeas entram na armadilha e pousam nas paredes da MosquiTRAP, elas aderem ao cartão adesivo. Em testes de laboratório e de campo, essa armadilha se mostrou efetiva e de baixo custo, o que a torna viável para operações em

larga escala. A MosquiTRAP também permite a identificação de vetores durante inspeções de campo (GAMA *et al.*, 2007).

2.4. Lectinas

As lectinas são uma classe de proteínas ou glicoproteínas estruturalmente diversas e que contêm pelo menos um domínio de ligação a carboidratos, tais como monossacarídeos e oligossacarídeos, aos quais se ligam com alta especificidade e de forma reversível (CORREIA *et al*., 2008). Apresentam uma ampla distribuição na natureza (RATANAPO et a*l*., 2001) e em plantas, as lectinas têm sido isoladas de sementes (SANTOS *et al*., 2009), folhas (NAPOLEÃO *et al*., 2012), flores (ITO, 1986), frutos (THAKUR *et al.*, 2007), entrecascas (NASCIMENTO *et al.*, 2008), rizomas (ALBUQUERQUE *et al.*, 2012), cerne (SÁ *et al*., 2009) e raízes (WANG & NG, 2006).

A detecção de lectinas em material biológico inicia-se a partir de ensaios de hemaglutinação (Figura 8A), na qual estas proteínas interagem com os carboidratos da superfície celular do eritrócito por meio dos seus sítios de ligação, formando ligações cruzadas entre as células (SANTOS *et al*., 2005). O ensaio da atividade hemaglutinante (AH) é comumente realizado pela técnica de diluições seriadas da amostra contendo lectina e posterior incubação com eritrócitos (SANTOS *et al.*, 2005). A presença de uma lectina na amostra, como agente aglutinante é confirmada através dos ensaios de inibição da AH (Figura 8B) com uma solução de carboidrato ou glicoproteína livre em solução (TRINDADE *et al*., 2006). A detecção, identificação e quantificação de lectinas também podem ser realizadas através de análise proteômica e de sequenciamento por espectrometria de massas para futuras aplicações em processos bioquímicos, imunológicos e toxicológicos (NASI *et al*., 2009).

Na purificação de lectinas estão envolvidas técnicas comuns a protocolos de isolamento de proteínas. A etapa inicial do isolamento consiste de preparações de extratos em água destilada (SANTOS *et al*., 2005), salina (KONOZY *et al*., 2003) ou em tampões (OLIVEIRA *et al*., 2002).

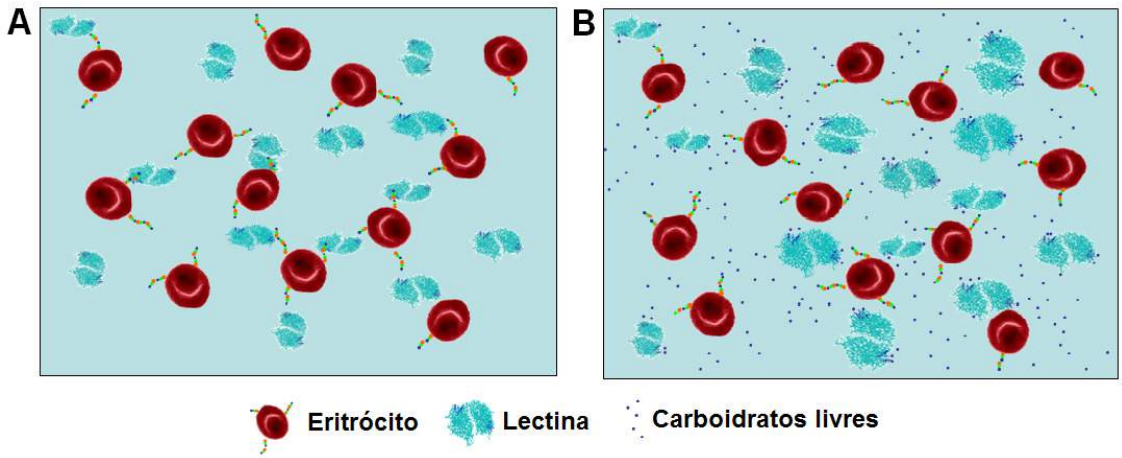

Figura 8. Representação da atividade hemaglutinante de lectinas (A) e inibição da aglutinação por carboidratos livres em solução (B).

Fonte: Paiva *et al*. (2010).

A purificação parcial de lectinas através de fracionamento salino utilizando o sulfato de amônio tornou-se um dos procedimentos mais utilizados, pois as proteínas possuem muitos grupos carregados e por isso, a sua solubilidade depende da concentração dos sais dissolvidos (PAIVA *et al*., 2011); a solubilidade aumenta com o acréscimo de sais (*salting in*) e volta a decrescer à medida que mais sal é adicionado além do limiar de saturação (*salting out*).

Após o fracionamento salino, as lectinas são normalmente submetidas a processos de diálise em membranas semipermeáveis, seguida de processos cromatográficos, tais como troca iônica (LAM & NG, 2008), gel filtração ou exclusão molecular (REGO *et al*., 2002; JUNG *et al*., 2007; POHLEVEN *et al*., 2009) e de afinidade (TATENO *et al*., 2003; SANTANA *et al*., 2008).

A caracterização é realizada por meio da determinação de diferentes propriedades físico-químicas da lectina e envolve métodos diversos como inibição da AH por carboidratos e/ou glicoconjugados (YANG *et al.*, 2007), avaliação da AH com eritrócitos de diferentes espécies de animais (por exemplo: coelho, galinha, sistema sanguíneo humano A, B, AB e O), em presença de íons e em diferentes valores de pH e temperatura (SANTOS *et al*., 2009). Técnicas eletroforéticas, mono ou bidimensional, são eficientes para definir a natureza da carga líquida da proteína e o peso molecular das subunidades, bem como para avaliar a pureza da preparação obtida (NASI *et al*., 2009).

2.4.1 Aplicações biotecnológicas e atividades biológicas

A específica interação das lectinas com glicoconjugados em solução ou na superfície celular dota estas moléculas de diversas atividades biológicas e as tornam ferramentas valiosas em diferentes aplicações biotecnológicas (CORREIA *et al*., 2008). Lectinas possuem várias atividades incluindo antimicrobiana, antitumoral, reconhecimento de carboidratos ou glicoconjugados presentes na superfície das células de diferentes animais, como também ação inseticida (PAIVA *et al.*, 2011a).

Lectinas purificadas podem ser utilizadas para diversos fins. Podem ser utilizadas em estudos citoquímicos e histoquímicos para detecção de resíduos glicosilados em superfícies teciduais de humanos e animais (PEDINI *et al*., 2002), como moléculas de reconhecimento para diferenciação de tumores malignos e benignos (GORELIK *et al*., 2001), como moléculas bioadesivas no endereçamento de drogas (BIES *et al*., 2004), indução de apoptose celular (LIU *et al*., 2009) e isolamento de glicoconjugados quando imobilizados em suportes insolúveis (FRANCO-FRAGUAS *et al*., 2003; BANERJEE *et al*., 2004). Assim, são proteínas amplamente versáteis.

As lectinas de plantas têm sido escolhidas para estudos das bases moleculares nos eventos de reconhecimento dos processos de infecções virais, bacterianas, fúngicas e parasíticas; endereçamento de células e componentes solúveis; fertilização, metástases, crescimento e diferenciação celular (LORIS *et al*., 1998; KEYAERTS *et al*., 2007). Algumas lectinas de plantas estimulam o sistema imune por ativação não específica de células T ou atuam influenciando a divisão celular. As lectinas também são usadas em procedimentos de diagnóstico e estudos das funções do sistema imune, tais como indução de linfócitos, produção e proliferação de interferons e citocinas, asma e inflamação e outros efeitos imunoestimulatórios (CARLINI & GROSSI-DE-SÁ, 2002; STAUDER & KREUSER, 2002).

2.4.1.1 Lectinas com atividade inseticida

Tem sido descrita a atividade inseticida de lectinas de plantas contra diversas espécies de insetos de diferentes ordens, tais como Coleoptera, Diptera, Homoptera, Lepidoptera e Isoptera. O mecanismo de ação inseticida das lectinas de plantas não está completamente elucidado. Tem sido sugerido que a resistência à degradação por proteases e a ligação a glicoconjugados da superfície de células epiteliais do intestino do inseto sejam dois pré-requisitos básicos para as lectinas exercerem seus efeitos deletérios, interferindo nas funções digestivas, protetoras ou secretórias do intestino. Tem sido demonstrado que algumas lectinas ligam-se às vilosidades do epitélio intestinal dos insetos, promovendo uma disfunção das células epiteliais, responsáveis pela assimilação de nutrientes para as células e absorção de substâncias potencialmente perigosas. Outro efeito descrito é a desestabilização do metabolismo do inseto devido à interferência das lectinas nas funções enzimáticas pela ligação às porções glicosiladas das enzimas digestivas dos insetos. Lectinas ligadoras de quitina têm sido estudadas como agentes inseticidas, e essa atividade tem sido atribuída à

ligação destas proteínas à matriz peritrófica, perturbando sua síntese e integridade e afetando, então, indiretamente o mecanismo regulatório das enzimas (PAIVA *et al.*, 2011a, 2012).

As lectinas isoladas das sementes de *Canavalia brasiliensis* (ConBr) e *Cratylia floribunda* (CFL) apresentaram efeitos deletérios contra *Callosobruchus maculatus* (Coleoptera: Bruchidae) e *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae), reduzindo a porcentagem de emergência de adultos e ainda foram resistentes às enzimas digestivas destes insetos (FREITAS *et al*., 2011). As lectinas isoladas da entrecasca (MuBL), cerne (MuHL) e folha (MuLL) de *M. urundeuva*, do líquen *Cladonia verticillaris* (ClaveLL), das raízes secundárias de *Bauhinia monandra* (BmoRoL), de cladódios de *Opuntia fícus-indica* (OfiL) e da entrecasca de *Crataeva tapia* (CrataBL) apresentaram atividade inseticida contra operários e soldados de *Nasutitermes corniger* (SÁ *et al.*, 2008; SILVA *et al.*, 2009; NAPOLEÃO *et al.*, 2011; PAIVA *et al.*, 2011b; SOUZA *et al.*, 2011; ARAÚJO *et al.*, 2012).

Extratos salinos, frações proteicas e lectinas purificadas do cerne (MuHL), da casca (MuBL) e da folha (MuLL) de *M. urundeuva* promoveram a mortalidade do quarto estágio larval de *Aedes aegypti* (SÁ *et al*., 2009; NAPOLEÃO *et al.*, 2012). MuLL foi resistente à degradação por proteases intestinais e apresentou efeito inibitório sobre a atividade de tripsina e estimulatório sobre α-amilase larvais (NAPOLEÃO *et al.*, 2012).

2.4.1.2 Efeitos da irradiação gama em lectinas

As radiações são ondas eletromagnéticas ou partículas que se propagam com uma determinada velocidade, contendo energia, carga elétrica e magnética. Podem ser geradas por fontes naturais ou por dispositivos construídos pelo homem. Possuem energia variável desde valores pequenos até muito elevados. Os raios gama podem ser originados de fontes radioativas naturais e artificiais, bem como de reações nucleares, tendo ampla aplicação nas

ciências. A radiação gama, com mais energia do que os raios-X, é originada a partir de fontes de isótopos radioativos, como o césio-137 ou cobalto-60, e é reconhecida pela Organização Mundial de Saúde como uma técnica de conservação de alimentos que melhora a segurança alimentar sem alterar a qualidade toxicológica, biológica ou nutricional dos alimentos (OMS, 1981). O código legislativo recomenda e autoriza diferentes tipos de radiação em doses de Kilogray (kGy, joule por kilograma). A irradiação gama promove segurança, eficiência e é utilizada para desinfecção, esterilização ou redução de microrganismos, como também aumento de meia vida dos alimentos (SOMMERS, 2004).

As radiações ionizantes do tipo gama causam mudanças na função e integridade de biomoléculas, incluindo proteínas, por dois meios distintos: primeiro pela interação direta com as proteínas e segundo pela formação de produtos a partir de radiólises da água. Dentre as modificações causadas em proteínas pela irradiação em solução aquosa e em presença de oxigênio, pode-se citar: carbonilação, oxidação de cadeias laterais dos resíduos de aminoácidos, cisão, fragmentação, formação de dímeros de tirosina, desenovelamento e ligação cruzada com formação de agregados moleculares (LEE & SONG, 2002; ZBIKOWSKA *et al.*, 2006).

A irradiação de lectinas de plantas em quantidades miligramas tem sido utilizada como um método alternativo de redução ou eliminação de alergenicidade de alimentos (VAZ *et al*., 2013). A lectina isolada da casca de *Sebastiania jacobinensis* (SejaBL), apresentou alterações estruturais após irradiação gama, e ensaios de hemaglutinação mostraram que a lectina foi estimulada pela radiação em baixas doses (0,1 kGy), enquanto que altas doses, acima de 1 kGy, ocorreu perda significativa de atividade (VAZ *et al*., 2011).

Outra aplicação da radiação gama, está relacionada à inativação ou fragmentação da estrutura molecular de moléculas tóxicas, tal como a lectina isolada do veneno de *Bothrops leucurus* (BIL). A irradiação causou o desenovelamento seguido de agregação da lectina,

provenientes de mudanças estruturais da proteína, resultando na perda de sua propriedade de ligação a carboidratos e ação citotóxica (NUNES *et al*., 2012).

2.5 A espécie *Moringa oleifera*

A família Moringaceae possui um único gênero denominado *Moringa*, constituído apenas por quatorze espécies, incluindo a *Moringa oleifera*. A moringa é uma planta tropical, perene, de porte arbóreo, entre 7 e 12 m de altura e originária do continente asiático, no noroeste da Índia. Com baixo custo de produção, foi introduzida no Brasil para ornamentação e arborização de ruas e praças, sendo conhecida popularmente como lírio branco, quiabo-de-quina ou simplesmente moringa (MATOS, 2002; MARACAJÁ *et al.*, 2010). É muito cultivada devido à adaptação a regiões de secas prolongadas, sobrevivendo a grandes períodos em solos pobres e com baixo teor de umidade (MCCONNACHIE *et al*., 1999; SOUSA, 2001).

Muitas propriedades têm sido identificadas em diferentes partes de *Moringa oleifera* (Figura 9): as folhas, frutos verdes, flores e sementes possuem valor alimentar devido à presença de quantidades representativas de cálcio, ferro, proteínas e também podem ser utilizadas como suplemento alimentar por apresentarem potássio, vitaminas do complexo B e cobre. As sementes de moringa apresentam compostos bioativos com ação coagulante, e essa propriedade está relacionada à presença de diferentes proteínas coagulantes que participam do processo de tratamento da água, através da remoção da turbidez, tornando-a própria para consumo humano (JOLY, 1998; OKUDA *et al.*, 2001; SANTOS *et al.*, 2009).

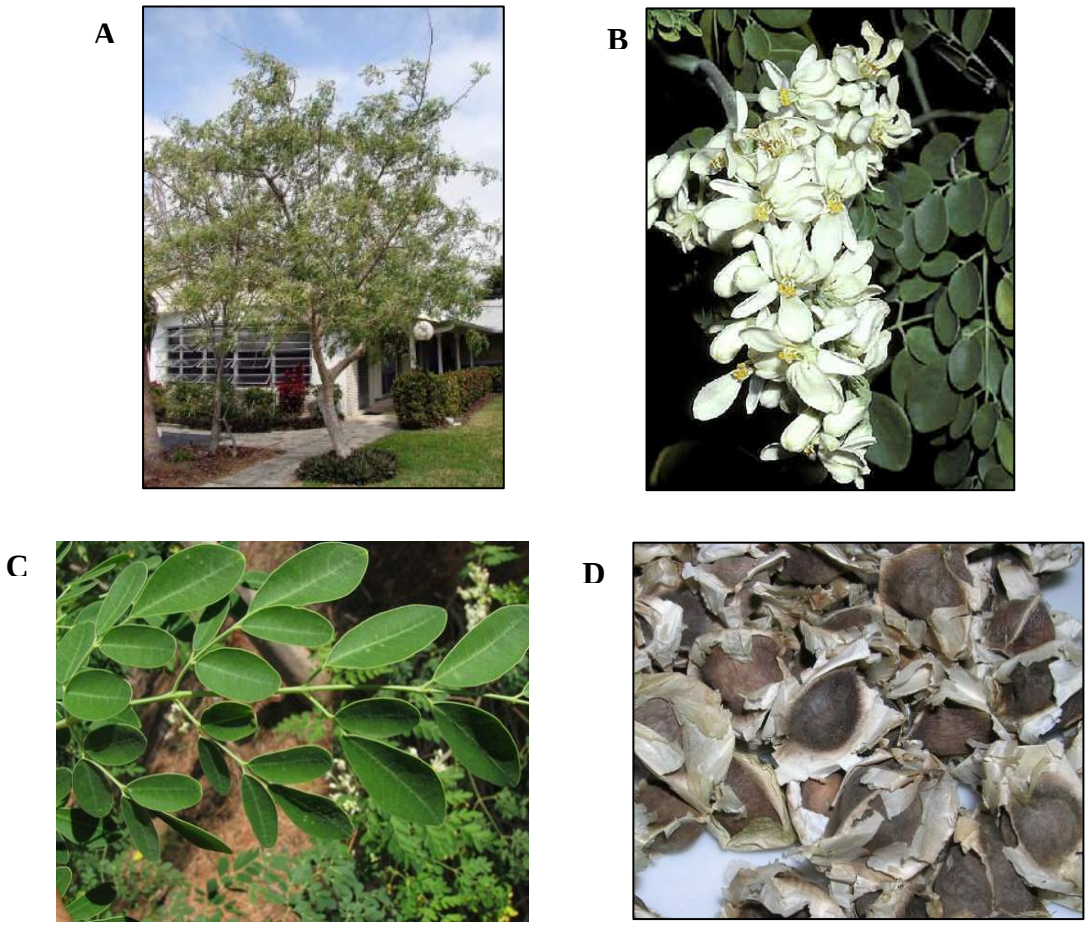

Figura 9. *Moringa oleifera*. (A) Aspecto geral. (B) Flores. (C) Folhas. (D) Sementes.

Fonte: (A) http://www.kqm.com.br/blog.php?post=189 (B) http://www.tumblr.com/tagged/moringa%20oleifera

(C) http://naturezadivina.org.br/comunidade/wp-content/uploads/2010/11/3048469273_8581a1df61.jpg

(D) Foto: Nataly D.L. Santos (2008)

2.5.1 Lectinas de sementes de *Moringa oleifera*

Três lectinas foram identificadas em sementes de *M. oleifera* sendo denominadas MoL (do inglês *M. oleifera lectin*), cMoL (do inglês *coagulant M. oleifera lectin*) e WSMoL (do inglês *water-soluble M. oleifera lectin*). MoL é uma lectina catiônica formada por subunidades de 7,1 kDa que foi isolada por cromatografias em DEAE-Celulose e CM-Sephadex (KATRE *et al.*, 2008). A lectina cMoL, também de natureza catiônica (PI teórico: 11,97), é uma proteína composta por 101 resíduos de aminoácidos e estrutura α/β que

apresentou atividade coagulante em modelo de água turva com caolin (SANTOS *et al.*, 2009; LUZ *et al.*, 2013). As lectinas cMoL e WSMoL apresentam características estruturais diferentes, como especificidade aos monossacarídeos, peso molecular e carga elétrica (SANTOS *et al.*, 2009; ROLIM *et al.*, 2011; PAIVA *et al.*, 2011). Ambas, são lectinas ligadoras de quitina com atividade inseticida (COELHO *et al.*, 2009; OLIVEIRA *et al.*, 2011).

WSMoL apresentou atividades antibacteriana e coagulante, sendo capaz de promover a sedimantação de microorganismos presentes na água (FERREIRA *et al.*, 2011). Estudo avaliando a genotoxicidade dessa lectina revelou que WSMoL não apresentou efeitos mutagênico nem promoveu quebras na molécula de DNA nas concentrações de 0,0125 a 0,8 μg/ml, indicando a segurança de seu uso para tratar água para consumo humano (ROLIM *et al.*, 2011). WSMoL possui efeito larvicida (LC_{50} de 0,197mg/mL) sobre o quarto estágio (L4) de *A. Aegypti* (COELHO *et al.*, 2009).

3. OBJETIVOS

3.1. Objetivo Geral

- ✓ Investigar os efeitos de WSMoL quanto aos efeitos sobre as taxas de oviposição e eclosão de ovos de *A. aegypti*, à eficácia como agente atraente para uso em armadilha de captura de fêmeas e a alterações em suas propriedades em resposta à irradiação gama.

3.2. Específicos

- ✓ Purificar WSMoL de acordo com procedimento previamente estabelecido.
- ✓ Determinar o efeito de preparações contendo WSMoL (extração, fração e lectina isolada) sobre a oviposição de fêmeas de *A. aegypti* em condições de laboratório.
- ✓ Analisar WSMoL quanto à presença de compostos voláteis através de cromatografia de fase gasosa acoplada a espectrometria de massas (GC-MS).
- ✓ Determinar o efeito de preparações contendo WSMoL na taxa de eclosão de ovos frescos de *A. aegypti* provenientes do ensaio de oviposição em laboratório.
- ✓ Avaliar a atividade ovicida de preparações contendo WSMoL, determinando os valores de EC_{50} (concentração efetiva de proteínas necessária para reduzir em 50% o número de ovos eclodidos de *A. aegypti*).
- ✓ Investigar o efeito de WSMoL sobre o desenvolvimento e sobrevivência de embriões de *A. aegypti* em ovos frescos e estocados que não eclodiram.
- ✓ Avaliar o efeito de WSMoL sobre a oviposição de fêmeas de *A. aegypti* em condições de campo simulado utilizando ovitrampas.

