UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA
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Atividade hemaglutinante e larvicida *(Aedes aegypti)* na água tratada com sementes de *Moringa oleifera*

Juliene Soares Coelho

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Atividade hemaglutinante e larvicida *(Aedes aegypti)* na água tratada com sementes de *Moringa oleifera*

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

ORIENTADOR: Profª Drª Patrícia Maria Guedes Paiva
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Ata da defesa de dissertação da Mestranda **Juliene Soares Coelho,** realizada em 14 de fevereiro de 2007, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 14:20 minutos do dia 14 de fevereiro de 2007, foi aberto, no Auditório Prof. Marcionilo Lins/Depto. de Bioquímica, o ato de defesa de dissertação da mestranda **Juliene Soares Coelho**, aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos a Prof. Dra. Vera Lúcia de Menezes Lima, Coordenadora do curso supra citado, fez a apresentação da aluna, de sua orientadora, Profa. Dra. Patrícia Maria Guedes Paiva, da co-orientadora Profa. Dra. Sônia Pereira Leite, do Depto. de Histologia/UFPE e da Banca Examinadora composta pelos professores doutores: Patrícia Maria Guedes Paiva, na qualidade de Presidente, Luana Cassandra Breitenbach Barroso Coelho, Ranilson de Souza Bezerra, os três do Depto. de Bioquímica/CCB/UFPE, e Russolina Benedeta Zingali, do Instituto de Bioquímica Médica/UFRJ. Após as apresentações, a Profa. Patrícia Paiva convidou a aluna para a apresentação de sua dissertação intitulada: **"Atividade hemaglutinante e larvicida (*Aedes aegypti)* na água tratada com sementes de *Moringa oleifera*"** e informou que de acordo com o Regimento Interno do Curso, a candidata disporia de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas seria de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 30 (trinta) minutos. Após a apresentação da mestranda, a Sra. Presidente concedeu um intervalo de 10 minutos. Reiniciando os trabalhos, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, a Profa. Dra. Russolina Zingali, em seguida para o Prof. Dr. Ranilson Bezerra, e finalmente para a Profa. Dra. Luana Coelho os quais após agradecerem o convite, fizeram alguns comentários, deram sugestões e iniciaram suas respectivas argüições. Ao final das mesmas, os referidos professores deram-se por satisfeitos. Em seguida, a Sra. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Mestrado, na presença da Coordenadora do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 14 de fevereiro de 2007.

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LISTA DE ABREVIATURA

AS-PTA	Assessoria e Serviços a Projetos em Agricultura Alternativa
BTI	*Bacillus thuringiensis israelensis*
DEN-1, DEN-2, DEN-3 e DEN-4	Sorotipos do vírus da dengue
DNA	Ácido desoxirribonucléico
EBV-EA	Epstein-Barr Vírus – early antigen
FAO	Food and Agriculture Organization
L_1, L_2, L_3 e L_4	Estádios larvais do *Aedes aegypti*
MO 2.1	Fração de *Moringa oleifera*
MOC-DW	Coagulante isolado de extrato aquoso de *Moringa oleifera*
MOC-SC	Coagulante obtido da extração salina de *Moringa oleifer**a***
MoW	Água tratada com sementes de *Moringa*
PNCD	Programa Nacional de Controle da Dengue
SVS	Secretaria de Vigilância em Saúde
UNO	United Nations Organization
WHO	World Health Organization
WSMoAC	Composto antioxidante de *Moringa oleifera* solúvel em água
WSMoL	Lectina de *Moringa oleifera* solúvel em água

SUMÁRIO

RESUMO

Sementes de Moringa oleifera têm sido freqüentemente usadas como coagulante para o tratamento de água no Nordeste do Brasil, local com alta incidência de dengue. Lectinas constituem um grupo de proteínas heterogêneas capazes de reconhecer e ligar reversivelmente carboidratos e glicoproteínas. Inibidores de protease impedem a atividade enzimática pela formação de complexos estáveis com tripsina e/ou quimotripsina. Lectinas e inibidores de tripsina com atividade inseticida têm sido descritos. O objetivo deste trabalho foi avaliar na água tratada com sementes de *M. oleifera*, a partir das atividades hemaglutinante (AH), inibidora de tripsina e larvicida. Água destilada foi tratada com 1, 3, 6 ou 15 sementes de *M. oleifera* (MoW). Ensaio para lectina usou eritrócitos de coelho. MoW foi avaliada para atividade inibidora de tripsina usando N-α-benzoil-DL-arginil-ρ-nitroanilida (BAPNA). Bioensaio larvicida foi feito usando larvas do vetor da dengue, *Aedes aegypti.* Desenvolvimento, mortalidade e aspectos morfológicos das larvas foram analisados. MoW contém AH e não foi capaz de inibir tripsina. Redução significante do desenvolvimento larval foi observado após 24, 48 e 72 h de incubação com MoW. Larvas no 4º estádio (L4) foram detectadas somente no controle, MoW_1 e MoW_3. Significante ($p<0.0001$) taxa de mortalidade foi detectada na preparação lectínica mais ativa, MoW_{15} (45%). Diferenças morfológicas nas larvas em L4 incubadas com MoW1 foram observadas quando comparadas ao controle por microscopia ótica invertida. Os resultados obtidos sugerem a avaliação das sementes de *M. oleifera* para o controle do vetor da dengue. A presença de AH na preparação ativa sobre as larvas do *A. aegypti* pode indicar o envolvimento de lectina no efeito larvicida de MoW.