- ✓ Investigar a eficácia de WSMoL em aumentar a taxa de captura de fêmeas por meio de armadilha adesiva (MosquiTRAP^{TM}).
- ✓ Determinar a volatilidade de WSMoL utilizando olfatômetro horizontal de dupla escolha.
- ✓ Avaliar a persistência do efeito de WSMoL sobre a oviposição em condições de campo simulado.
- ✓ Submeter WSMoL a irradiação gama com ^{137}Cs em doses de 10 mGy e 10 Gy.
- ✓ Caracterizar WSMoL irradiada ou não irradiada quanto ao perfil em cromatografia de exclusão molecular.
- ✓ Investigar WSMoL irradiada quanto a atividade hemaglutinante, larvicida, ovicida e estimulante de oviposição sobre *A. aegypti*.

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5. CAPÍTULO 1

Oviposition-stimulant and ovicidal activities of *Moringa oleifera* lectin on *Aedes aegypti*

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Oviposition-Stimulant and Ovicidal Activities of *Moringa oleifera* Lectin on *Aedes aegypti*

Nataly Diniz de Lima Santos[1], Kézia Santana de Moura[1], Thiago Henrique Napoleão[1], Geanne Karla Novais Santos[2], Luana Cassandra Breitenbach Barroso Coelho[1], Daniela Maria do Amaral Ferraz Navarro[2], Patrícia Maria Guedes Paiva[1]*

1 Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Cidade Universitária, Recife, Pernambuco, Brazil, **2** Departamento de Química Fundamental, Centro de Ciências Exatas e da Natureza, Universidade Federal de Pernambuco, Cidade Universitária, Recife, Pernambuco, Brazil

Abstract

Background: Natural insecticides against the vector mosquito *Aedes aegypti* have been the object of research due to their high level of eco-safety. The water-soluble *Moringa oleifera* lectin (WSMoL) is a larvicidal agent against *A. aegypti*. This work reports the effects of WSMoL on oviposition and egg hatching of *A. aegypti*.

Methodology/Principal Findings: WSMoL crude preparations (seed extract and 0–60 protein fraction), at 0.1 mg/mL protein concentration, did not affect oviposition, while *A. aegypti* gravid females laid their eggs preferentially (73%) in vessels containing isolated WSMoL (0.1 mg/mL), compared with vessels containing only distilled water (control). Volatile compounds were not detected in WSMoL preparation. The hatchability of fresh eggs deposited in the solutions in the oviposition assay was evaluated. The numbers of hatched larvae in seed extract, 0–60 protein fraction and WSMoL were 45±8.7 %, 20±11 % and 55±7.5 %, respectively, significantly ($p<0.05$) lower than in controls containing only distilled water (75–95%). Embryos were visualized inside fresh control eggs, but not within eggs that were laid and maintained in WSMoL solution. Ovicidal activity was also assessed using stored *A. aegypti* eggs. The protein concentrations able to reduce the hatching rate by 50% (EC50) were 0.32, 0.16 and 0.1 mg/mL for seed extract, 0–60 protein fraction and WSMoL, respectively. The absence of hatching of stored eggs treated with WSMoL at 0.3 mg/mL (EC99) after transfer to medium without lectin indicates that embryos within the eggs were killed by WSMoL. The reduction in hatching rate of *A. aegypti* was not linked to decrease in bacteria population.

Conclusions/Significance: WSMoL acted both as a chemical stimulant cue for ovipositing females and ovicidal agent at a given concentration. The oviposition-stimulant and ovicidal activities, combined with the previously reported larvicidal activity, make WSMoL a very interesting candidate in integrated *A. aegypti* control.

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* E-mail: ppaivaufpe@yahoo.com.br

Introduction

Aedes aegypti Linnaeus is a domestic, daytime mosquito that breeds preferably in water storage containers, discarded plastic vials, and automobile tyres [1]. This mosquito has great importance in tropical and subtropical countries, since it is the vector of yellow and dengue fevers. Currently, dengue is the vector-borne disease that spreads most rapidly worldwide, with over two-fifths of the world's population at risk of infection [2,3]. The absence of an effective vaccine makes the control of vector population the only way to minimize dengue spreading.

Worldwide, synthetic chemicals, mainly pyrethroids, carbamates and organophosphates are used to control adults, larvae, pupae and eggs of *A. aegypti*. These compounds pose high environmental risks, due to adverse effects on human and non-target organisms [4,5]. In addition, their widespread usage has lead to mosquito resistance, compromising the effectiveness of control strategies [6]. It was also demonstrated that the presence of residual herbicides in ecosystems can reduce the sensitiveness of mosquito larvae to insecticides [7]. Due to their biodegradability, insecticides extracted from plants have been considered environmentally friendly substitutes for synthetic insecticides [8].

Plant extracts, secondary metabolites, essential oils and lectins (carbohydrate-binding and hemagglutinating proteins) have been shown to exert deleterious effect on *A. aegypti*, delaying development, impairing growth and digestive enzyme activities, reducing egg hatching and larval survival, as well as deterring feeding and oviposition activities [9–15]. The deleterious effects of lectins on insects have been associated with interaction of lectin with *N*-acetylglucosamine residues of chitin, a structural component in

insects being found in peritrophic matrices, epidermal cuticles and tracheas [16].

Oviposition behavior of mosquitoes is a useful tool in determining female preference for oviposition sites. The acceptance or rejection by gravid females of a site for oviposition can be related to chemosensory cues (semiochemicals) which the insects detect through specialized cuticular structures, sensilla and chemosensory neurons in antennae, mouthparts, wing margins and legs [17]. Olfactory sensilla detect airborne volatiles, while gustatory sensilla respond to the treatment with chemicals of low-volatility [17].

Oviposition repellents have been the object of research designed to discover ways to eliminate oviposition sites and ward off *A. aegypti* gravid females from human hosts. Monoterpenes and sesquiterpenes present in essential oils act as repellant to females [18,19]. Compounds that elicit increased *A. aegypti* oviposition have also been searched, an attractive property that may be used in ovitraps in combination with larvicidal agents [20]. Secondary metabolites produced by fungus *Trichoderma viride* and infusions obtained by fermentation of organic materials (e.g. sod, hay, grass, leaves, senescent bamboo and pelletized plant-based animal feeds) were shown to act as oviposition stimulants to gravid *Aedes* and *Culex* mosquitoes [21–24].

A. aegypti eggs are the main dispersion form of mosquito. Eggs resist dry conditions and manage to survive for many months or years in adverse environments [25]. They also afford these insects to rapidly reconstitute populations, even if the other stages have been eliminated by control measures. Thus, it is important to search for insecticides that act also against this developmental stage of the mosquito. Different plant species have been investigated for ovicidal activity on *A. aegypti*. Organic solvent extracts, as well as essential oils have been found to impair egg hatching [14,26,27].

Moringa oleifera Lamarck is a tree widely cultivated throughout the tropics and subtropics. In developing countries, its seeds are used to treat water for human consumption. Its seeds also contain a water-soluble lectin (WSMoL), which showed similarity with M02.1 and M02.2 (identification number gi|127215) proteins [9]. Using cell-free plasmid DNA and *Salmonella typhimurium* assays, a study on genotoxicity of WSMoL demonstrated that the lectin was non-mutagenic, indicating its safety for use in water treatment [28]. This lectin also acted as coagulant and antibacterial against *Staphylococcus aureus*, and effectively reduced lake water bacteria growth [29]. WSMoL exerted larvicidal activity (LC_{50} of 0.197 mg/mL) against *A. aegypti* fourth-stage larvae. The larvae treated with WSMoL lost the underlying epithelium, and showed increased gut lumen and hypertrophic segments [9]. Due to the high solubility in water and larvicidal activity against *A. aegypti*, WSMoL may become a potential candidate in *A. aegypti* control.

This work evaluated the effects of crude preparations (seed extract and 0–60 protein fraction) containing WSMoL and isolated lectin on *A. aegypti* oviposition. The hatchability of the freshly laid eggs obtained from the oviposition assay as well as of stored eggs was assessed. The presence of volatiles in WSMoL solution and the possibility that the effect of WSMoL on hatching involved embryo death and reduction of bacteria population in incubation medium were investigated. In addition, fresh and stored eggs from control and WSMoL treatments were examined on a stereomicroscope.

Methods

Breeding and rearing of A. aegypti in laboratory

The mosquitoes and eggs used were obtained from the colony (Rockfeller strain) maintained at the *Laboratório de Ecologia Química* of the *Universidade Federal de Pernambuco* (Recife, Brazil) since 2003. The insectary room was kept at 27±1°C, 78±2% relative humidity and 14:10 (light/dark) photoperiod. The larvae were reared in plastic bowls containing water and cat food (Whiskas®). Adult mosquitoes were reared in cages (30×30×30 cm) covered with a fine mesh cloth and fed a 10% glucose solution. Females took blood meal from chicken blood acquired from local farms and dispensed from a common artificial feeder. The eggs laid by females were collected and used to restart the cycle for maintaining the colony or used to assess ovicidal activity.

Crude preparations of WSMoL

M. oleifera (Family Moringaceae) has the vernacular names "moringa" in Portuguese, "árbol del ben" in Spanish, and horseradish tree or drumstick in English. Seeds were collected from 10–15-year-old trees in Recife City, State of Pernambuco, northeastern Brazil, and stored at −20°C. Voucher specimen (number 73,345) is archived at the herbarium Dárdano de Andrade Lima (Instituto Agronômico de Pernambuco, Recife, Brazil).

Crude preparations of WSMoL were obtained according to Coelho et al. [9]. Seeds were milled to a fine powder and homogenized (10 g) with distilled water (100 mL) using a magnetic stirrer for 16 h at 4°C. After filtration through gauze and centrifugation (9,000 g, 15 min, 4°C), the clear supernatant (seed extract) was submitted to protein precipitation using ammonium sulphate at saturation of 60% according to Green and Hughes [30]. The 0–60 precipitate collected after centrifugation (9,000 *g*, 15 min, 4°C) was dialyzed (3.5 kDa cut-off membrane) against distilled water (4 h) for use in bioassays and additionally with 0.15 M NaCl (4 h) for use in chromatography step. The dialysed fraction corresponded to the 0–60 protein fraction. The presence of residual ammonium sulphate in the 0–60 protein fraction was evaluated adding 50 µL of this sample to 0.5 mL of a 10 mg/mL barium chloride solution acidified to pH 4.0 with 1.0 M HCl. The formation of precipitates indicates that there is ammonium sulphate in sample [31].

Isolation of WSMoL

WSMoL was isolated according to the procedure described by Coelho et al. [9]. The 0–60 protein fraction was loaded (40 mg of proteins) onto a chitin column (7.5×1.5 cm) equilibrated (0.3 mL/min flow rate) with 0.15 M NaCl, a salt concentration which does not interfere on *A. aegypti* oviposition [32]. After washing with the equilibrating solution, WSMoL was recovered by elution with 1.0 M acetic acid and dialysed (3.5 kDa cut-off membrane) against distilled water (4 h) at 4°C for eluent elimination. The presence of residual sodium chloride in WSMoL was evaluated according to the classic Mohr's method for determination of chloride by precipitation titration with silver nitrate. The detection limit of the method was 5×10^{-4} M.

Protein and carbohydrate contents

The protein concentration was determined according to Lowry et al. [33] using bovine serum albumin (31.25–500 µg/mL) as standard. Carbohydrate concentration was determined according to Dubois et al. [34] using mannose (10–500 µg/mL) as standard.

Hemagglutinating activity

Hemagglutinating activity of seed extract, 0–60 protein fraction and erythrocytes treated with glutaraldehyde [36]. One hemagglutination unit (titer) was defined as the reciprocal of the highest dilution of the sample promoting full agglutination of erythrocytes [11]. Specific hemagglutinating activity (unit WSMoL was assessed aiming to quantify and monitor lectin activity. The assay was performed in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho [35] using suspension (2.5% v/v) of rabbit /mg) was defined as the ratio between the titer and protein concentration.

Oviposition assay

Oviposition assay was performed according to Navarro et al. [32]. Oviposition assays were carried out using *A. aegypti* gravid females 3 days after blood meal. Twenty-five females were placed in a bioassay cage (33×21×30 cm) containing two glass vessels (10 cm diameter), each containing 50 mL of distilled water and placed at diagonally opposite corners of the cage. Aliquot (1 mL, 5.1 mg/mL of protein) of seed extract, 0–60 protein fraction or WSMoL was added to a vessel, resulting in a final protein concentration of 0.1 mg/mL. The same volume of distilled water was added to the control vessel. A disk shaped piece of filter paper (18 cm in diameter) folded into a cone was placed covering the inside of each vessel, to provide a support for oviposition (Figure 1). The females were maintained at 27±0.5°C with 73±0.4% relative humidity for 14 h in the dark. After this period, eggs in oviposition paper sheets were manually counted using a stereomicroscope (Leica M80). The oviposition response was expressed as follows: % oviposition = 100× [(number of eggs in sample vessel) / (number of eggs in sample and control vessels)]. For each treatment (seed extract, 0–60 protein fraction or WSMoL), three independent experiments were performed in quintuplicate, on different dates.

Volatiles analysis by headspace analysis

WSMoL preparation was enclosed within a glass vessel from which the air was drawn for 30 min by a battery-operated membrane pump (ASF Thomas) at a constant flow rate of 200 mL/min through sorbent traps containing a mixture of 0.05 g of Tenax TA (80/100 mesh, Macherey-Nagel 706318) and 0.05 g of Carbopack X (20/40 mesh, Supleco 1–0435). Blanks corresponded to the air drawn from empty vessel. The traps were eluted with 150 µL acetone, which was kept under −24°C refrigeration until analysis.

The presence of trapped volatiles was analyzed by combined gas-chromatography-mass spectrometry (GC-MS) on a Thermo Finnigan Voyager Mass Spectrometer coupled with a Thermo Trace GC 2000 (Thermo Fisher Scientific) equipped with a CP-Wax 52CB column (Varian; 30 m×0.25 mm×0.25 µm). The sample eluted from traps (1.0 µL) was injected in the column in splitless mode, and the temperature of the inlet was 250°C. GC oven temperature was set at 60°C for 3 min, increased by 2.5°C/min to 240°C, and then held steady for 10 min. Helium carrier gas flow was maintained at a constant pressure of 100 kPa. The MS interface was 200°C, and mass spectra were taken at 70 eV in EI mode) with a scanning speed of 0.5 scan/s from m/z 20–350.

Hatchability of fresh eggs laid on seed extract, 0–60 protein fraction and WSMoL

Aiming to determine if *M. oleifera* seed preparations affect hatchability, fifty fresh eggs from each vessel from oviposition assays (including controls) were selected considering their integrity using a stereomicroscope and placed again in the same solutions where they were deposited. The number of hatched larvae present in solution was determined after 144 h of incubation at 28°C. For each treatment (seed extract, 0–60 protein fraction or WSMoL), three independent experiments were performed in quintuplicate on different dates.

Ovicidal assay using stored eggs

Ovicidal assay was performed according to Prajapati et al. [37]. A. aegypti eggs stored for 3 months at 28°C were selected considering their integrity using a stereomicroscope. Seed extract was diluted in filtered tap water to provide test solutions with protein concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 mg/mL. The same procedure was performed to provide test solutions of 0–60 protein fraction (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL of protein) and purified WSMoL (0.03, 0.05, 0.08, 0.1, 0.13, and 0.15 mg/mL). The final volume of each ovicidal assay was 20 mL of test solution and contained 50–60 eggs.

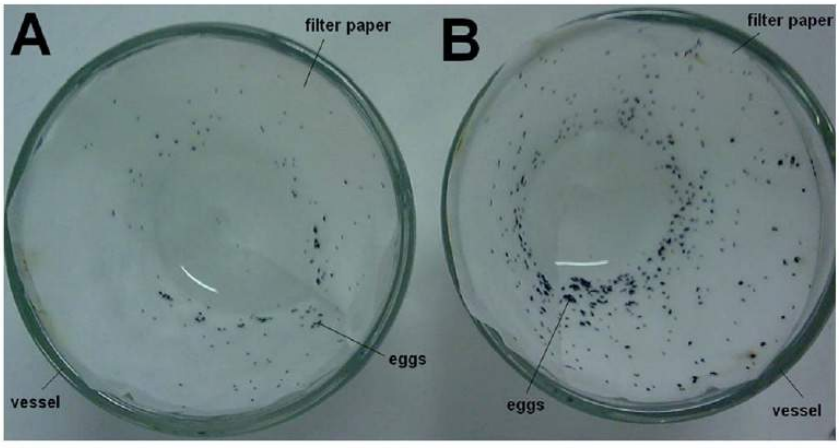

Figure 1. Top view of glass vessels used in oviposition assay. A filter paper cone was placed covering the inner of each vessel to provide a support for oviposition. Next, the vessels were filled with distilled water (A, control) or WSMoL at 0.1 mg/mL in distilled water (B).
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Controls contained distilled water, in a volume equivalent to that of sample used to achieve each concentration, completed to 20 mL with filtered tap water. The number of hatched larvae was determined after 72 h of incubation at 28°C. Three independent experiments were run in triplicate.

Aiming to evaluate whether WSMoL inhibits hatching and/or kills embryos, stored eggs submerged for 72 h in a 0.3 mg/mL lectin solution were rinsed with distilled water and transferred to another vessel filled with filtered tap water. The hatching of larvae was assessed after 24 and 48 h.

Evaluation of bacteria population in ovicidal assays medium

Fresh and stored eggs were incubated with WSMoL at 0.1 and 0.3 mg/mL, respectively. After 144 (fresh eggs) or 72 h (stored eggs), the media (50 µL) from treatments or controls were smeared on petri dishes containing nutrient agar. The plates were incubated at 37°C for 16 h. After, the number of bacterial colony forming units (CFU) was determined.

Visualization of embryos in fresh and stored eggs from ovicidal assays

Fresh and stored eggs were incubated with WSMoL at 0.1 and 0.3 mg/mL, respectively. After 144 h (fresh) or 72 h (stored), the eggs were placed in solution of 5% sodium hypochlorite until all the chorion had dissolved, leaving the vitelline membrane intact [38]. The eggs were then rinsed with distilled water to prevent complete dissolution by sodium hypoclorite and visualized in a Leica KL300 stereomicroscope (Leica Microsystems, Wetzlar, Germany). Fresh eggs incubated with water and stored eggs not incubated with any solution were also observed.

Statistical analysis

Standard deviations (s.d.) 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 ± s.d. Significant differences between treatment groups were analysed by Student's *t*-test (significance at $p<0.05$) using Origin 6.0 program. The effective concentrations required to reduce the hatching of *A. aegypti* eggs by 50% and 99% (EC_{50} and EC_{99}) in 72 h were calculated by probit analysis with a reliability interval of 95% (degrees of freedom of 5, 4 and 4 for tests with seed extract, 0–60 protein fraction and WSMoL, respectively) using the computer software IBM SPSS Statistics (IBM Corporation, New York, USA).

Results

In seed extract, protein concentration was 4.1 mg/mL and specific hemagglutinating activity was 15. In 0–60 protein fraction, protein concentration was 21.0 mg/mL and specific hemagglutinating activity was 97. No precipitate formation was observed after addition of 0–60 protein fraction to acidified barium chloride solution, revealing that no residual ammonium sulphate remained in the sample. WSMoL was isolated by chitin chromatography and was the most hemagglutinating preparation (specific hemagglutinating activity of 2,915). The results from Mohr's method did not reveal the presence of chloride ions in solution, indicating absence of residual sodium chloride content. Carbohydrate contents in seed extract, 0–60 protein fraction were 0.2 and 0.008 mg/mL, respectively and no detected in WSMoL.

The effects of *M. oleifera* seed preparations on *A. aegypti* oviposition are shown in Fig. 2A. The difference between the amount of eggs deposited in seed extract and control vessels was not significant ($p>0.05$), showing that this preparation was not able to attract or repel *A. aegypti* females. Similarly to the results found for seed extract, *A. aegypti* gravid females did not show ($p>0.05$) preference or rejection for vessels containing the 0–60 protein fraction. The number of eggs laid in vessels containing WSMoL was significantly ($p<0.05$) higher than that laid in control vessels, showing that the lectin exerts an oviposition-stimulant activity.

Volatile compounds were not detected in WSMoL preparation by dynamic headspace analysis since chromatograms from GC-MS did not reveal the presence of any such compound.

The effect of *M. oleifera* preparations on *A. aegypti* eggs from oviposition assay (fresh eggs) was evaluated aiming at determining if the hatchability of eggs laid on lectin solution would be affected. Fig. 2B shows that hatching rates of fresh eggs from seed extract, 0–60 protein fraction and WSMoL assays at concentration of 0.1 mg/mL were 45%, 20% and 55%, respectively. Significant reductions ($p<0.05$) in hatching rate of fresh eggs were observed after 144-h incubation with seed extract, 0–60 protein fraction and WSMoL in comparison with their respective controls.

The hatching rate of stored eggs was also reduced after treatment with seed extract, 0–60 protein fraction and WSMoL in a dose-dependent manner (Fig. 3). The values of EC_{50} are shown in Table 1. When eggs submerged for 72 h in WSMoL at EC99 (0.3 mg/mL) were transferred to medium containing only tap water, the hatching rate was zero after 24 and 48 h. The absence of dead larvae in WSMoL solutions at 0.03, 0.05, 0.08, 0.1, 0.13, 0.15 mg/mL and no hatching of eggs treated with WSMoL at EC_{99} reflect the ovicidal, not the larvicidal activity of lectin.

The numbers of CFU from ovicidal assays using fresh and stored eggs incubated with WSMoL were $1.4\times103\pm28$ and $1.3\times103\pm73$ CFU/mL, respectively. Media from controls of assays with fresh and stored eggs presented $1.5\times103\pm135$ and $1.3\times103\pm94$ CFU/mL, respectively.

The visualization on stereomicroscope of fresh eggs deposited in distilled water (control) or WSMoL (0.1 mg/mL) and maintained for 72 h in these same solutions revealed the presence of embryo head inside eggs from control treatment (Figure 4A), but the embryo could not be visualized through the vitelline membrane in eggs from WSMoL treatment (Figure 4B). Embryos were visualized in stored eggs incubated with WSMoL for 72 h and from control (Figure 4C and 4D).

Discussion

The results obtained for WSMoL isolation were similar to those obtained by the protocol previously described by Coelho et al. [9]. The lower values of specific hemagglutinating activity in seed extract and 0–60 protein fraction, in comparison with isolated lectin, indicate the lowest concentration of WSMoL. The measurement of the biological activity of a protein is essential to assure that it is active before determination of a biological activity. In lectinology, the hemagglutinating assay is the classic tool to assess the carbohydrate-binding property of a lectin, making sure that it is active. Increased specific hemagglutinating activity reveals lectin concentration and purification [39].

Ammonium sulphate precipitation is a rapid and inexpensive method broadly used to concentrate proteins. This salt does not affect structure and function, and can be easily removed from the protein solution by exhaustive dialysis [9–11,30,34,40]. The absence of precipitate species after treatment of 0–60 protein fraction with acidified barium chloride solution proved the absence of residual ammonium sulphate in this preparation.

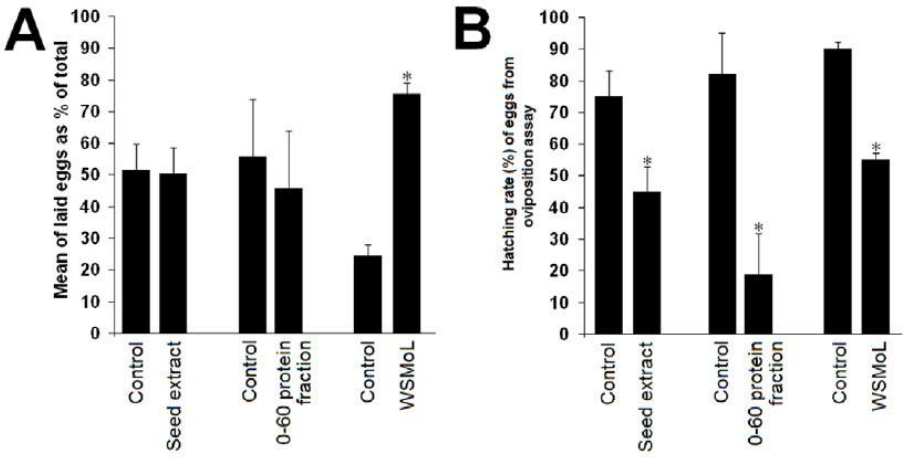

Figure 2. Effect of *M. oleifera* seed preparations (0.1 mg/mL of protein) on oviposition and egg hatching of *Aedes aegypti*. (A) Mean of eggs laid by *A. aegypti* gravid females in distilled water and *M. oleifera* seed preparations. The oviposition response was evaluated by double-choice bioassays. Three distinct assays ("control vs. seed extract", "control vs. 0–60 protein fraction" and "control vs. WSMoL") were performed separately, each one with its respective control. The oviposition response was expressed as: % oviposition = 100× [(number of eggs in sample vessel) / (number of eggs in sample and control vessels)]. (B) Hatching rate (%) of eggs laid by gravid females during the oviposition assays in distilled water and *M. oleifera* seed preparations. (*) indicates significant differences ($p<0.05$) between control and test groups.
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The results from oviposition assays revealed that WSMoL at 0.1 mg/mL with specific hemagglutinating of 2,915 acted as a chemical cue for *A. aegypti* gravid females, eliciting increased oviposition response. The absence of oviposition-stimulant activity in seed extract and 0–60 protein fraction was probably due to the lowest specific hemagglutinating activity of these preparations. To the best of our knowledge, there are no reports of oviposition deterrent, attractive or stimulant response from mosquitoes induced by plant lectins. The 0.1 mg/mL (or 100 ppm) concentration was selected, since this value is often used in oviposition bioassays [18,41,42].

Prabhu et al. [43] reported that methanolic extract from *M. oleifera* seeds (0.5, 1.0 and 2.0 mg/cm2) repelled *Anopheles stephensi*. The authors observed a decrease in number of bites in arms of human volunteers treated with the extract. The fact that the seed extract used here did not exert repellent activity reveals that repellent compounds were not extracted in water or that, if present at all, concentrations were below the detection threshold by females.

Oviposition-stimulant activity of WSMoL can be due to gustatory stimuli, since non-volatile chemicals such as proteins are perceived by gustatory system of insects [44]. The stimulus in oviposition can be unchained by WSMoL adsorption onto gustatory sensillas of *A. aegypti* female, as reported in a study on the detection of sex pheromones and protein kairomones by *Glossina* spp. and *Diadromus pulchellus*, respectively [45,46]. Volatile compounds were not detected by dynamic headspace analysis in WSMoL preparation indicating that, if present, they occurred in trace amounts. Olfactory stimuli by volatile compounds were described for selection of oviposition sites by *Culex quinquefasciatus*, *A. aegypti* and *Aedes triseriatus* [47].

WSMoL from chitin column was dialysed against distilled water aiming to eliminate acetic acid used in elution as well as residues of 0.15 M NaCl (0.85%) used in washing steps of chromatography. TianFu et al. [48] reported that acetic acid (0.001 mg/L) acted as an oviposition attractant to *Aedes albopictus* gravid females. In the present study, GC-MS analysis did not identify acetic acid in WSMoL. Thus, the effect of lectin preparation was not due to contamination by this compound. Chloride ions were not detected by Mohr's method in WSMoL, indicating that the oviposition-stimulant effect of lectin cannot be linked to salt contamination. Also, Navarro et al. [32] demonstrated that NaCl at 0 to 5% concentrations did not interfere on *A. aegypti* oviposition.

Frings and Hamrum [49] reported that sugars can be detected through contact receptors present in labella of *A. aegypti* mosquito. Thus, *M. oleifera* preparations were evaluated for presence of these compounds. The results reveal that carbohydrates from *M. oleifera* seed preparations were not attractive components, since WSMoL, which showed the highest oviposition rate, was free of carbohydrates. WSMoL can be included in the group of non-volatile proteins with oviposition-stimulant activity on *A. aegypti*.

Since the embryogenesis of *A. aegypti* eggs is completed between 77–96 h and acquisition of egg resistance occurs 48 h after post-oviposition [25,50] the effect on hatchability of eggs from oviposition assay was assessed. The hatching rates were determined after 144 h to guarantee that the eggs had a reasonable time to complete embryonic development. The results showed that besides stimulating oviposition by females, WSMoL at 0.1 mg/mL was also able to impair hatching, an advantage if it will be used in ovitraps.

The effect of WSMoL preparations on stored eggs was also evaluated during an incubation period of 72 h. Incubation periods between 72 and 120 h have been used to assess ovicidal activity on eggs which have already completed their embryogenesis [51–56]. The detection of lowest EC_{50} value for WSMoL suggests that the lectin is an active principle in seed extract and 0–60 protein fraction against stored eggs, although a proportional correlation between increase in specific hemagglutinating activity and ovicidal effect (decrease in EC_{50}) was not observed. The absence of hatching of stored eggs treated with WSMoL at 0.3 mg/mL (EC_{99}) after transfer to medium without lectin indicates that embryos within the eggs were killed by WSMoL. The absence of contaminant traces of acetic acid, sodium chloride and ammonium sulphate in 0–60 protein fraction and WSMoL assure that the

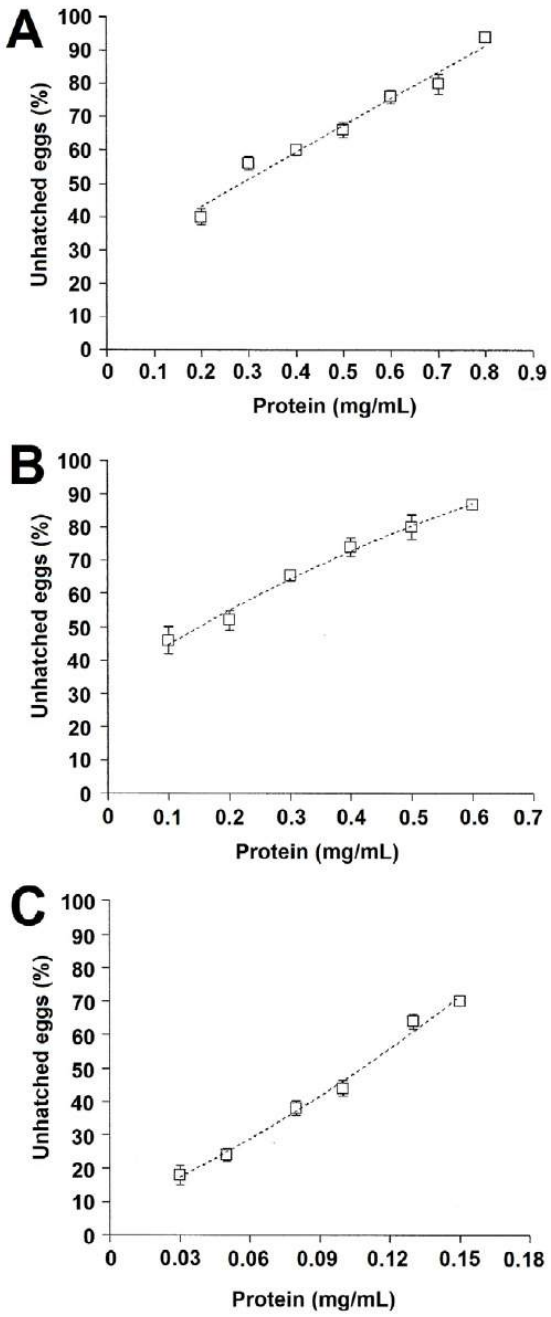

Figure 3. Percentage of unhatched stored eggs after incubation with seed extract (A), 0–60 protein fraction (B) and WSMoL (C) for 72 h.
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adverse effects of these preparations on hatching rates were not due to residues of these chemicals.

It has been reported that the presence of bacteria is a hatching-stimulant factor for *A. aegypti* eggs and in the absence of bacteria the hatchability is lower than 5% [57]. Since it is known that

Table 1. Ovicidal activity on *A. aegypti* stored eggs of *M. oleifera* seed extract, 0–60 protein fraction and WSMoL.

Sample	EC_{50} (mg/mL) [a]	EC_{99} [a]
Seed extract	0.32 [0.25–0.37]	1.18 [1.05–1.38]
0–60 protein fraction	0.16 [0.1–0.22]	1.14 [0.96–1.44]
WSMoL	0.10 [0.09–0.12]	0.30 [0.25–0.38]

[a]Effective concentrations of proteins required to reduce in 50% (EC_{50}) and 99% (EC_{99}) the hatching of *A. aegypti* eggs in 72 h calculated by probit analysis with a reliability interval of 95%. Values in square brackets represent the lower and upper endpoints for reliability interval.
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WSMoL exerts antibacterial effect [29], it was evaluated whether the lower hatching rate could have been also a consequence of reduction in bacteria population. The numbers of CFU/mL in media from ovicidal assays using WSMoL was not lower than that detected in media from control assays. This result reveals that, in this case, lower hatching rates in WSMoL treatment were not linked to decrease in bacterial population. So, it is possible to conclude that impairment of egg hatching by WSMoL was due to embryo death.

It was also evaluated if the sensitiveness of freshly laid eggs to *M. oleifera* preparations was different from that of stored eggs. According to probit analysis of results from ovicidal assays using stored eggs, the egg hatching rates after treatment with seed extract, 0–60 protein fraction and WSMoL at concentration of 0.1 mg/mL would be 65%, 54% and 50%, respectively. Comparison of data from ovicidal assays using fresh and stored eggs and protein concentration of 0.1 mg/mL reveals that stored eggs were less sensitive to seed extract and 0–60 protein fraction, while WSMoL reduced similarly the hatching rates of fresh and stored eggs. These findings indicate the presence of other ovicidal agents in seed extract and 0–60 protein fraction that were eliminated in the WSMoL isolation procedure. Ferreira et al. [58] did not detect ovicidal activity from aqueous extract from dehulled *M. oleifera* seeds (5.2 mg/mL) using stored eggs. The authors attributed this fact to the high resistance of eggs. Differently, WSMoL showed similar ovicidal activity against fresh and stored eggs, indicating that the completion of embryogenesis and egg maturation processes was not accompanied by reduction in sensitiveness to lectin. This is an interesting and advantageous property concerning *A. aegypti* control strategies targeting the eggs, the most resistant stage of mosquito life cycle.

The EC_{50} of WSMoL on stored eggs was lower than values determined for essential oils from *Juniperus macropoda*, *Zingiber officinale* and *Pimpinella anisum*, whose EC_{50} ranged from 0.15 to 0.18 mg/mL and leaf extracts of *Eclipta alba*, whose EC_{50} ranged from 0.10 to 0.20 mg/mL [27,37]. The ovicidal activity of WSMoL was compared with those from essential oils and plant extracts because there are no previous reports on ovicidal activity of lectins on insects.

Ovicidal compounds are able to interrupt embryo development, impair the survival of larva inside the egg or block egg hatching [27]. Fresh eggs from control treatment showed embryogenesis in progress while impairment of embryo development was detected in fresh eggs treated with WSMoL, reflecting the ovicidal activity.

Chitin is present in oocytes, eggshell and eggs of *A. aegypti* and this polysaccharide has an important role in egg viability [50]. The ovicidal activity of WSMoL may be linked to binding of lectin to chitin present in eggshells, blocking the hatching process by promoting disruption of this structure. Also, the fact that stored

Figure 4. Visualization of *A. aegypti* fresh and stored eggs exposed or not to WSMoL in stereomicroscope (x80) after clearing of the heavily pigmented chorion with 5% sodium hypochlorite. (A) Fresh egg laid and maintained in distilled water (control). (B) Fresh egg laid and maintained in WSMoL (0.1 mg/mL). (C) Stored egg which was not treated with any solution. (D) Stored egg that did not hatch after incubation with WSMoL at EC_{99} (0.3 mg/mL) for 72 h. (h) head; (a) abdomen.
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eggs transferred to water after incubation with WSMoL for 72 h did not hatch reveals that embryos, observed inside eggs using a stereomicroscope, are dead. It is possible that WSMoL gets inside the egg, interfering in embryo development through the same mechanisms which kill *A. aegypti* larvae.

The previously described larvicidal activity of WSMoL, combined with its oviposition-stimulant and ovicidal activities described here make this lectin a very interesting candidate for use in integrated mosquito control programs. The WSMoL concentration in which oviposition-stimulant (0.1 mg/mL) and ovicidal (EC_{50} of 0.1 mg/mL) activities were detected was lower than that in which the lectin promotes larvicidal activity (LC_{50} of 0.197 mg/mL). This finding is interesting, because if WSMoL is applied in an *A. aegypti* breeding site aiming at killing the larvae, it will also attract gravid females and will impair the hatching of new eggs laid by females.

In summary, WSMoL is a sustainable and environmentally friendly alternative for *A. aegypti* control, since one same concentration of WSMoL acts as a chemical stimulant cue for ovipositing females and ovicidal agent at the same time. Further studies will be performed on oviposition response of gravid females under field conditions as well as on the development of effective formulations for WSMoL and its large-scale production based on genetic engineering techniques.

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Author Contributions

Conceived and designed the experiments: NDLS THN DMAFN PMGP. Performed the experiments: NDLS KSM THN GKNS. Analyzed the data: NDLS KSM THN DMAFN PMGP. Contributed reagents/materials/analysis tools: LCBBC DMAFN PMGP. Wrote the paper: NDLS THN GKNS LCBBC DMAFN PMGP.

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6. CAPÍTULO 2

Evaluation of the *Moringa oleifera* seed lectin in traps for capture of *Aedes aegypti* eggs and mosquitoes under semi-field conditions

ARTIGO A SER SUBMETIDO AO PERIÓDICO "PLOS Neglected Tropical Diseases"

(Fator de impacto: 4.693)

Evaluation of the *Moringa oleifera* seed lectin in traps for capture of *Aedes aegypti* eggs and mosquitoes under semi-field conditions

Nataly Diniz de Lima Santos[a], Kelly da Silva Paixão[b], Priscila Barbi Trindade[b], Mariele Ribeiro Pinto[b], Luana Cassandra Breitenbach Barroso Coelho[a], Thiago Henrique Napoleão[a], Álvaro Eduardo Eiras[b], Daniela Maria do Amaral Ferraz Navarro[c], Patrícia Maria Guedes Paiva[a,*]

[a]*Departamento de Bioquímica-CCB, Universidade Federal de Pernambuco, Recife, PE 50670-420, Brazil.*

[b]*Departamento de Parasitologia-ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil.*

[c]*Departamento de Química Fundamental-CCEN, Universidade Federal de Pernambuco, Recife, PE 50670-901, Brazil.*

*Corresponding author. Tel: +558121268540; fax: +558121268576.

E-mail address: ppaivaufpe@yahoo.com.br

Abstract

Background: The water-soluble lectin from *Moringa oleifera* seeds (WSMoL) is a larvicidal, ovicidal and oviposition-stimulant agent against *Aedes aegypti* under laboratory conditions. This work investigated the effect of WSMoL in traps for capture of *A. aegypti* eggs and females under semi-field conditions as well as the involvement of female olfactory sensilla in the response toward WSMoL.

Methodology/Principal Findings: WSMoL was isolated according to procedure previously described using chitin chromatography. The bioassays were performed in large cages (2.5 x 2.5 x 2.0 m) at the campus of the *Universidade Federal de Minas Gerais*, Belo Horizonte, Brazil. Two traps for capture of eggs (ovitrap) or adult mosquitoes (MosquiTRAP^{TM}) were placed in each cage being one filled with WSMoL (0.1 mg/mL) and another tap water (negative control). Infusion of *Panicum maximum* leaves was used as positive control. Forty gravid females were then released on each cage. After 2 h (for oviposition) or 3 h (for female capture), the traps were removed and the number of eggs or females was counted. Olfactometry assay was performed aiming to investigate the involvement of olfactory sensilla in the response of females toward WSMoL. WSMoL showed oviposition-stimulant effect (65±14%) similar ($p<0.05$) to that promoted by *P. maximum* infusion (67±11%). The efficiency of MosquiTRAP^{TM} in capture gravid females was not increased by WSMoL. The olfactometry assay indicated that the response of females to WSMoL did not involve olfactory sensilla.

Conclusions/Significance: WSMoL was effective for capture of eggs when used in ovitraps under semi-field conditions; this property, together with the ovicidal and larvicidal activities of this lectin, makes it as a candidate with excellent characteristics for *A. aegypti* control.

Introduction

Blood-feeding arthropods are transmitters of several infectious agents such as bacteria, protozoan, helminthes and viruses [1]. The mosquito *Aedes aegypti* is the vector of the viruses that cause yellow, chikungunya and dengue fevers, which occur mainly in tropical and subtropical regions. The dengue fever is included in the group of neglected tropical human diseases, which comprises infirmities most commonly associated with poverty and are responsible for the morbidity and/or mortality of millions each year [2].

The dengue virus (DENV) is the most widespread arbovirus being endemic in over 100 countries and causing 50 million infections yearly [3]. Infection with the serotypes DENV-1 (five genotypes), DENV-2 (six genotypes), DENV-3 (five genotypes), or DENV-4 (four genotypes) may be asymptomatic or cause mild febrile illness, dengue fever, or severe dengue. Although many advances have been reached in regard to the development of vaccines and anti-virals, there is still no vaccine and specific treatment available for dengue fever [4,5].

The control of *A. aegypti* populations is essential in combating dengue fever spreading. Studies have focused on monitoring the *A. aegypti* abundance, which is nearly associated with environmental fluctuations [6] as well as on the application of insecticides against eggs, larvae, pupae and adults of the mosquito. Most vector control programs rely on the use of chemical insecticides but resistance development by the insect has increased. In this scenario, studies have investigated plant-derived compounds including secondary metabolites, lectins and protease inhibitors as alternative insecticides [7-9].

Different types of traps have been used to control or monitor the density of *A. aegypti* populations. Ovitraps are usually containers dark in color which can be filled with water or other solution and contains a wooden paddle serving as support for females laid the eggs. The ovitraps are mainly considered for monitoring vector populations or using as repellents in

reservoirs to prevent mosquito breeding [10]. The Mosquitrap™ is a sticky trap designed by Eiras [11] to capture gravid *A. aegypti* females aiming monitoring the species density. This trap is based upon the behavior of females to choose a potential breeding site and combines visual (black color) and olfactory (attractant solution) stimuli.

Lectins, carbohydrate-binding proteins, are found in *M. oleifera* seeds. Coelho et al. [12] and Santos et al. [8] reported that the water-soluble *M. oleifera* lectin (WSMoL) killed *Aedes aegypti* fourth-stage larvae (LC_{50} of 0.197 mg/mL), impaired the hatching of *A. aegypti* stored eggs by killing the embryos, showed oviposition-stimulant activity and prevented the development of embryos in the eggs laid by *A. aegypti* gravid females. These results indicated WSMoL as a very interesting candidate for use in integrated *A. aegypti* control. Since novel control strategies require confirmation under field conditions before they can be operationally deployed [13], this work evaluated the efficacy of WSMoL when used in traps for capture of *A. aegypti* eggs or females under semi-field conditions.

Methods

Plant material

M. oleifera (Family Moringaceae) is popularly known as ‘‘moringa” in Portuguese, “árbol del ben” in Spanish, and horseradish tree or drumstick in English. Seeds of *M. oleifera* were collected in Recife, Pernambuco, Brazil, and stored at -20 °C. A voucher specimen is deposited number 73,345 at the herbarium ‘‘Dárdano de Andrade Lima” from the *Instituto Agronômico de Pernambuco* (Recife, Brazil).

Aedes aegypti maintenance in laboratory

The insects colony (F9) used in semi-field experimental area are maintained in the *Laboratório de Ecologia Química de Insetos Vetores* at the *Universidade Federal de Minas Gerais* (Belo Horizonte, Minas Gerais, Brazil) since 2000. The rearing room is kept under 27 ± 1ºC, 75-80% humidity and 12:12 light-dark photoperiod. The larvae were kept in plastic bowls with water and fed with ornamental fish food (Goldfish®). The adult mosquitoes (males and females) were kept in fine screen cages (30x30x30 cm, Bug-Dorm 1®, Mega View Science Education Services Co. Ltd. Taiwan) and fed with a glucose solution (10% sucrose). Adult females aged 10 to 20 days received blood meals and were separated in a selection cage consisting of an acrylic box (50 x 50 x 50 cm). This cage contained a small electrical fan at the back, which served as a ventilation system that allows the odor of a human hand to direct females to fly toward a breeding cage placed inside the acrylic box [14]. Females with 3-4 days post blood meal were used in the experiments [15].

Isolation of WSMoL

WSMoL was isolated according to the procedure described by Coelho et al. [12]. Ten grams of *M. oleifera* seed powder were homogenized with distilled water (100 mL) for 16 h at 4 °C using a magnetic stirrer. After filtration through gauze and centrifugation (9,000 *g*, 15 min, 4ºC), the clear supernatant (seed extract) was submitted to protein precipitation for 4 h at 28 ºC using ammonium sulphate at 60%-saturation [16]. The 0-60% precipitate was collected after centrifugation (9,000 *g*, 15 min, 4 ºC) and dialyzed (3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (4 h). The dialyzed fraction corresponded to the 0-60 protein fraction.

The 0-60 protein fraction was loaded (40 mg of protein) onto a chitin column (7.5 x 1.5 cm) equilibrated (0.3 mL/min flow rate) with 0.15 M NaCl. After washing with the equilibrating solution, WSMoL was eluted from the column with 1.0 M acetic acid and

dialyzed (3.5 kDa cut-off membrane) against distilled water (4 h) at 4°C for eluent elimination. The protein concentration was determined according to Lowry et al. [17] using bovine serum albumin (31.25–500 µg/mL) as standard.

For use in bioassays, WSMoL was diluted in tap water to the concentration of 0.1 mg/mL, the same at which this lectin showed oviposition-stimulant effect under laboratory conditions [8].

Hemagglutinating activity

Hemagglutinating activity was assessed in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho [18] using 2.5% (v/v) suspension of rabbit erythrocytes treated with glutaraldehyde [19]. One hemagglutination unit ($titer^{-1}$) was defined as the reciprocal of the highest sample dilution that promotes full agglutination of erythrocytes. Specific hemagglutinating activity (unit/mg) was defined as the ratio between the hemagglutinating activity units and protein concentration (mg/mL).

Semi-field area

The semi-field area (14 x 7 x 3.5 m) used in this work was built at the *Instituto of Ciências Biológicas* from the *Universidade Federal de Minas Gerais*, Belo Horizonte, Minas Gerais, Brazil. All details are described by Roque and Eiras [15]. The experimental area contains eight test cages (2.5 x 2.5 x 2 m) with walls and ceiling made of white voile fabric. A potted plant (*Spathiphyllum*) was placed at the center of each cage to function as a resting place for the mosquitoes. Temperature and humidity throughout the experiments were monitored by a thermo-hygrometer placed in the center of the experimental area. The experiments were carried out in the morning (9 a.m to 11 a.m) and in the afternoon (3 p.m. to 5 p.m.) from January to March 2012.

Assays using ovitraps

The oviposition traps (ovitraps) used in the tests consisted of a black plastic cylinder container (12 x 10 cm diameter; 1 L capacity) with a wooden paddle (12.5 x 2.5 cm) as the oviposition substrate which was fixed vertically inside of the ovitrap with a metallic clip. The ovitraps were filled with 300 mL of 0.1 mg/mL WSMoL (test), 10% (w/v) gray infusion of *Panicum maximum* (positive control) or tap water (negative control) and assays were carried out using *A. aegypti* gravid females 3 days after blood meal. Forty females were placed in each cage containing a pair of ovitraps (infusion vs. tap water or WSMoL vs. tap water). Eight replications were performed with 120 min of duration [15].

Assays using traps for mosquitoes

The MosquiTRAP™ (Version 1.0, Ecovec Ltda.) consisted of a black plastic cylinder container (16 x 11cm) with a sticky card for capture of mosquitoes and a screen that prevents mosquitoes entering in contact with the liquid with which the trap is filled [11]. This trap was used to evaluate the efficacy of WSMoL as an attractant for capturing *A. aegypti* females under semi-field conditions. The traps were filled with 300 mL of 0.1 mg/mL WSMoL (test), positive control 10% (w/v) gray infusion of *Panicum maximum* [20] or tap water (negative control). Forty gravid *A. aegypti* females were released in the center of each cage containing a pair of traps (infusion vs. tap water or WSMoL vs. tap water). Eight replications were performed for each one. The number of captured females on sticky card was evaluated in the cages after 180 min.

Olfactometer assay

Involvement of olfactory receptors in attractiveness of *A. aegypti* females by WSMoL was evaluated in a horizontal dual choice olfactometer system [21]. Figure 1 schematizes the

system used. A release cage containing 10 females was attached to the main tube of the olfactometer and it was allowed that the mosquitoes acclimatize with the clean air flushing for 15 min. Next, a vessel containing WSMoL (0.1 mg/mL) was introduced into one of the two parallel sample (stimulus) chambers, at the same time as another vessel containing only tap water was introduced in the other chamber (blank control). The mosquitoes were then released from the cage and allowed 1 min to respond, after which the rotating doors (indicated as arrows in Figure 1) were closed trapping them inside the different parts of the olfactometer. WSMoL was tested in balanced and randomized block designs. Fifteen replications were carried out.

Statistical analysis

The insect's responses in semi-field experiments were analyzed using *t-test* or *Mann Whitnney test*. The number of insects that responded to stimuli in the olfactometer bioassays was converted into a percentage and then the mean percentages (± s.d.) were calculated.

Results and discussion

The effects of WSMoL on dengue mosquito *A. aegypti* under laboratory conditions were investigated by Coelho et al. [12] and Santos et al. [8]. This lectin shows larvicidal and ovicidal activities as well as is a chemical stimulant cue for ovipositing females. This work evaluated WSMoL in traps for capture of *A. aegypti* eggs and females under semi-field conditions.

A. aegypti gravid females laid a higher ($p<0.05$) number of eggs in ovitraps containing 0.1 mg/mL WSMoL than in ovitraps containing only tap water (Figure 2A). This result reveals that the oviposition-stimulant effect of WSMoL observed in laboratory was also

detected under semi-field conditions. WSMoL showed similar efficiency in attract ovipositing gravid females than the *P. maximum* infusion used as positive control (Figure 2B).

Temperature, relative humidity of the air, light, and wind are some factors that can interfere on *A. aegypti* oviposition behavior. Roque and Eiras [15] when calibrating the semi-field area used in this experiment, observed that the intervals of 25–30 °C and 65–70% humidity were the most adequate for conduction of experiments. The assays used in the present paper were performed only when the temperature and humidity were inside these intervals and thus the possibility of false positives or negatives due to interference of environmental changes can be discharged.

Ovitraps usually contain a wooden paddle that serves as an oviposition substrate since the *A. aegypti* females prefer laid the eggs on rough substrates [22]. The most of eggs found in ovitraps containing WSMoL was laid by females on the liquid surface (Figure 3A) while in the negative control ovitraps there was no significant difference ($p>0.05$) between the number of eggs laid on the liquid surface and on the paddle (Figure 3A). These results are probably linked to the fact that the oviposition-stimulant effect of WSMoL involves interactions between lectin and contact receptors (gustatory sensilla), which are found in legs and mouth parts of mosquitoes. In this sense, the females would need to touch the liquid surface in order to recognize WSMoL as a chemical cue for oviposition. Distinctly a higher number of eggs were laid on paddle in ovitraps containing *P. maximum* infusion (Figure 3B) and this fact may be related to the fact that volatile compounds, in addition to non-volatile chemicals, has been reported to act as attractants in plant infusions [23].

It was reported that volatile compounds were not detected in WSMoL preparations [8] and this is in agreement with the hypothesis that oviposition response by females involves contact sensilla. In order to verify this assumption, an olfactometer assay was performed. The results demonstrated that the most of females released were inactive (59.5 ± 10.3%), i.e., did

not leave the release cage showing no flight activity. Among the females that flied (active) the most remained in the main tube (30.5 ± 8.9%) and did not reach any of choice tubes. This result reveals that gravid females were not able to perceive the presence of WSMoL from a distance through their olfactory sensilla.

The responses of *A. aegypti* to volatile compounds through olfactory sensilla have been well studied but informations on responses to chemicals mediated by gustatory receptors as well as molecular structures of these receptors are scarce. Melo et al. [24] identified the receptor *AaOr7* which is found in both olfactory and gustatory organs. Recently, gustatory neurons were characterized for the first time as contact sensilla on the labella of *A. aegypti* females for response to insect repellents [25]. The authors detected sensilla responses to the insect repellents DEET, picaridin, citronellal, and IR3535 and suggested that these contact receptors are probably involved in detection of toxic or otherwise deleterious stimuli. It has been reported that infusion of *Quercus alba* leaf mediated oviposition of *Aedes triseriatus* and *Aedes albopictus* through contact with non-volatile arrestants [26]. Also, an alfalfa hay infusion was reported to contain non-volatile chemicals that arrested females at the liquid surface and stimulate oviposition [27].

The fact that the most of eggs laid in ovitraps containing WSMoL were found on liquid surface is interesting since this lectin also possess ovicidal activity by blocking the embryo development or killing the embryos inside the eggs [8]. Thus the eggs laid on ovitraps containing this lectin would have their hatching unviable.

The results using MosquiTRAP^{TM} revealed that WSMoL did not increase the efficiency of this trap in capturing females in comparison with the traps containing only tap water (Figure 5A). However, the number of trapped females was significantly higher ($p<0.05$) when the *P. maximum* infusion was used (Figure 5B). These results are probably linked to the fact that, in this kind of trap, the mosquitoes cannot enter in contact with the liquid surface

and thus the attractant needs to act on females from a distance. In this way, the females were probably not able to detect the presence of WSMoL through their gustatory sensilla while in traps with *P. maximum* infusion the capture increases due to attraction by volatile compounds.

In conclusion, WSMoL acted efficiently as an oviposition-stimulant for optimization of ovitraps under semi-field conditions but was not effective when used in traps for adults. The mechanisms linked to WSMoL detection by females does not involve olfactory stimuli and are probably linked to gustatory sensilla (contact receptors). Further studies are now needed on the detection of WSMoL receptors on the female body as well as on the development of effective formulations for WSMoL and its large-scale production.

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Author Contributions

Conceived and designed the experiments: NDLS KSP THN AEE DMAFN PMGP. Performed the experiments: NDLS KSP PBT MRP. Analyzed the data: NDLS KSM THN

DMAFN PMGP. Contributed reagents/materials/analysis tools: LCBBC AEE DMAFN PMGP. Wrote the paper: NDLS KSP THN LCBBC DMAFN PMGP.

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Figure captions

Figure 1. Schematic representation of the olfactometer system. (A) Release cage. (B) Main tube. (C) Choice tubes. (D) Sample (stimulus) chambers. Each solid arrow indicates the localization of a rotating door. The dashed arrow indicated the air flow direction. A clean air flush is generated by a compressor and directed from sample chambers to release cage in order to carry any odor present in the sample.

Figure 2. Mean of eggs laid by *A. aegypti* gravid females in ovitraps containing tap water (control), 0.1 mg/mL WSMoL (A) or 10% (w/v) *P. maximum* infusion (B). The oviposition response was evaluated by double-choice bioassays. Two distinct assays ("control vs. WSMoL" and "control vs. *P. maximum* infusion") were performed separately. The oviposition response was expressed as: % oviposition = 100 x [(number of eggs in sample ovitrap) / (number of eggs in sample and control ovitrap)]. (*) indicates significant differences ($p<0.05$) between control and test groups.

Figure 3. Mean of eggs laid by *A. aegypti* gravid females on liquid surface or wooden paddles in ovitraps containing tap water (control), 0.1 mg/mL WSMoL (A) or 10% (w/v) *P. maximum* infusion (B). The percentages were expressed as: % eggs = 100 x [(number of eggs on liquid surface or paddle) / (number of eggs laid in the ovitrap)]. (*) indicates significant differences ($p<0.05$) between control and test groups.

Figure 4. Mean of gravid *A. aegypti* gravid females trapped using MosquiTRAP^{TM} containing distilled water (control), 0.1 mg/mL WSMoL (A) or 10% (w/v) *P. maximum* infusion (B). The trapping efficacy was evaluated by double-choice bioassays. Two distinct assays ("control vs.

WSMoL" and "control vs. *P. maximum* infusion") were performed separately. The oviposition response was expressed as: % trapping = 100 x [(number of females in sample MosquiTRAP^{TM}) / (number of females in sample and control MosquiTRAP^{TM})]. (*) indicates significant differences ($p<0.05$) between control and test groups.

Figure 1

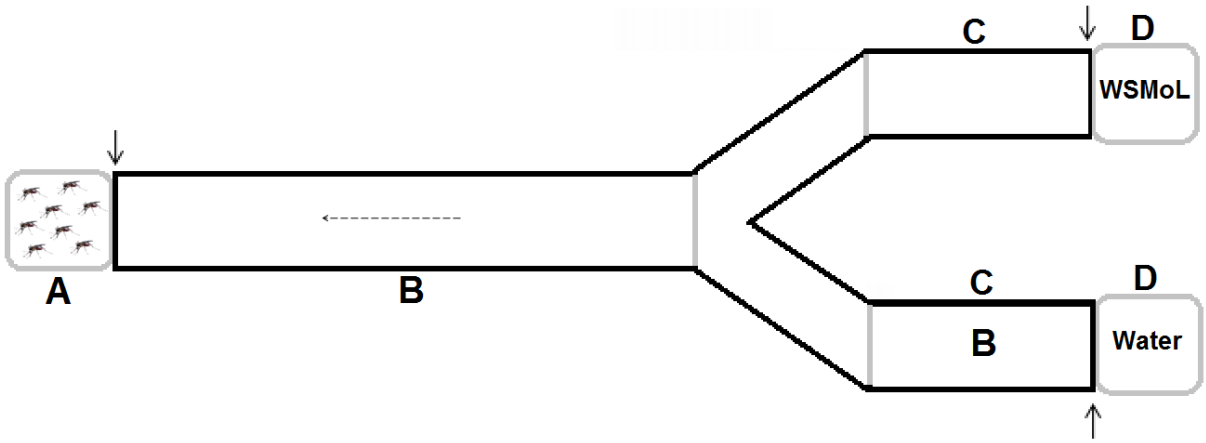


Figure 2

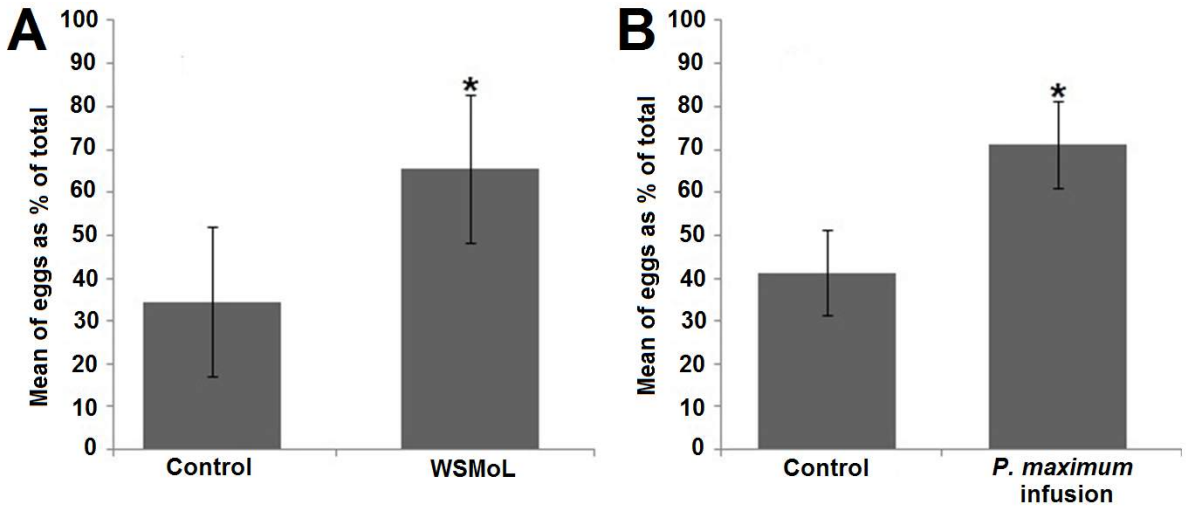


Figure 3

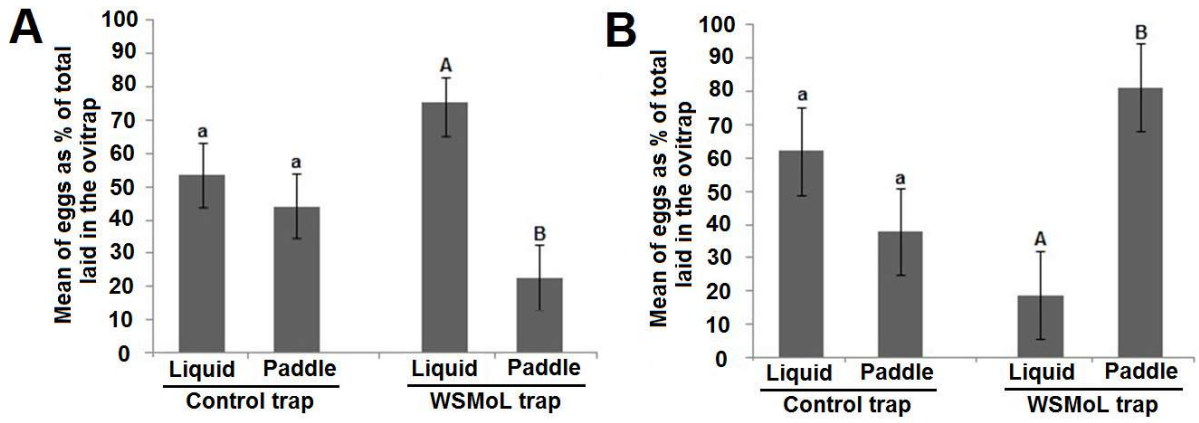
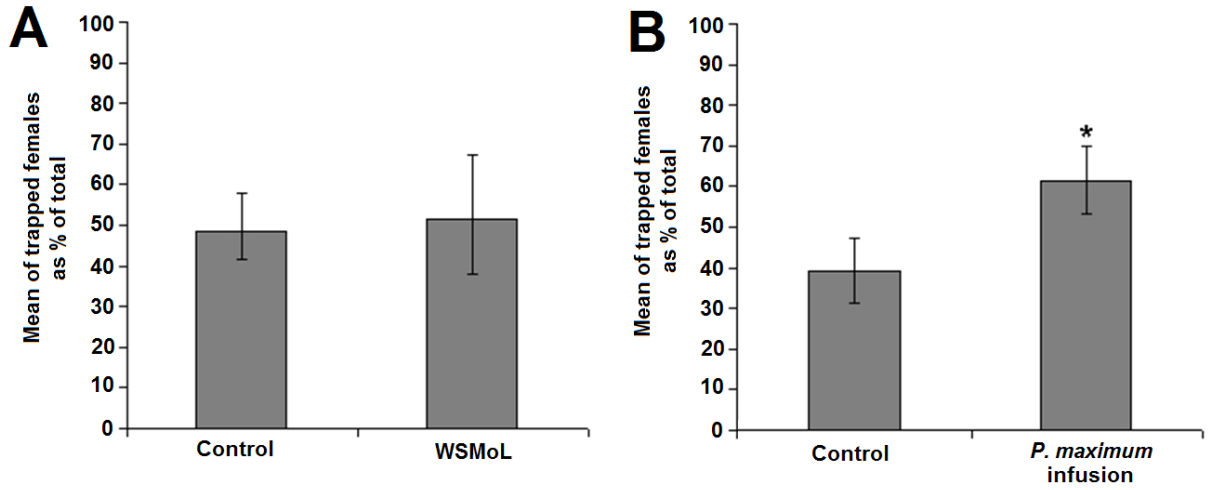


Figure 4



7. CAPÍTULO 3

Effect of gamma irradiation of *Moringa oleifera* seed lectin on its larvicidal, ovicidal and oviposition-stimulant activities against *Aedes aegypti*

ARTIGO A SER SUBMETIDO AO PERIÓDICO "Parasites & Vectors"

(Fator de impacto: 2.940)

Effect of gamma irradiation of *Moringa oleifera* seed lectin on its larvicidal, ovicidal and oviposition-stimulant activities against *Aedes aegypti*

Nataly Diniz de Lima Santos[1], Thiago Henrique Napoleão[1], Clayton Augusto Benevides[2], Lidiane Pereira de Albuquerque[1], Emmanuel Viana Pontual[1], Luana Cassandra Breitenbach Barroso Coelho[1], Daniela Maria do Amaral Ferraz Navarro[3], Patrícia Maria Guedes Paiva[1,*]

[1]Departamento de Bioquímica-CCB, Universidade Federal de Pernambuco, Recife, PE 50670-420, Brazil.

[2]Centro Regional de Ciências Nucleares (CRCN/NE), Comissão Nacional de Energia Nuclear, Ministério da Ciência, Tecnologia e Inovação, Recife, PE 50740-540, Brazil.

[2]Departamento de Química Fundamental-CCEN, Universidade Federal de Pernambuco, Recife, PE 50670-901, Brazil.

*Correspondence: ppaivaufpe@yahoo.com.br

Abstract

Background: The water-soluble lectin from *Moringa oleifera* seeds (WSMoL) was larvicidal agent against *A. aegypti* as well as showed ovicidal effect on fresh and stored eggs and oviposition-stimulant activity on gravid females. This work evaluated the effect of low doses (10 mGy and 10 Gy) of gamma irradiation on the activity of WSMoL on survival of larvae and eggs of *A. aegypti* as well as on the oviposition behavior of gravid females.

Results: Protein concentration in WSMoL samples was not affected after irradiation at 10 mGy and 10 Gy, revealing that there was no loss of protein molecules by precipitation. The specific hemagglutinating activity of WSMoL increased after irradiation with the lowest dose used (10 mGy) while the sample irradiated with 10 Gy showed the same hemagglutinating activity of non-irradiated lectin. The irradiation of WSMoL with 10 mGy resulted in potentialization of larvicidal effect and ovicidal activity on stored eggs while the lectin irradiated with 10 Gy showed no larvicidal and ovicidal effects. The oviposition-stimulant effect of WSMoL was damaged after the lectin was submitted to both doses of gamma irradiation. On the other hand, the hatchability of the eggs laid on vessels containing 10 mGy-irradiated lectin was more affected than in vessels with non-irradiated WSMoL.

Conclusion: In conclusion, a very low dose of gamma irradiation (10 mGy) can be used to improve the deleterious effects of WSMoL on *A. aegypti* larvae and eggs while a higher dose (10 Gy) was able to abolish all the insecticidal effects of this lectin on this mosquito species.

Keywords: *Moringa oleifera*, gamma radiation, lectin irradiation, larvicidal effect, ovicidal activity, oviposition-stimulante, dengue mosquito, disease control, dengue fever.

Background

It is estimated that about 40% of the global population in some 100 countries are exposed to several types of vector-borne diseases that result in thousands of deaths annually [1]. Among these diseases are the arboviruses, which are transmitted by arthropod vectors. The mosquito *Aedes aegypti* (Diptera: Culicidae) is considered the main vector of the viruses that cause dengue and yellow fevers. Dengue fever is considered as a priority public health problem in Brazil and measures to control the mosquito population remains as the main component in disease prevention programs in endemic countries [2].

Pesticides currently used for mosquito control are known to cause environmental pollution with residual effects, and their indiscriminate use has led to selection of resistant individuals (Pluempanupat et al., 2013). Phytochemicals with mosquitocidal potential have been studied as suitable alternative insecticides to replace synthetic insecticides in mosquito control programs due to their larvicidal, pupicidal, and adulticidal effects, biodegradability and general lower toxicity than non-natural chemicals [3,4]

Lectins – carbohydrate-binding proteins – isolated from plants have shown deleterious effects on *A. aegypti* [5-7]. The water-soluble lectin from *Moringa oleifera* seeds (WSMoL) was larvicidal agent against *A. aegypti* as well as showed ovicidal effect on fresh and stored eggs and oviposition-stimulant activity on gravid females; this last was also detected under semi-field conditions [5,8,9]. It was reported that the larvicidal activity of WSMoL was probably linked to binding of lectin to glycoconjugates and peritrophic matrix at insect midgut causing disruption of gut epithelium while the ovicidal effect (absence of hatching) was probably due to blocking of embryo development in fresh eggs and death of mature embryo still inside the stored eggs. In addition WSMoL act as a chemical cue for ovipositing

A. aegypti females probably by acting through interaction with contact (gustatory) sensilla of insects and not through olfactory sensilla [5,8,9].

Gamma radiation is able to alter the molecular structure of several biomolecules. Depending on the doses applied, these alterations may led to improvement in bioactivity as well as may fragmentize and inactive the biomolecule. For example, low dose (1 kGy) of gamma irradiation increased the allergenic potential of the lectin concanavalin A [10] while high irradiation doses (10 and 25 kGy) suppressed the allergenicity of lectin from *Cratylia mollis* seeds [11]. Modifications caused by irradiation of proteins in aqueous solution and presence of oxygen include protein carbonylation, oxidation of side-chain groups (including hydrophobic amino acyl residues and –SH groups), protein scission, backbone fragmentation, dityrosine formation, unfolding and cross-linking with formation of high molecular weight aggregates [12,13].

Doses up to 1 kGy are considered low for gamma irradiation of proteins and are used to control food-borne pathogens and reduce microbial load and insect infestation, for example [14]. The objective of this study was to evaluate the effect of low doses (10 mGy and 10 Gy) of gamma irradiation on the activity of WSMoL on survival of larvae and eggs of *A. aegypti* as well as on the oviposition behavior of gravid females. The study was conducted in order to evaluate if gamma irradiation would improve lectin effects and thus, the biological properties of irradiated WSMoL and non-irradiated lectin sample were compared.

Methods

Plant material

Seeds of *M. oleifera* (known as ‘‘moringa” in Portuguese, “árbol del ben” in Spanish, and horseradish tree or drumstick in English) were collected in Recife, Pernambuco, Brazil.

The seeds were powdered and the meal stored at -20 °C. A voucher specimen is deposited number 73,345 at the herbarium ‘‘Dárdano de Andrade Lima” from the *Instituto Agronômico de Pernambuco* (Recife, Brazil).

Isolation of WSMoL

WSMoL was isolated according to the procedure described by Coelho et al. [5]. *M. oleifera* seed powder (10 g) was homogenized with distilled water (100 mL) for 16 h at 4 °C using a magnetic stirrer. Next, the homogenate was filtered through gauze and centrifuged at 9,000 *g* for 15 min at 4 ºC. The resulting clear supernatant (seed extract) was treated with ammonium sulphate at 60%-saturation for 4 h at 28 ºC [15] and the precipitate (0-60% fraction) obtained after centrifugation (9,000 *g*, 15 min, 4 ºC) was collected. The 0-60% fraction was dialyzed (3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (4 h) before loaded (40 mg of proteins) onto a chitin column (7.5 x 1.5 cm) equilibrated (0.3 mL/min flow rate) with 0.15 M NaCl. After washing with the equilibrating solution, WSMoL was eluted from the column with 1.0 M acetic acid. WSMoL was dialyzed (3.5 kDa cut-off membrane) against distilled water (4 h) at 4 °C for eluent elimination. The protein concentration was determined according to Lowry et al. [16] using bovine serum albumin (31.25–500 µg/mL) as standard.

Irradiation of WSMoL

WSMoL samples (0.37 mg/mL) in distilled water were irradiated at a rate of 1.79 Gy/h for final doses of 10 mGy (20 s) and 10 Gy (20089 s). Irradiation was conducted under atmospheric O_2 using a ^{137}Cs irradiator model 28-8A (J.L. Shepherd & Associates, San Fernando, California, USA).

Hemagglutinating activity

Non-irradiated and irradiated WSMoL samples were evaluated for hemagglutinating activity (HA) as described by Paiva and Coelho [17]. The assays were performed using 2.5% (v/v) suspension of rabbit erythrocytes treated with glutaraldehyde [18]. One hemagglutination unit (titer^{-1}) was defined as the reciprocal of the highest sample dilution that promotes full agglutination of erythrocytes. Specific hemagglutinating activity (unit/mg) was defined as the ratio between the hemagglutination units and protein concentration (mg/mL).

Gel filtration chromatography

Non-irradiated and irradiated WSMoL were chromatographed on a Hiprep 16/60 Sephacryl S-100 column (16 mm x 60 cm) coupled to a ÄKTA Prime system (GE Healthcare, Sweden) pre-equilibrated at 24 ºC with 0.15 M NaCl. Sample (2.0 mL containing 0.7 mg of protein) was injected and eluted with 0.15 M NaCl at a flow rate of 0.5 mL/min. Fractions of 2.0 mL were collected. The molecular mass standards phosphorylase b (97000 Da), albumin (66000 Da), ovalbumin (45000 Da), carbonic anhydrase (30000Da), trypsin inhibitor (20100 Da), and α-lactalbumin (14400 Da) were similarly chromatographed.

Assays with *Aedes aegypti*

Eggs, larvae and females

The *A. aegypti* colony (Rockfeller strain) maintained at the *Laboratório de Ecologia Química* of the *Universidade Federal de Pernambuco* (Recife, Brazil) since 2003 was used in the experiments. The insectary room was kept at 27±1°C, 78±2% relative humidity and 14:10 (light/dark) photoperiod. Adult mosquitoes were reared in cages (30 x 30 x 30 cm) covered

with a fine mesh cloth and fed a 10% glucose solution. Females took blood meal from chicken blood acquired from local farms and dispensed from a common artificial feeder. The eggs laid by females were collected and used to restart the cycle and originate larvae or used to assess ovicidal activity. The larvae were reared in plastic bowls containing water and cat food (Whiskas®). When reaching the early fourth-stage (L_4) the larvae were selected and used in larvicidal assay.

Larvicidal assay

Larvicidal assay corresponded to an adaptation of the World Health Organization [19] method and previously used by Coelho et al. [5] to determine larvicidal activity of WSMoL. Groups of 20-25 L_4 were exposed to samples containing non-irradiated or irradiated WSMoL at 0.1 mg/mL. Each assay had a final volume of each assay was 20 mL and was achieved in triplicate. Three independent experiments were run. Mortality rates (%) were determined after 24 h of incubation at 27 °C and 12-12 (light-dark) photoperiodism. The control contained only distilled water.

Ovicidal assay

Ovicidal assay was performed according to the method previously used by Santos et al. [8] to determine ovicidal activity of WSMoL. *A. aegypti* eggs stored for 3 months at 28 ºC were selected considering their integrity using a stereomicroscope. The sample (non-irradiated or irradiated WSMoL) was diluted in filtered tap water to provide test solutions at 0.1 mg/mL. The final volume of each assay was 20 mL of test solution and contained 50-60 eggs. Controls contained filtered tap water and a volume distilled water equivalent to that used to dilute samples. The number of hatched larvae was determined after 72 h of incubation at 28 ºC. Next the treated eggs were rinsed with distilled water and transferred to another vessel

filled with only filtered tap water. The hatching of larvae was assessed after 48 h. Three independent experiments were run in triplicate.

Oviposition assay

Oviposition assay was performed according to the method previously used by Santos et al. [8] to determine the effect of WSMoL on oviposition by gravid females. For each assay, twenty-five *A. aegypti* gravid females (3-days after blood meal) were placed in a bioassay cage (33 x 21 x 30 cm) containing two glass vessels (10 cm diameter) placed at diagonally opposite corners of the cage. One of the vessels was filled with 50 mL of distilled water and the other with 50 mL of sample (non-irradiated or irradiated WSMoL at 0.1 mg/mL). Next, a disk shaped piece of filter paper (18 cm in diameter) folded into a cone was placed covering the inside of each vessel, to provide a support for oviposition. The females were maintained at 27 ± 0.5 °C with 73 ± 0.4% relative humidity for 14 h in the dark. After this period, eggs in oviposition paper sheets were manually counted using a stereomicroscope (Leica M80). The oviposition response was expressed as follows: % oviposition = 100 x [(number of eggs in sample vessel) / (number of eggs in sample and control vessel)]. The assays were performed in quintuplicate.

The hatchability of eggs laid by *A. aegypti* gravid females on control and test vessels was evaluated. Fifty fresh eggs from each vessel from oviposition assays (including controls) were selected using a stereomicroscope and placed again in the same solutions where they were deposited. The number of hatched larvae present in solution was determined after 144 h of incubation at 28 ºC.

Statistical analysis

Standard deviations (s.d.) 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 ± s.d. Significant differences between treatment groups were analysed by Student´s *t*-test (significance at $p<0.05$) using Origin 6.0 program.

Results and discussion

Moringa oleifera seeds contain the bioactive lectin WSMoL that shows coagulant activity promoting decrease in water turbidity, antibacterial action reducing bacterial contamination in ambient water and larvicidal, ovicidal and oviposition-stimulant activities on *A. aegypti* [5,8,20]. In this study we evaluated if gamma irradiation would improve the deleterious effects of WSMoL on *A. aegypti* larvae and eggs and its oviposition-stimulant activity. Radiation doses in the range used in this work (10 mGy and 10 Gy) are very lower than those reported to cause severe structural modification in proteins [10,11,21,22] and were selected in order to avoid total unfolding or fragmentation of lectin. On the other hand, doses of 10 kGy or higher have been reported causing loss of protein content as well as inactivation and/or fragmentation of lectins [11,21].

Protein concentration in WSMoL samples was not affected after irradiation at 10 mGy and 10 Gy, revealing that there was no loss of protein molecules by precipitation. The specific hemagglutinating activity of WSMoL increased after irradiation with the lowest dose used (10 mGy) while the sample irradiated with 10 Gy showed the same hemagglutinating activity of non-irradiated lectin (Table 1). These results indicate that 10 mGy-irradiation induced a lectin conformation that allows a better interaction between the sugar and the carbohydrate-binding sites. Similarly to WSMoL, the lectin from *Sebastiania jacobinensis* bark had its

hemagglutinating activity stimulated by a radiation dose of 100 Gy, which is also considered low [23]. Several types of structural modifications may occur during irradiation but drastic alterations such as unfolding and aggregation are usually not observed at low-dose range [10].

The hemagglutinating activities of lectins show variable degree of resistance to gamma irradiation. Our results revealed that the hemagglutinating activity of WSMoL was preserved even after exposure to the dose of 10 Gy. Indeed the ability in agglutinate erythrocytes of other lectins was also not affected even after irradiation with higher doses; for example, the hemagglutinating activity of the lectins from *Bothrops leucurus* venom and *Viscum album* mistletoe were stable toward irradiation at 1 kGy and 5 kGy, respectively [21,24].

Gel filtration chromatography of non-irradiated and 10 mGy-irradiated WSMoL showed main protein peaks of 63 kDa (Figure 1A and 1B). The profile of WSMoL irradiated with 10 Gy showed two unseparated peaks corresponding to molecular masses of 55 and 59 kDa (Figure 1C). This result may indicate the presence of WSMoL molecules with altered structure after irradiation with the highest dose. In addition peaks with very low molecular mass (< 2 kDa) were detected in chromatographic prolifes of irradiated samples (Figures 1B and 1C) which can be result of fragmentation.

Interestingly, the increase in specific hemagglutinating activity of WSMoL after irradiation with 10 mGy was accompanied by a potentialization of larvicidal effect and ovicidal activity on stored eggs (Table 1) while the lectin irradiated with 10 Gy showed no larvicidal and ovicidal effects. It is probable that the 10 mGy dose caused minor alterations in WSMoL structure that resulted in improvement of these biological properties while the irradiation with 10 Gy, although not resulted in loss of hemagglutinating activity, lead to more severe alterations in lectin structure resulting in loss of insecticidal effects. The data found for non-irradiated WSMoL are in according to that reported by Coelho et al. [5] and Santos et al.

[8], with the lectin at 0.1 mg/mL concentration causing minimal larval mortality and reduction in 50% of hatching rate of stored eggs.

It has been suggested that the larvicidal effect of lectins involves interactions between lectin and glycoconjugates present at the digestive tract of insect [25]. In this sense, the improvement of carbohydrate binding property of WSMoL evidenced by hemagglutinating activity assay may be related with the highest larvicidal activity of 10 mGy-irradiated WSMoL in comparison with non-irradiated lectin. On the other hand, the no alteration of hemagglutinating activity after irradiation with 10 Gy failed to ensure that the insecticidal properties were not damaged. Further studies are needed in order to evidence the relationships between these changes in a structural basis.

The oviposition-stimulant effect of WSMoL was damaged after the lectin was submitted to both doses of gamma irradiation since there were no significant differences between the number of eggs deposited by females in control and test vessels (Figure 2A). These results suggest that the recognition of WSMoL by females as a chemical cue requires a specific lectin conformation which was affected by irradiation with minimal doses. On the other hand, the hatchability of the eggs laid on vessels containing 10 mGy-irradiated lectin was more affected than in vessels with non-irradiated WSMoL (Figure 2B), similarly to observed with stored eggs.

In conclusion, a very low dose of gamma irradiation (10 mGy) can be used to improve the deleterious effects of WSMoL on *A. aegypti* larvae and eggs while a higher dose (10 Gy) was able to abolish all the insecticidal effects of this lectin on this mosquito species.

Competing interests

The authors declare that they have no competing interests.

Authors's contributions

Conceived and designed the experiments: NDLS, THN, DMAFN and PMGP. Performed the experiments: NDLS, THN, CAB, LPA and EVP. Analyzed the data: NDLS, THN DMAFN and PMGP. Contributed reagents/materials/analysis tools: LCBBC, DMAFN and PMGP. Wrote the paper: NDLS, THN, EVP, LCBBC, DMAFN and PMGP.

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Figure captions

Fig. 1. Gel filtration chromatography of non-irradiated WSMoL (A), 10 mGy-irradiated WSMoL (B) and 10 Gy-irradiated WSMoL (C) on a Hiprep 16/60 Sephacryl S-100 column coupled to ÄKTA prime system. Samples (0.7 mg of protein) were injected and eluted (2.0 mL fraction) with 0.15 M NaCl.

Fig. 2. Effect of non-irradiated and irradiated WSMoL (0.1 mg/mL of protein) on oviposition and egg hatching of *Aedes aegypti*. (A) Mean of eggs laid by *A. aegypti* gravid females in distilled water and lectin samples. The oviposition response was evaluated by double-choice bioassays. Three distinct assays ("control vs. non-irradiated WSMoL", "control vs. 10-mGy irradiated WSMoL" and "control vs. 10-Gy irradiated WSMoL") were performed separately, each one with its respective control. The oviposition response was expressed as: % oviposition = 100 x [(number of eggs in sample vessel) / (number of eggs in sample and control vessels)]. (B) Hatching rate (%) of eggs laid by gravid females during the oviposition assays in distilled water and lectin samples. (*) indicates significant differences ($p<0.05$) between control and test groups.

Table 1. Hemagglutinating activity and insecticidal effects on larvae and stored eggs of *Aedes aegypti* of irradiated and non-irradiated WSMoL.

Lectin sample	SHA	Larval mortality (%)	Inhibition of egg hatching (%)
Non-irradiated	5535 a	11.6 ± 2.8 a	50.0 a
Irradiated			
10 mGy	44281 b	40.0 ± 5.0 b	100.0 b
10 Gy	5535 a	3.3 ± 2.8 c	0.0 c

Figure 1

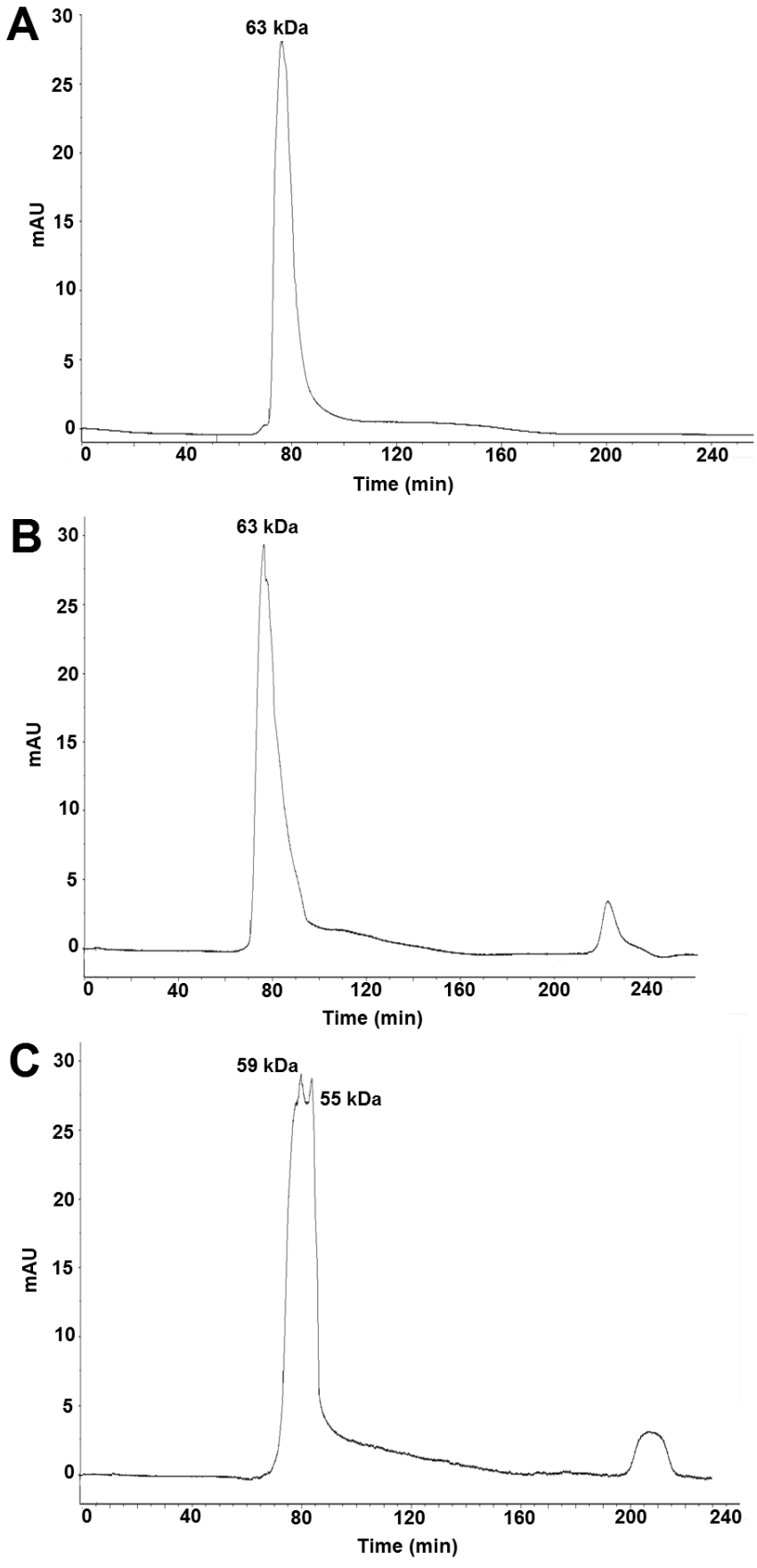
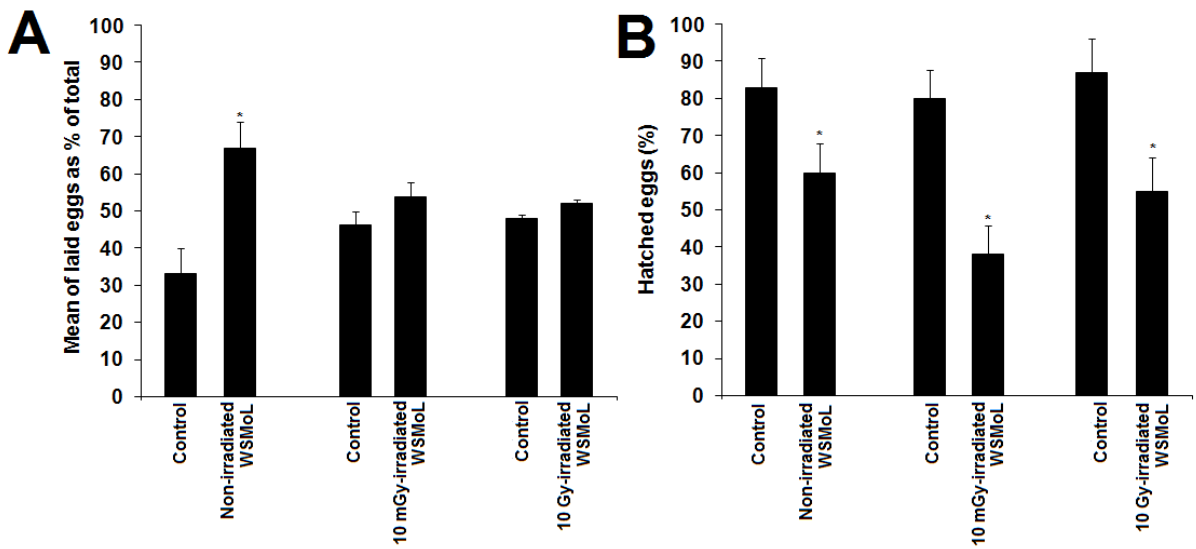

A
63 kDa
mAU
Time (min)
B
63 kDa
mAU
Time (min)
C
59 kDa
55 kDa
mAU
Time (min)

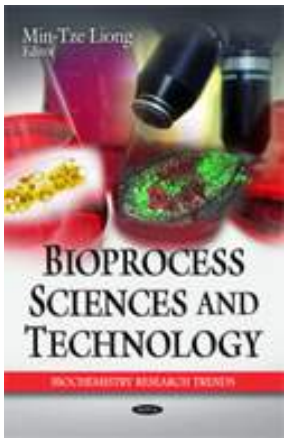
Figure 2


A
Mean of laid eggs as % of total
100
90
80
70
60
50
40
30
20
10
0
*
Control
Non-irradiated WSMoL
Control
10 mGy-irradiated WSMoL
Control
10 Gy-irradiated WSMoL
B
Hatched eggs (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Control
Non-irradiated WSMoL
Control
10 mGy-irradiated WSMoL
Control
10 Gy-irradiated WSMoL

8. CAPÍTULO 4

Plant compounds with *Aedes aegypti* larvicidal activity and other biological properties

Capítulo de revisão publicado no livro "Bioprocess Sciences and Technology", publicado pela Nova Science Publishers, Inc., New York (ISBN: 978-1-61122-950-9)



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Chapter 11

PLANT COMPOUNDS WITH *AEDES AEGYPTI* LARVICIDAL ACTIVITY AND OTHER BIOLOGICAL PROPERTIES

P. M. G. Paiva[1,*], T. H. Napoleão[1], N. D. L. Santos[1], M. T. S. Correia[1], D. M. A. F. Navarro[2] and L. C. B. B. Coelho[1]

[1]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife, Pernambuco, Brazil.
ppaivaufpe@yahoo.com.br

[2]Departamento de Química Fundamental, Centro de Ciências Exatas e da Natureza, Universidade Federal de Pernambuco, Cidade Universitária, 50670-901, Recife, Pernambuco, Brazil

ABSTRACT

Chemical insecticides to control *Aedes aegypti* adversely affect the environment and also promote emergence of resistant mosquitoes. Plant compounds with damage effect on immature stage forms or adult insects can be an alternative to synthetic insecticides. This chapter shows essential oils, organic solvent extracts and lectins from plants as enviro-friendly agents against *A. aegypti* and other arthropods. Extraction, concentration and isolation of plant compounds are discussed; effect of essential oils, organic solvent extracts or lectins (hemagglutinating proteins) on *A. aegypti* larval development and survival as well as other biological properties are reported. Larvicidal mechanisms proposed for essential oils, organic solvent extracts and lectins are also reported. The chapter contributes to the study of alternative strategies to control arthropods. Additionally, it reports the medicinal and industrial uses of plant compounds with biotechnological applications.

1. *AEDES AEGYPTI*: VECTOR MOSQUITO OF ETIOLOGIC AGENTS FROM YELLOW FEVER, DENGUE FEVER AND HEMORRHAGIC DENGUE FEVER

A. aegypti Linn. belongs to the Phylum Arthropoda, Class Hexapoda, Order Diptera, Culicidae Family and its common name is dengue mosquito. *A. aegypti* is a mosquito with diurnal habit, with a preference to oviposit in clean water.

The control of *A. aegypti* can be directed to larvae (four instars: L1, L2, L3 and L4), pupa and adult insects. Prevention of infestation by larvae elimination is easier than control of adult insects since the larvae are confined in containers. The effect of plant compounds on *A. aegypti* larvae can be investigated by evaluation of larval development [1] and detection of larval mortality [2].

2. ESSENTIAL OILS: VOLATILE COMPOUNDS FROM PLANTS

Essential oils are complex compounds synthesized as secondary metabolites by buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark; they are characterized by two or three major volatile compounds (20–70%), lipid soluble and soluble in organic solvents [3]. The main chemical constituents found in essential oils are phenols, aldehydes, ethers, esters, alcohols, ketones, benzenoids, phenylpropanoids and terpenes. Isoprene is the building block of terpene and a number of authors use the designation "terpene" to include all terpenoids. Limonene, alpha-pinene, menthol, citronellal and citral (Figure 1) are good examples of monoterpenes (two isoprene units, C10).

Menthol is the main constituent in the essential oil of *Mentha piperita* and camphor was isolated from the essential oil in the wood of *Cinnamomum camphora* [4, 5]. The phenols carvacrol, 2-ethyl-4,5-dimethylphenol and thymol were isolated from shoots of *Thymus vulgaris* and eugenol is present in flower buds of *Eugenia caryophyllata* [6, 7]. Other chemical constituents of *T. vulgaris* essential oil are 1-octen-2-ol (alcohol), alpha-trimethyldodecane (hydrocarbon) and (Z)-3-hexenyl butyrate (ester). Benzyl benzoate (ester benzenoid) and octanal, nonanal and decanal (aldehydes) are found in essential oils of *Licaria canella* and oranges, respectively [8, 9].

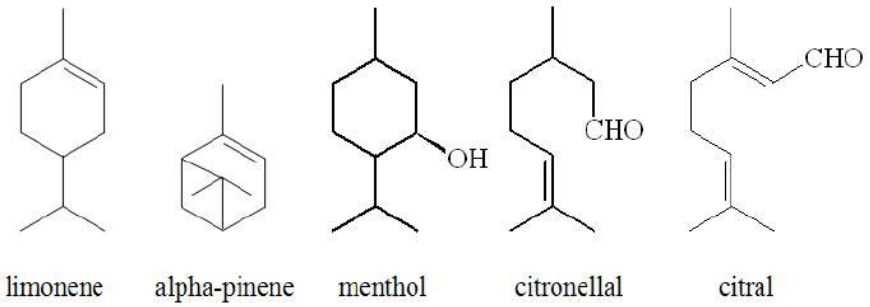

Figure 1. Monoterpene constituents of essential oils.

2.1. Methods for the Extraction, Concentration and Isolation of Compounds

The most useful extraction methods for obtaining essential oils from tissue plant are based on distillation. In distillation with cohobation, a solvent such as water is recycled in the process, returning to the still [10]. In hydro-distillation, the plant material is fully dipped in the water and placed into a still that has a heat source at the bottom and the top is connected to a condenser (Figure 2A); the disadvantage of this technique is that the thermally unstable compounds may decompose if remain into direct contact with the heat source. In steam distillation, the essential oil is isolated using a water boiler tank to produce steam; the steam passes through a flask containing the plant material and the oil is collected and separated to the oil receiver (Figure 2B). Hydro-steam distillation occurs when the vapor is generated below the plant material, which is suspended in the still on a support.

Enfleurage uses odorless animal fat to capture volatile compounds from the aromatic parts of plant; essential oil is obtained after washing the fat with alcohol or heating. Modern enfleurage uses vegetal fat instead of animal fat. Hot maceration extraction is very similar to this method, but is faster and uses hot fat or oil in which the plant material is immersed [10].

Microwave-assisted extraction has been used for the extraction of chemical constituents in plant material [11]; a small matrix particle size and high dielectric solvent can enhance the microwave-assisted extraction. The improved extraction yield, reduced extraction time and minimal amount (or even absence) of solvent are the greatest advantages of method. Solvent-free microwave extraction (Figure 3A) was used for isolation of essential oils from three aromatic herbs [12]; the advantage of this extraction method is the short extraction time and substantial energy savings. Microwave-assisted hydro-distillation (MAHD, Figure 3B) was used for extraction of essential oil from *Cinnamomum iners* leaves [13] and MAHD method using an adapted domestic microwave oven with the connection of a round glass flask in the oven to a condenser outside the oven was used for extraction of essential oils from *Satureja hortensis* and *Satureja montana* [14].

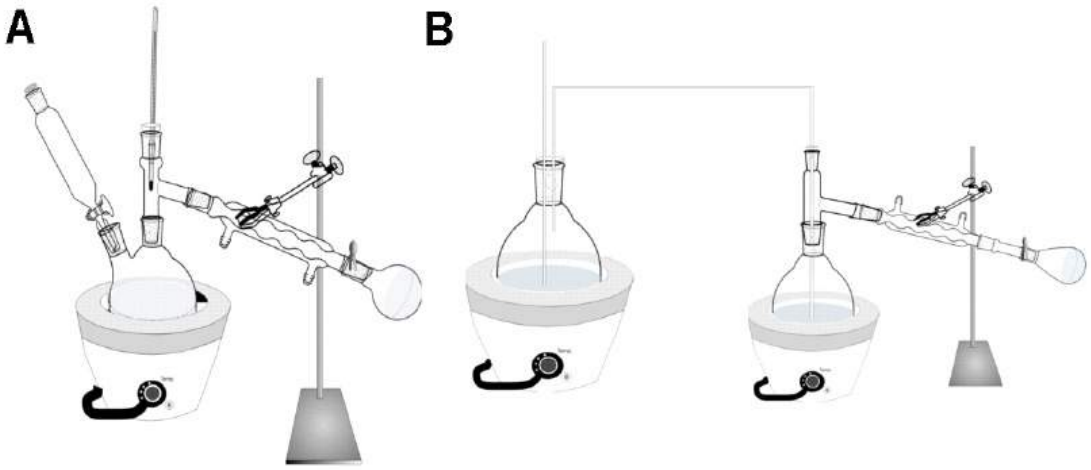

Figure 2. Laboratorial apparatus to extract essential oils by methods based on distillation. Hydro-distillation (A) and steam distillation (B).

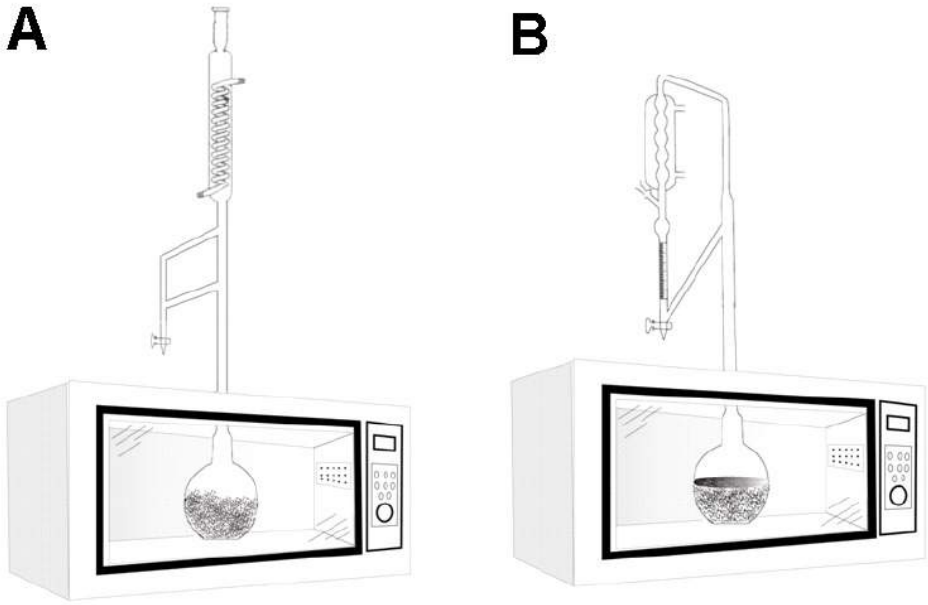

Figure 3. Laboratorial apparatus for solvent-free microwave-assisted extraction (A) and microwave assisted with Clevenger extraction (B).

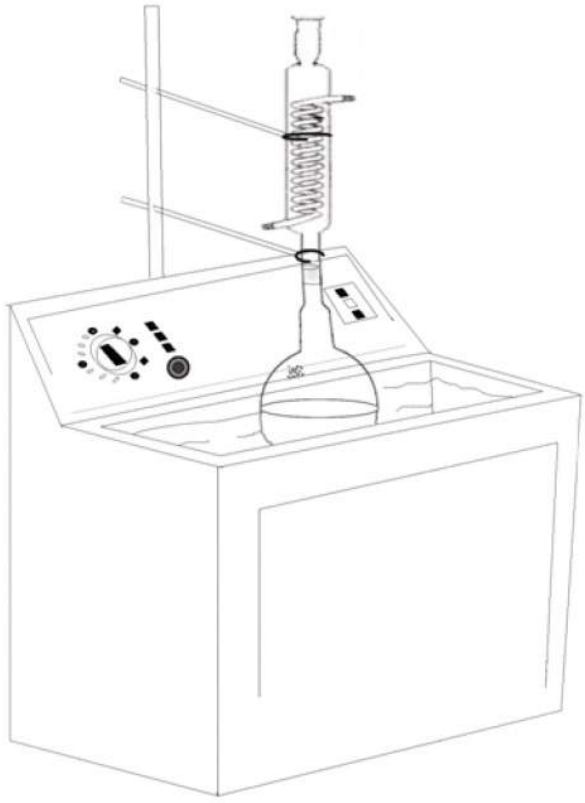

Figure 4. Simple ultrasound-assisted extraction apparatus with ultrasound bath, round glass flask and Liebig condenser.

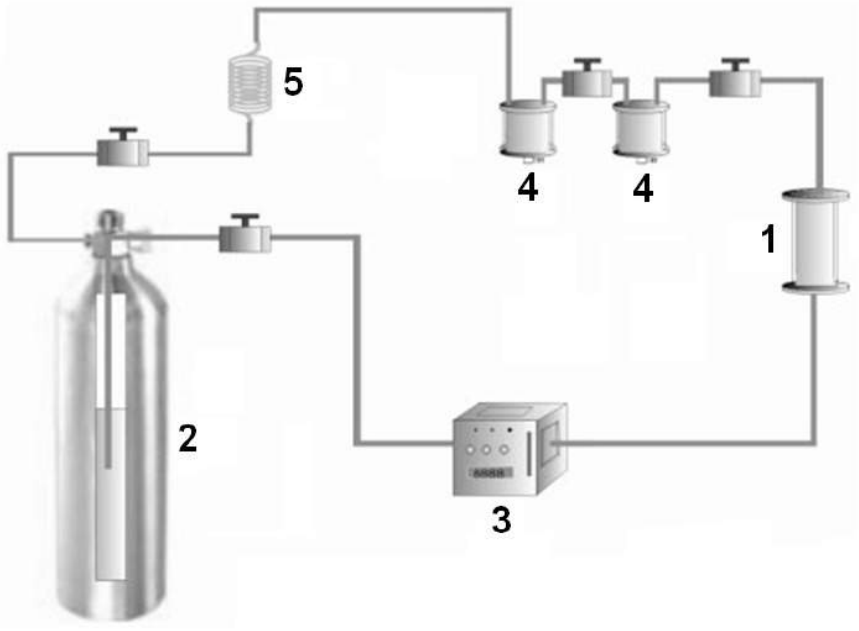

Figure 5. Supercritical fluid extraction apparatus. The raw plant material is placed in an extractor recipient (1) and the supercritical fluid is produced from a reservoir (2) connected to a powerful pump (3); the system is pressurized and controlled by valves. After passing through the extractor, the fluid and dissolved compounds are transported to separators (4). The dissolution of the organic matter is precipitated by a decrease inpressure and the product is collected via a valve located at the bottom of the separators. A condenser (5) regenerates the fluid, which is returned to the fluid reservoir.

Figure 4 shows a simple and open-system ultrasound-assisted extraction apparatus for extracting essential oils constituted by ultrasound bath, round glass flask and Liebig condenser [15]. Ultrasound-assisted extraction is more advantageous for the extraction of thermally unstable molecules.The advantage of ultrasound is that it provides a greater penetration of the solvent into the cell components and improves the transfer of mass from plant cells to the solvent. The ultrasound extraction applied to a large-scale is limited due to the higher costs; another disadvantage is that ultrasound can produce free radicals that can react with active molecules present in the plant, during ultrasound extraction.

Supercritical fluid extraction (Figure 5) has become attractive due to the lack of an organic solvent and extraction occurs near room temperature, thereby avoiding the thermal degradation of bioactive compounds. The most often employed supercritical fluid is carbon dioxide (CO_2) due to its moderate supercritical fluid conditions (*T*C=31.06 °C and *P*C=73.81 bar), high capacity to solubilize organic compounds under supercritical conditions and the fact that it is non-toxic, non-inflammable and inexpensive.

2.2. Larvicidal Activity against *Aedes aegypti*

Essential oils are permeable to cellular membrane due to their lipophilic nature; cell alterations in plasma membrane, cytoplasm and nucleus are suggested to be responsible by their cytotoxic effects [3]. Essential oils with larvicidal activity on *A. aegypti* are a mixture of

active compounds and, in general, the oil is more efficient in promoting larvae mortality than isolated constituents.

Larvae treated with essential oils from leaves of *Chloroxylon swietenia* (LC_{50} of 16.5 $\mu g.mL^{-1}$), *Lippia gracilis* (LC_{50} of 98 ppm), *Hyptis fruticosa* (LC_{50} of 502 ppm) and *Hyptis pectinata* (LC_{50} pf 366 ppm) showed abnormal wagging and later died. The sesquiterpenes pregeijerene, geijerene and germacrene D isolated from *C. swietenia* were the active principles with LC_{50} of 28.3, 43.4, and 63.6 $\mu g.mL^{-1}$, respectively; the major constituent of *L. gracilis* oil was carvacrol (LC_{50} of 70 ppm) although the most active (LC_{50} of 37 ppm) constituent was *R*-limonene [16, 17].

Larvicidal activity on L3 was reported for essential oils from leaves of *Piper permucronatum*, *Piper hostmanianum*, *Piper humaytanum*, and *Piper gaudichaudianum* with LC_{50} values of 36, 54, 121 and 156 $\mu g.mL^{-1}$, respectively. The oils from *P. permucronatum* and *P. hostmanianum* showed high content of arylpropanoids [18]. Essential oil from *Lippia sidoides* and its hydrolate were strongly active on *A. aegypti* L3 promoting 100% mortality in 1-5 min (pure oil and hydrolate), 20 min (1:5 dilution), and 24 h (1:10 and 1:20 dilutions). In addition, the hydrolate showed considerable persistence remaining active even after 60 days and did not cause adverse effects on mice. Thymol was identified as the active principle promoting 100% mortality at 0.017% (w/v) in 1 ½ h [19]. *Mentha piperita* essential oil (peppermint oil) was also larvicidal for L3. The bioassay was performed applying the oil in an enamel containing water; the oil remained in the surface as a layer. Peppermint oil caused 100% mortality in 24 h at 4 $mL.m^{-2}$ and reduction in adult emergence [20].

Larvicidal activity on *A. aegypti* L4 was detected to essential oils from *Apium graveolens* (LC_{50} of 42.07 ppm), *Carum carvi* (54.62 ppm), *Curcuma zedoaria* (31.87 ppm), *Foeniculum vulgare* (49.32 ppm) and *Zanthoxylum limonella* (24.61 ppm). The authors observed that, in addition to mortality, the exposure to the oils provoked restlessness, sluggishness, tremors, convulsions and larvae paralysis [21]. Essential oil from foliage of *Tagetes patula* was also able to kill *A. aegypti* L4 (LC_{50} of 13.57 ppm); larvae showed restlessness, abnormal wagging and did not develop into pupae [22]. Essential oils from *Pimpinella anisum* seeds (LC_{95} of 115.7 $\mu g.mL^{-1}$) and *Zingiber officinale* rhizome (193.9 $\mu g.mL^{-1}$) also were toxic on L4 [23].

Larvicidal activity against L4 has been found in multiple compounds isolated from essential oils. The most abundant constituents (*E*)- or (*Z*)-asarone and patchouli alcohol from *Piper marginatum* leaf, stem and inflorescence oils were toxic on L4 with LC_{50} of 23.8, 19.9, and 19.9 ppm, respectively [24]. Essential oil from *Dendropanax morbifera* flowers contain the larvicidal agents (LC_{50} of 62.32 ppm) γ-elemene, tetramethyltricyclohydrocarbon, β-selinene, α-zingibirene, 2-isopropyl-5-methylbicylodecen and β-cubebene, while α-pinene and α-terpinene were suggested as the main larvicidal compounds of *Eucalyptus camaldulensis* and *Eucalyptus grandis* oils which LC_{50} were 14.7 $\mu g.mL^{-1}$ and 32.4 ppm, respectively [25, 26, 27]. Fourth instar larvae were also susceptible to *Ocimum sanctum* essential oil (LC_{50} of 85.11 ppm), which main components are oxygenated monoterpenes and sesquiterpene hydrocarbons [28].

2.3. Other Biological Properties

Essential oils with larvicidal activity on *A. aegypti* are also harmful to other mosquito's species (Table 1). Essential oil from *Cinnamomum osmophloeum* leaf was larvicidal on L4 of

Aedes albopictus (LC_{50} of 40.8 $\mu g.mL^{-1}$) and the active constituents were benzaldehyde and trans-cinnamaldehyde; the last was also toxic to fourth-instar larvae of *Culex quinquefasciatus* (lymphatic filariasis vector) and *Armigeres subalbtus* [29]. Essential oils from *Apium graveolens*, *Carum carvi*, *Curcuma zedoaria*, *Foeniculum vulgare*, and *Zanthoxylum limonella* are larvicidal agents on both fourth instar larvae of *A. aegypti* and *Anopheles dirus* although larvae showed different susceptibility to them; *A. graveolens, C. carvi*, *Z. limonella* oils were mainly effective on *A. aegypti* while the others were on *A. dirus* [21]. Similarly, larvae of *Anopheles stephensi* (malaria vector) were more susceptible than that of *A. aegypti* to oils from *Chloroxylon swietenia* leaf and stem [16].

Table 1. Plant essential oils able to kill arthropod species

Arthropod	Plant
Acari	
Tetranychus urticae	*Micromeria fruticosa*, *Nepeta racemosa*, *Origanum vulgare*
Diptera	
Aedes aegypti	*Apium graveolens*, *Carum carvi*, *Chloroxylon swietenia*, *Curcuma zedoaria*, *Dendropanax morbifera*, *Eucalyptus camaldulensis*, *Eucalyptus grandis*, *Foeniculum vulgare*, *Hyptis fruticosa*, *Hyptis pectinata*, *Lippia gracilis*, *Lippia sidoides*, *Mentha piperita*, *Ocimum sanctum*, *Pimpinella ansium*, *Piper gaudichaudianum*, *Piper hostmanianum*, *Piper humaytanum*, *Piper marginatum*, *Piper permucronatum*, *Tagetes patula*, *Zanthoxylum limonella*, *Zingiber officinale*
Aedes albopictus	*Cinnamomum osmophloeum*
Anopheles dirus	*Apium graveolens*, *Carum carvi*, *Curcuma zedoaria*, *Foeniculum vulgare*, *Zanthoxylum limonella*
Anopheles stephensi	*Chloroxylon swietenia*, *Eucalyptus tereticornis*, *Mentha piperita*, *Tagetes patula*
Armigeres subalbtus	*Cinnamomum osmophloeum*, *Mentha piperita*
Culex quinquefasciatus	*Cinnamomum osmophloeum*, *Tagetes patula*
Isoptera	
Coptotermes formosanus	*Calocedrus macrolepis*, *Chamaecyparis obtusa*, *Cryptomeria japonica*
Homoptera	
Bemisia tabaci	*Micromeria fruticosa*, *Nepeta racemosa*, *Origanum vulgare*

References: [16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 31, 32].

Essential oils, beyond the larvicidal activity, may interfere with other stages of the insect life cycle; ovicidal, oviposition-deterrent activity, pupicidal and adulticidal activities have also been reported. Essential oils showed repellent activity on Coleoptera, Diptera, Isoptera, Lepidoptera, Phthiraptera and Thysanoptera; mixed oils are more efficient than pure oils probably due to synergistic effect among particular compounds [30]. *Mentha piperita* oil has insecticidal potential against *A. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* by its strong repellent action against adult mosquitoes; loss of fertility and fecundity of adults emerged from larvae exposed to oil [20].

Essential oils can promote termite and acari mortality (Table 1) as well as termite and tick repellency. Essential oils of *Calocedrus macrolepis* var. *formosana* (heartwood), *Chamaecyparis obtusa* var. *formosana* (leaf) and *Cryptomeria japonica* (sapwood) showed anti-termitic activity on termite *Coptotermes formosanus* due to the toxicity and repellent action [31]. Essential oil vapors from leaves of aromatic plants *Micromeria fruticosa*, *Nepeta racemosa* and *Origanum vulgare* showed acaricidal activity (95% mortality at 120 h) on *Tetranychus urticae* (adult and nymphal stage) and insecticidal action (100% mortality at 120 h) against *Bemisia tabaci* (Homoptera), pests of greenhouse vegetables and ornamentals [32]. Essential oils of *Dianthus caryophyllum* and *Cymbopogon nardus* (citronella) have repelling properties on *Aedes* species and tick *Ixodes ricinus*; phenylethanol (*D. caryophyllum*) and citronellol (oil of citronella) were the most active compounds [33, 34].

3. Organic Solvent Extracts

Larvicidal plant extracts have been obtained from bark, fruits, leaves, seeds, rhizomes and woods. These organic solvent extracts can constitute an economically feasible strategy for the control of insects; some of them can be associated with other control methods to obtain a relatively lasting effect.

3.1. Preparation of Extracts

Organic solvent extracts from plant tissues, in general, are prepared using air-dried material in order to obtain reasonable extraction yields. Cutting, breaking, powdering and cryogenic milling are convenient methods to increase tissue surface area; cryogenic milling, in addition, minimizes the losses of volatile organic compounds [35]. Vegetal tissues with high lipid, pigment and polyphenol contents can be treated before extraction procedure to eliminate these components; defatting can be made using non-polar organic solvents such as hexane; pigment and polyphenol can be removed by extracting them with acetone, methanol, petroleum ether and detergents.

Extraction with organic solvents is the separation process of a constituent from plant material; the component is removed from a phase and brought to another immiscible liquid phase. Solute will be more concentrated in the phase in which it has higher solubility; solvent is chosen according to the characteristics of constituents being extracted. Polar compounds like sugars and alkalis will dissolve in polar solvents (such as organic acids), intermediate or low polar compounds (tannins, phenolics, and terpenes) will dissolve in solvents of

intermediate polarity (such as methanol, ethanol, acetone, and dichloromethane) and non-polar compounds (oils, resins, and waxes) are extracted using solvents of low polarity (such as hexane and chloroform). Plant tissue can be sequentially extracted with different solvents in increasing order of polarity or a unique extract obtained using a polar solvent is later partitioned in different fractions using solvents in increasing order of polarity.

The time and temperature of extraction is variable; thermal instability of some compounds may limit the temperature of extraction. Steam distillation is a technique used for extraction of volatile plant compounds and the compounds obtained are separated in the oil and water phases; vegetal tissue is continuously flushed with steam and volatile compounds are taken up by the vapor phase [35].

Maceration and percolation are useful methods to obtain organic solvent extracts when the compounds of interest show high solubility in the solvent. Maceration consists of soaking the plant material in the solvent, using agitation or heat to increase the solubility of the compounds and the rate of mass transfer. A single maceration step does not result in the total extraction of the compounds and for this reason residues are generally re-extracted in two or three more steps.

Soxhlet extractor is used when the compound of interest shows low solubility in organic solvent or the compound is more soluble in water than in the organic solvent. In a conventional Soxhlet extractor, the plant tissue is placed in a thimble-holder which is gradually filled with the solvent present in a distillation flask and there is a reflux of the solvent in an intermittent process. The liquid that overflows returns to the distillation flask through a siphon and carries the substances that have been extracted. This operation is repeated until the extraction is complete. Soxhlet extraction can be coupled to high-pressure, automated, ultrasound-assisted, and microwave-assisted systems intended to automating the extraction and shortening percolation time by using different forms of energy [36].

3.2. Larvicidal Activity against *Aedes aegypti*

The damage effect of organic solvent plant extracts on *A. aegypti* larvae can be detected by evaluation of larval development in presence of extracts; ethanolic extract from *Melia azedarach* leaf promoted delay in *A. aegypti* larval development and larvae needed twice than usual the number of days to complete the third instar [37].

Bioassays for larvicidal activity of organic extracts are performed using defined larval instars. Larvicidal mechanisms proposed are antichitinogenic activity, toxic effect on larvae nervous system and changes in digestive tract including total or partial destruction of cells, high cytoplasmic vacuolation, increased subperitrophic space, cell hypertrophy, and apparent epithelial stratification [38, 39, 40].

Larvicidal methanolic extracts of *Albizzia amara* and *Ocimum basilucum* leaves [41] were more harmful to L1 (LC_{50} of 5.243% and 3.734%, respectively) than L2 (6.480% and 4.154%), L3 (7.106% and 4.664%) and L4 (7.515% and 5.124%) instars. Dichloromethane extracts of *Aristolochia triangularis*, *Baccharis cordifolia*, *Eupatorium hecatanthum*, *Pterocaulon purpurascens*, *Xanthium spinosum*, and *Abuta grandifolia* were active on L2 [42]; the efficiency (LC_{50}) of extracts to promote larval mortality varied from 2.6 (*A. grandifolia*) to 373.3 $\mu g.mL^{-1}$ (*B. cordifolia*). Acetone fraction obtained with petroleum ether extract of *Argemone mexicana* seeds was also larvicidal for *A. aegypti* L2 in laboratory and

under field conditions at concentrations of 25, 50, 100 and 200 ppm; larvae were more sensitive under field conditions [39].

Ethanol was able to extract compounds with larvicidal activity on L3 and L4 [38, 43, 44] from *Magonia pubescens* (stem bark), *Spondias mombin* (seed), *Annona muricata* (leaf), *Bauhinia cheilantha* (wood), *Caesalpinia echinata* (leaf and wood), *Operculina macrocarpa* (tuber), *Eugenia uniflora* (leaf), *Annona crassiflora* (root, LC_{50} of 0.71 $\mu g.mL^{-1}$) and *Annona glabra* (seeds, LC_{50} of 0.06 $\mu g.mL^{-1}$). Methanolic extract from *Atlantia monophylla* leaves harmful to L4 (LC_{50} of 0.09 $mg.L^{-1}$) was safe to aquatic mosquito predators [45]. Petroleum ether extracts of *Jatropha curcas*, *Pedilanthus tithymaloides*, *Phyllanthus amarus*, *Euphorbia hirta* and *Euphorbia tirucalli* showed larvicidal activity on L4 with LC_{50} of 11.34, 76.61, 113.40, 424.94, and 5.52 ppm, respectively. Based on these results, the authors indicated *E. tirucalli* extract as an ideal potential larvicide against *A. aegypti* [46].

Several plant compounds have been identified as larvicidal agent on *A. aegypti* larvae including tectoquinone (LC_{50} of 3.3 $\mu g.mL^{-1}$) from methanolic extract of *Cryptomeria japonica* sapwood; cubebol (LC_{50} of 60.1 $\mu g.mL^{-1}$) from ethanolic extract of *C. japonica* wood; cordiaquinones (LC_{100} ranging of 12.5 to 25 $\mu g.mL^{-1}$) from dichloromethane extract of *Cordia curassavica* root; limonoids from ethanolic extracts of *Melia azedarach* and *Azadirachta indica* fruit endocarps; dicentrine from ethanolic extract of *Ocotea valloziana valloziana* trunk bark (LC_{50} of 213.70 $\mu g.mL^{-1}$) and saponins from methanolic extract of *Balanites aegyptiaca* callus [40, 47, 48, 49, 50, 51]. Larvicidal organic solvent extracts also can be harmful to larvae before pupation and *A. aegypti* adult [37, 39, 41, 45]. Pupicidal activity was detected in leaf extracts from *Melia azedarach, Albizzia amara, Ocimum basilicum* and *Atlantia monophylla.* Acetone fraction from petroleum ether extract of *Argemone mexicana* induced reduction in blood meal utilization and fecundity as well as sterility of first generation eggs [39].

3.3. Other Biological Properties

Organic solvent extracts are effective in killing mosquitoes other than *A. aegypti* (Table 2). Tectoquinone from methanolic extract of *Cryptomeria japonica* sapwood is a larvicidal agent (LC_{50} of 5.4 $\mu g.mL^{-1}$) against L4 larvae of *Aedes albopictus*; petroleum ether extracts from *Jatropha curcas*, *Pedilanthus tithymaloides*, *Phyllanthus amarus*, *Euphorbia hirta*, and *Euphorbia tirucalli* were lethal for L4 larvae of *Culex quinquefasciatus* and methanolic extract from *Atlantia monophylla* leaves was pupicidal for *Anopheles stephensi* and *C. quinquefasciatus* [45, 46, 49].

Table 2. Plant organic solvent extracts able to kill arthropod species

Arthropod	Plant
Acari	
Panonychus citri	*Boenninghausenia sessilicarpa*
Coleoptera	
Tribolium confusum	*Mantisalca duriaei*, *Rhaponticum acaule*

Diptera	
Aedes aegypti	*Abuta grandifolia, Albizzia amara, Annona crassiflora, Annona glabra, Annona muricata, Argemone mexicana, Aristolochia triangularis, Atlantia monophylla, Azadirachta indica, Baccharis cordifolia, Balanites aegyptiaca, Bauhinia cheilantha, Caesalpinia echinata, Cryptomeria japonica, Eugenia uniflora, Eupatorium hecatanthum, Euphorbia hirta, Euphorbia tirucalli, Jatropha curcas, Magonia pubescens, Melia azedarach, Pedilanthus tithymaloides, Phyllanthus amarus, Pterocaulon purpurascens, Ocimum basilucum, Ocotea valloziana, Operculina macrocarpa, Spondias mombin, Xanthium spinosum*
Aedes albopictus	*Cryptomeria japonica*
Anopheles subpictus	*Centella asiatica*
Anopheles labranchiae	*Calotropis procera, Cotula cinerea*
Anopheles stephensi	*Atlantia monophylla*
Bactrocera oleae	*Citrus aurantium*
Ceratitis capitata	*Citrus aurantium*
Culex quinquefasciatus	*Atlantia monophylla, Curcuma aromatica, Euphorbia hirta, Euphorbia tirucalli, Jatropha curcas, Pedilanthus tithymaloides, Phyllanthus amarus*
Culex tritaeniorhynchus	*Centella asiatica*
Hemiptera	
Nilaparvata lugens	*Euphorbia kansui*
Homoptera	
Pterochloroides persicae	*Rhamnus dispermus*
Isoptera	
Nasutitermes corniger	*Myracrodruon urundeuva*
Ixodida	
Haemaphysalis bispinosa	*Centella asiatica*
Lepidoptera	
Spodoptera littoralis	*Ocimum basilicum, Origanum majorana, Salva officinalis*

References: [37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 60, 89].

Insecticidal activity has also been reported for methanolic extracts of *Ocimum basilicum*, *Origanum majorana* and *Salvia officinalis* on third instar larvae of the moth *Spodoptera littoralis* (LC_{50} of 0.17, 0.36, and 0.47 %, respectively); petroleum ether extract from *Curcuma aromatica* rhizome was a larvicidal agent against *Culex quinquefasciatus* (LC_{50} of 11.42 ppm) and active principles identified were 9-oxoneoprocurcumenol and neoprocurcumenol [52, 53, 54]. Table 2 shows other insects that were affected by organic solvent extracts including flies (*Bactrocera oleae* and *Ceratitis capitata*), aphids (*Pterochloroides persicae*), bugs (*Nilaparvata lugens*), and beetles [55, 56, 57, 58]. The flavanone pinocembrin from *Flourensia oolepis* ethanolic extract showed antifeedant activity (ED_{50} 7.98 $\mu g.cm^{-2}$) on *Epilachna paenulata* [59].

Plant extracts insecticidal on Diptera insects are harmful to other arthropods (Table 2). Methanolic extracts of *Centella asiatica* leaves showed larvicidal effect against *Anopheles subpictus* and *Culex tritaeniorhynchus* as well as adulticidal acitivity on ticks *Haemaphysalis bispinosa* and *Paramphistomum cervi* [60]. Toluene extract from *Artemisia abrotanum* leaves showed repelling property on *A. aegypti* and nymphs of tick (*Ixodes ricinus*); eugenol was identified as the constituent of highest activity [34].

Saponin and thujone were identified as active molluscicidal compounds in ethanolic extracts from *Saraca asoca* bark and *Thuya orientalis* leaf [61]. The alkaloid dicentrine is known as an antitumor agent on human hepatoma cell line HuH-7 [62].

4. LECTINS: CARBOHYDRATE-BINDING PROTEINS

The term lectin (originated from the Latin *lectus* that means selected) was introduced by Boyd and Shapleigh in 1954, because of the ability of these proteins to bind carbohydrates selectively. Lectins interact with glycoconjugates present on cell surface inducing agglutination and the term agglutinin is also used to designate lectin; hemagglutinating activity occurs when the lectin binds to carbohydrate from erythrocyte surface promoting a network among them (Figure 6A). Plant lectins have been isolated from bark, cladodes, flowers, leaves, rhizomes, roots and seeds.

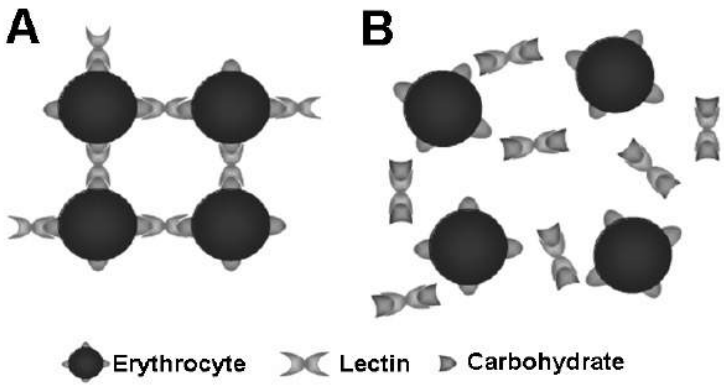

Figure. 6. Schematic representation of erythrocyte network promoted by lectin binding to surface carbohydrate (A) and inhibition of hemagglutinating activity by free carbohydrate (B).

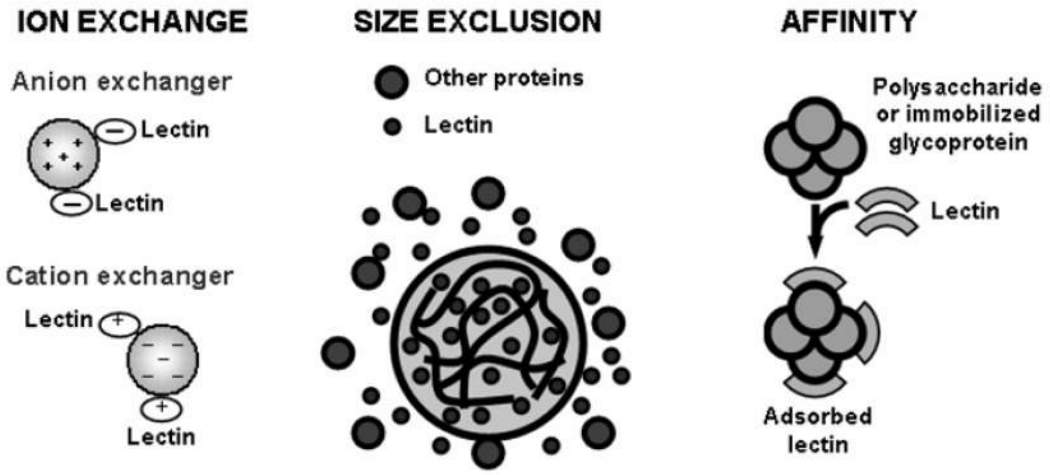

Figure 7. Schematic representation of chromatographic methods used for lectin isolation.

4.1. Methodologies for Extraction, Concentration and Purification

The fragmentation of plant tissue is performed before the extraction step and aims to promote greater solubilization by increasing contact surface between the lectin and extraction solution. Treatment of crude extract with ammonium sulfate [63] promotes differential protein precipitation since proteins rich in hydrophilic amino acids are more soluble than those rich in hydrophobic amino acids and require highest concentration of ammonium sulfate to precipitate from the solution. Fractionation of lectin can also be obtained by heating the crude extract at elevated temperatures (thermal precipitation); denatured proteins precipitate and thermo-stable lectins remain in solution [64].

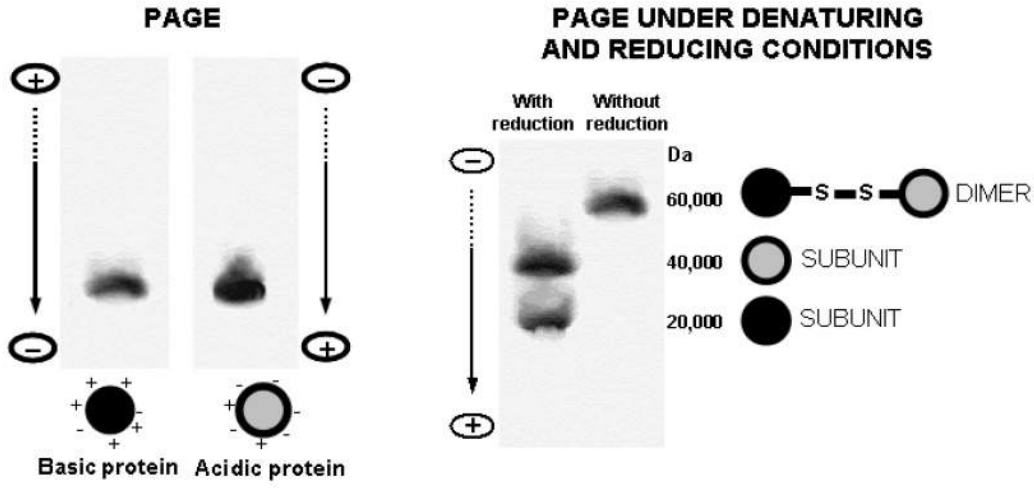

Figure 8. Schematic representation of polyacrylamide gel electrophoresis (PAGE) for native proteins and PAGE in presence of sodium dodecyl sulfate and reducing agent.

Lectin present in a mixture of proteins can be isolated by chromatographic methods (Figure 7). Ion exchange chromatography is able to separate proteins that differ in isoelectric points. Basic and acidic lectins bind, respectively, to matrices negatively (cation exchange column) and positively (anion exchange column) charged and lectin desorption can be made using stepwise or linear gradients of pH or NaCl. Size exclusion chromatography is based on percolation of a protein sample in the pores of inert matrices of controlled porosity with subsequent separation of the components by differential elution, according to the molecular size. The separation criterion in affinity chromatography is the property of lectins to bind specifically to carbohydrates. The lectin adsorbed on affinity matrix is recovered by elution with solution containing the hapten saccharide, or changing pH and ionic strength of chromatographic medium.

Lectin carbohydrate specificity can be easily defined by hemagglutinating activity assay when it is done in the presence of different carbohydrates. The lectin binding to carbohydrate free in solution is not able to interact with glycoconjugates from surface of erythrocytes and the hemagglutinating activity is inhibited (Figure 6B).

Electrophoretic methods are efficient tools for structural characterization and purity evaluation of lectins. Polyacrylamide gel electrophoresis (PAGE) for native proteins reveals the protein charge nature; in the presence of sodium dodecyl sulfate and reducing agent the protein is unfolded in its constituent subunits and the molecular mass is defined (Figure 8). Protein is usually detected on gel by Coomassie Brilliant Blue, amido black or silver staining. Electrophoresis is also efficient for identification and characterization of carbohydrate moiety from glycosylated lectins.

4.2. Larvicidal Activity against *Aedes aegypti*

Insect gut contains the peritrophic matrix which is constituted by proteins, glycoproteins, proteoglycans, and chitin. The matrix separates gut lumen contents from digestive epithelial cells; matrix integrity is essential for insect survival by important roles in digestive processes as well as insect protection from invasion by microorganisms and parasites. It has been suggested that chitin at peritrophic membrane, glycosylated receptors at the stomach epithelial cell surface as well as sugar moiety of glycosylated digestive enzymes are also targets for lectin binding [65, 66, 67 68, 69].

Recently, three lectins with detrimental action on *A. aegypti* larvae were reported. One lectin was isolated from *Moringa oleifera* seeds, the so-called water-soluble *M. oleifera* lectin (WSMoL); two were obtained from *Myracrodruon urundeuva* bark (MuBL) and heartwood (MuHL). *M. oleifera* belongs to the Moringaceae family and some of its vernacular names are ‘‘moringa” in Portuguese, ‘‘árbol del ben” in Spanish, and horseradish tree in English. Seeds of *M. oleifera* are widely used in developing countries as a natural coagulant to treat water for human consumption. *M. urundeuva* belongs to the Anacardiaceae family and its vernacular names are “aroeira do sertão” in Portuguese and “urundel” in Spanish. The plant is broadly distributed in Brazil and its heartwood is considered a hardwood very resistant to termites. WSMoL, MuBL and MuHL were purified from tissue extracts by ammonium sulphate precipitation followed by chromatography on chitin column; the hemagglutinating activity of these lectins was inhibited by *N*-acetylglucosamine.

Aqueous extracts of 1, 3, 6 and 15 seeds of *Moringa oleifera* (SE1, SE3, SE6 and SE15) delayed *A. aegypti* larval development (L1 to L4) and the effect was more pronounced in SE6 and SE15 [70]. Figure 9 shows that after 24 h a greater number of L1 was found in treatment with *M. oleifera* extracts than in distilled water (negative control); the life cycle at 72 h has reached the last larval stage (L4) only in control, SE1 and SE3. The extracts SE6 and SE15, with quantity of seeds higher than SE1 and SE3, prolonged mainly L1 and L2 instars revealing a correlation between larval stage and sensitivity to the active principle.

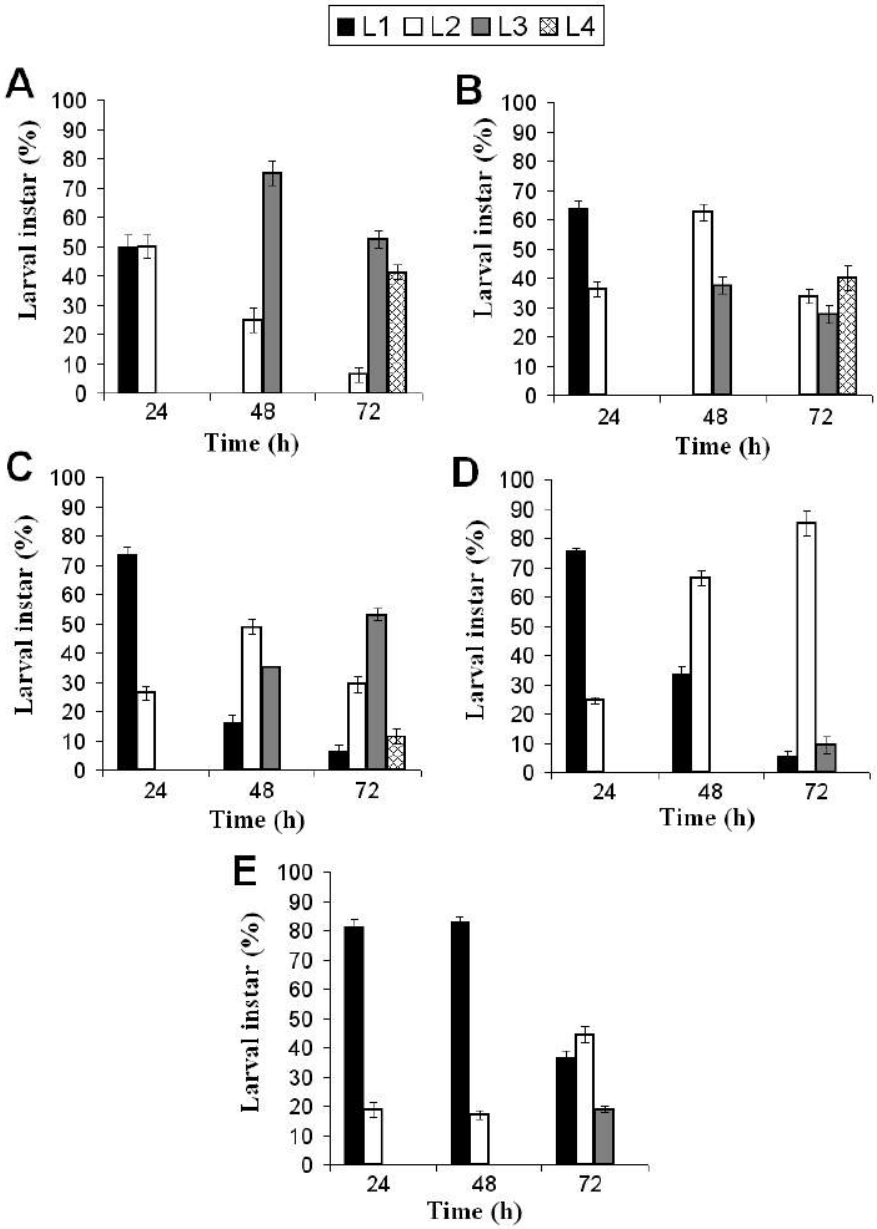

Figure 9. Larval instars (%) in incubation with control (A) and seed extracts SE1 (B), SE3 (C), SE6 (D) and SE15 (E). The bars are the mean ± S.D.

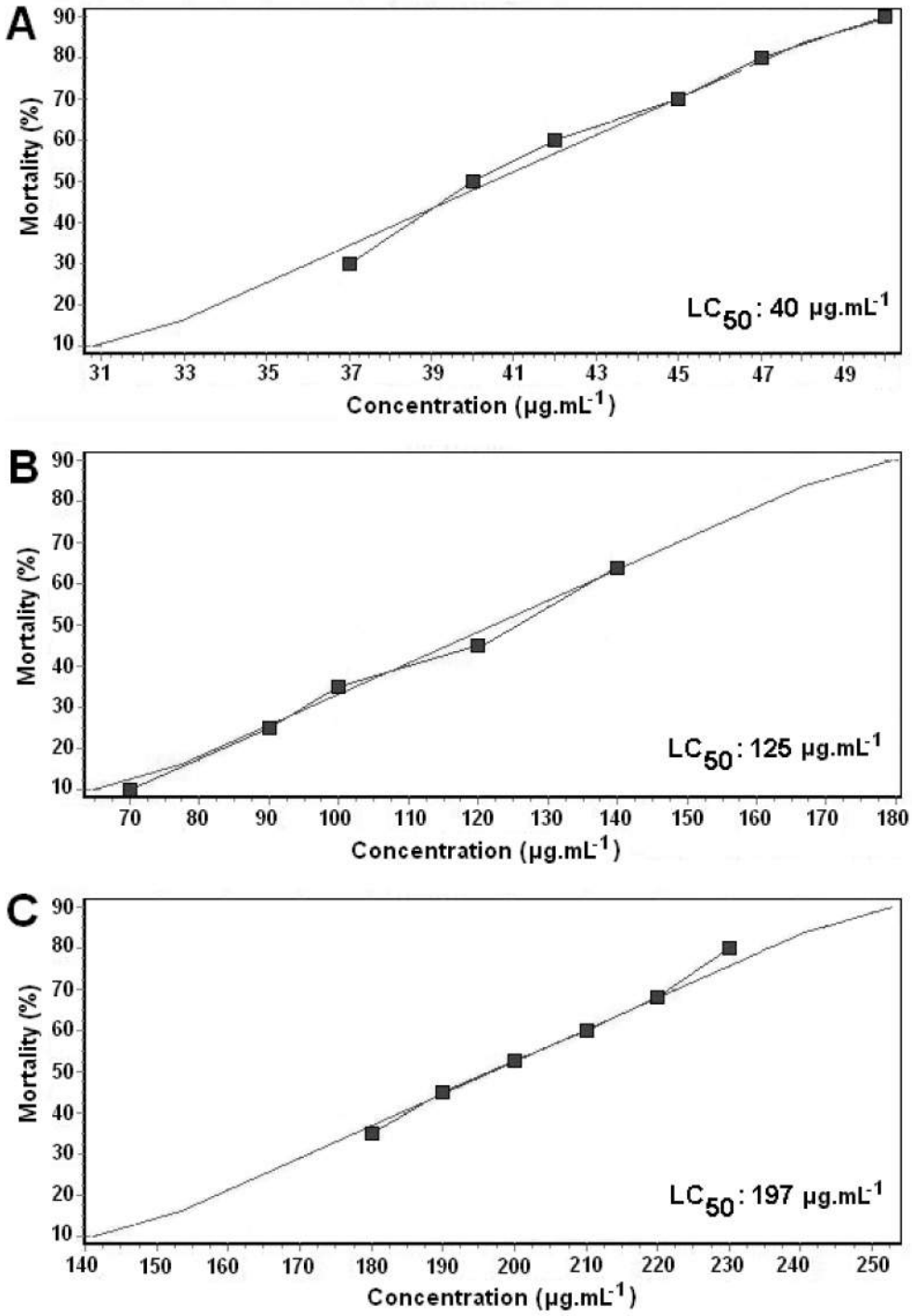

Figure 10. Mortality of *A. aegypti* L4 in incubation with lectins from *Myracrodruon urundeuva* heartwood (A) and bark (B) as well as *Moringa oleifera* seeds (C). Lethal lectin concentration required to kill 50% (LC_{50}) of larvae in 24 h was determined by probit analysis with a reliability interval of 95%.

Table 3. Insecticidal plant lectins

Insect	Plant (lectin abreviation)
Coleoptera	
Callosobruchus maculatus	*Bauhinia monandra* (BmoLL), *Griffonia simplicifolia* (GSII), *Solanum tuberosum* (STA), *Triticum aestivum* (WGA)
Zabrotes subfasciatus	BmoLL
Diptera	
Aedes aegypti	*Myracrodruon urundeuva* (MuBL, MuHL), *Moringa oleifera* (WSMoL)
Bactrocera curcubitae	*Arisaema helleborifolium* (AHL)
Lucilia cuprina	*Canavalia ensiformis* (ConA), *Lens culinaris* (lentil lectin), *Triticum aestivum* (WGA)
Hemiptera	
Acyrthosiphon pisum	ConA
Nilaparvata lugens	ConA, *Galanthus nivalis* GNA
Homoptera	
Dysdercus cingulatus	*Allium sativum* (ASAL)
Lypaphis erysimi	ASAL
Hymenoptera	
Atta opaciceps	*Canavalia brasiliensis* (ConBr)
Isoptera	
Nasutitermes corniger	MuHL
Lepidoptera	
Anagasta kuehniella	*Annona coriacea* (ACLEC), BmoLL, KpLec
Corcyra cephalonica	ACLEC
Neuroptera	
Chrysoperla carnea	GNA

References: [68, 69, 70, 71, 74, 90, 91, 92, 93, 94, 95, 96, 97].

Moringa oleifera and *Myracrodruon urundeuva* lectins promoted mortality of L4 and the effect was proportional to the concentration of proteins (Figure 10); lethal concentrations of

WSMoL, MuBL and MuHL required to kill 50% of the larvae (LC_{50}) in 24 h were 0.197, 0.125 and 0.04 $mg.mL^{-1}$, respectively [70, 71]. The lectins were thermo-stable proteins. Evaluation by optical microscopy of dead L4 from WSMoL treatment showed morphological changes with hypertrophy of the segments, gut volume increase and absence of epithelial layer that delimits the gut. *M. oleifera* seed extract was also a larvicidal agent on *A. aegypti* L3 (LC_{50} of 1,260 $\mu g.mL^{-1}$) and evaluation of extract effect on non-target organisms revealed low toxicity on *Daphnia magna*, *Mus musculus* and *Rattus novergicus* [72].

4.3. Other Biological Properties

Plant lectins are toxic on several insect orders (Table 3). Lectin binding to glycosylated receptors at the surface of stomach epithelial cells of *Acyrthosiphon pisum* nymphs induced epithelial cell distention, enlargement, and shedding in the midgut region [73]. The involvement of different targets in the mechanism of insecticidal lectins justifies the fact that lectins of different specificities are harmful to the same insect species and also different insecticidal efficiency of lectins with similar carbohydrate-binding ability. Termites consume many types of food, for example humus and celullosic materials such as paper and wood; these insects are responsible for deterioration of wood, paintings, ancient books, monuments and buildings. *Cladonia verticillaris* lichen and *Myracrodruon urundeuva* heartwood lectins showed insecticidal activity on *Nasutitermes corniger* and are promising for population control of this insect pest [74, 75].

The availability of a great number of lectins with distinct carbohydrate specificities has resulted in the use of these proteins as tools in medical and biological research [76]. Lectins can be utilized to detection, isolation and characterization of glycoconjugates and to explore cellular surfaces binding to the carbohydrate moiety of glycoproteins or glycolipids projected from the cell. Lectin applications with therapeutic purposes have been suggested.

Lectin affinity adsorbents can be prepared by lectin immobilization to insoluble supports such as silica and agarose and these matrices are able to fractionate both nitrogen and oxygen-linked sugar chain glycoproteins [77, 78, 79, 80]. Lectin-Sepharose column can be used for isolation of glycosylated molecules; the method was able to isolate active trypsin inhibitor without structural modification and did not have the pitfalls (irreversible inhibitor-enzyme binding and/or partial inhibitor proteolysis) of inhibitor isolation by trypsin-Sepharose column [81, 82]. Lectin affinity matrices separate glycoproteins by natural pattern of glycosylation and therefore can be used for characterization of serum glycoproteins [83].

The high carbohydrate specificity of lectin does allow its use for diagnostic purposes. Lectin immobilized on gold nanoparticles with polyvinyl butyral, and adsorbed on the surface of gold electrodes can be used to detect lectin–sugar interaction. The modified electrode could be applicable to the construction of a biosensor to isolate glycoproteins present in human blood serum [84]. The system is also able to detect different glycoprotein patterns in the sera from patients infected by dengue fever and hemorrhagic dengue fever [85]. Peroxidase-labelled lectin and lectin-acridinium ester conjugate (chemiluminescent marker) did detect changes in glycosylation pattern in cell surface by differential recognition patterns (differential staining) of normal and neoplasic tissues [86, 87].

Lectins are potential therapeutic agents in cancer treatment and promote tumor growth reduction; since some antitumor lectins are toxic to normal cells they can be encapsulated into

liposomes. *Cratylia mollis* isolectins (Cramoll 1,4) showed antitumor activity against Sarcoma 180; however lectin hepatotoxicity was revealed by lymphocyte infiltration in the liver of treated animals. The lectin was then loaded into liposomes and the preparation was an antitumor agent more efficient (71%) than free Cramoll 1,4 (30%); liver abnormalities and morphological alterations were not detected in spleen or kidneys after treatment with Cramoll 1,4-loaded liposomes [88].

5. Conclusion

The search for environmental safety and biodegradability stimulates the evaluation of plant versatility for several biological properties. In general, *A. aegypti* mortality promoted by essential oils, solvent organic extracts and lectins is directly proportional to compound concentration and the first two stages of larval development are more sensible to plant compounds than following larval stages. Larvicidal mechanisms proposed for essential oils and organic solvent extracts are cell alterations, antichitinogenic activity and toxic effect on larvae nervous system. Changes in digestive tract is a sign that chitinous structures at the peritrophic matrix and/or receptors at epithelial cells present in *A. aegypti* midgut are the lectin targets; common characteristics of insecticidal lectins include thermo-stability, resistance to enzyme hydrolysis and chitin-binding ability. Plant tissues are sources of promising essential oils, solvent organic extracts and lectins for pharmaceutical and cosmetic uses, as well as control of arthropods.

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9. CONCLUSÕES

- ✓ WSMoL (0,1 mg/mL) apresentou efeito estimulante sobre a oviposição de *A. aegypti* em condições de laboratório e quando utilizada em armadilhas para captura de ovos (ovitrampas) em condições de campo simulado.
- ✓ A não-volatidade de WSMoL, a presença da maioria dos ovos depositados pelas fêmeas na superfície da solução de WSMoL nas ovitrampas, bem como os resultados obtidos no ensaio de olfatometria indicam que o mecanismo de percepção de WSMoL pelas fêmeas não envolve sensores olfativos, mas sensores de contato (gustatórios).
- ✓ A ação estimulante de oviposição de WSMoL em condições de campo simulado apresentou persistência por 5 dias.
- ✓ WSMoL (0,1 mg/mL) não foi efetiva em aumentar a eficiência de armadilhas (MosquiTRAP^{TM}) para captura de fêmeas grávidas.
- ✓ Os ovos depositados pelas fêmeas em solução do extrato de sementes, da fração proteica 0-60% e de WSMoL isolada, nos ensaios de oviposição em condições de laboratório, apresentaram eclodibilidade reduzida. Análise por microscopia óptica indica que WSMoL provavelment interfere no desenvolvimento do embrião em formação.
- ✓ Extrato, fração proteica 0-60% e WSMoL apresentaram ação ovicida sobre ovos estocados, com valores de EC_{50} de 0,28, 0,18 e 0,1 mg/mL de proteínas, respectivamente, indicando a lectina como princípio ativo das preparações brutas.
- ✓ A ausência de eclosão dos ovos tratados com WSMoL e análise por microscopia óptica indicam que WSMoL apresenta atividade ovicida por matar os embriões dentro dos ovos.
- ✓ Irradiação gama na dose de 10 mGy potencializou as atividades larvicida e ovicida de WSMoL e aboliu a atividade estimulante de oviposição.
- ✓ WSMoL é a primeira lectina com atividade ovicida sobre insetos descrita. O presente trabalho relata também, pela primeira vez, o efeito estimulante de oviposição de uma

lectina e demonstra a atuação de uma molécula fixa (não volátil) como uma pista química utilizada pelas fêmeas grávidas de *A. aegypti* para seleção do sítio de oviposição.

- ✓ Os dados evidenciam a aplicabilidade de WSMoL no controle de *A. aegypti*, constituindo uma molécula com grande potencial por interferir na sobrevivência de ovos e larvas desse mosquito, bem como no comportamento de fêmeas grávidas.