Palavras-chave: ***Aedes aegypti*; larvicidal activity; lectin; *Moringa oleifera*.**

ABSTRACT

Moringa oleifera seeds have been frequently used as coagulant for water treatment in Brazilian Northeast, where a high incidence of dengue fever. Lectins constitute a heterogeneous protein group capable of recognizing and reversibly binding to carbohydrates and glycoproteins. Protease inhibitors disrupt enzymatic action by formation of stable complexes with trypsin and/or chymotrypsin. Lectins and trypsin inhibitors with insecticidal activity have been described. The aim of this work was to evaluate hemagglutinating (HA), trypsin inhibitor and larvicidal activities, in the water treated with *M. Oleifera* seeds. Distilled water was treated with 1, 3, 6 or 15 seeds of *M. oleifera* (MoW). Lectin assay used rabbit erythrocytes. MoW was also evaluated by trypsin inhibitory activity using N-α-benzoyl-DL-arginyl-ρ-nitroanilide (BAPNA). Larvicidal bioassay was performed using larvae of dengue vector *Aedes aegypti.* Development, mortality and morphological aspects of larvae were analyzed. MoW contains HA and was not able to inhibit trypsin. Reduction of larvae development was observed after 24, 48 and 72 h of incubation with MoW. Fourth instar larvae (L4) were only detected in control, MoW_1 and MoW_3. Significant ($p<0.0001$) larval mortality was detected in the most active lectin preparation, MoW_{15}. Morphological changes in L4 incubated with MoW1 were demonstrated at light microscope level. The results obtained suggest the evaluation of *M. oleifera* seeds for the control of dengue vector. The presence of HA in MoW preparations active on *A. aegypti* larvae can be indicative of lectin involvement in MoW larvicidal effect.

Key-words: *Aedes aegypti*; larvicidal activity; lectin; *Moringa oleifera*.

1. Introdução

1.1 Sistemas de tratamento de água.

A água é um meio particularmente vulnerável a diversos tipos de contaminação, desde grandes descargas industriais à contaminação domésticas causadas pelo homem como usuário dela. A desinfecção da água tem sido praticada por milênios; existem indícios de que o uso de água fervida já era recomendado em 500 a.C., mas alguns historiadores julgam que esta prática foi adotada desde o começo da civilização (LABUSCH, 1971).

O propósito primário para a exigência de qualidade da água é a proteção à saúde pública. Os critérios adotados para assegurar essa qualidade têm por objetivo fornecer uma base para o desenvolvimento de ações que, se propriamente implementadas junto à população, garantirão a segurança do fornecimento de água através da eliminação ou redução à concentração mínima de constituintes na água perigosos à saúde (D'AGUILA *et al*., 2000).

O índice de doenças nas áreas rurais pode ser consideravelmente reduzido, caso a população tenha acesso à água potável. Entretanto, um dos maiores problemas das fontes particulares é a ausência de monitoramento da qualidade da água consumida (MISRA, 1975).

Antes da água potável ser distribuída aos consumidores ela sofre o processo de tratamento que envolve as etapas de coagulação, floculação, seguido de sedimentação, filtração e desinfecção, freqüentemente por cloração, que é o processo adotado mundialmente pela indústria de tratamento (KAWAMURA, 1991; AWWA, 1990; DEGREMONT, 1989; EDZWALD *et al*., 1989; DESJARDINS, 1988).

Muitos coagulantes são freqüentemente utilizados no processo convencional de tratamento da água para consumo humano. Estes podem ser coagulantes inorgânicos (sulfato de alumínio e sais de ferro), polímeros sintéticos orgânicos (derivados de poliacrilamida) ou

coagulantes que ocorrem naturalmente - quitosana e coagulantes microbianos (OKUDA *et al*., 1999). Entretanto, estudos têm indicado o envolvimento dos sais de alumínio na doença de Alzheimer (MILLER *et al*., 1984) e de problemas de saúde relacionados ao acúmulo, na água tratada, de alumínio residual (AWWA, 1990; LETTERMAN & DRISCOLL, 1988; QURESHI & MALMBERG, 1985). Problemas também surgem da reação do alumínio com a alcalinidade natural presente na água, o que conduz a uma redução no pH e a uma baixa eficiência no processo de coagulação na água fria (HAARHOFF & CLEASBY, 1988). Sais férricos e polímeros sintéticos são alternativos ao sulfato de alumino, mas com uso limitado, devido ao fato do impacto causado nos seres vivos não ser completamente conhecido (LETTERMAN & PERO, 1990). Monômeros de alguns polímeros sintéticos orgânicos, como a acrilamida, possuem propriedades carcinogênicas e neurotóxicas (McCOLLISTER *et al*., 1964).

Coagulantes naturais de origem vegetal e mineral estavam em uso no tratamento da água antes do advento dos sais químicos, mas eles não puderam competir eficazmente devido à falta de uma completa compreensão científica sobre sua eficácia e seus mecanismos de ação. Dessa forma, o uso destes ficou restrito apenas em áreas remotas dos paises em desenvolvimento (JAHN, 1981).

Em contrapartida, nos últimos anos, houve um ressurgimento do interesse em estudar as propriedades dos coagulantes de ocorrência natural; por serem presumivelmente pouco ofensivos à saúde quando comparados aos coagulantes de uso consagrado como os sais de alumínio e polímeros sintéticos orgânicos. Diversos estudos têm sido feitos para avaliar a performance das sementes de *Moringa oleifera* como um coagulante alternativo ou um auxiliar na coagulação e suas atividades biológicas (OKUDA *et al*., 1999).

1.2 *Moringa oleifera*

Moringa oleifera é uma planta tropical pertencente à família das *Moringaceae* (Figura1). Esta espécie destaca-se por apresentar um composto coagulante ativo que atua na clarificação de águas barrentas (OKUDA *et al.,* 2001; JAHN, 1988). *M. oleifera* tem a Índia como país de origem, apesar de ser amplamente cultivada nas regiões tropicais de todo o mundo, onde cresce rapidamente, sendo capaz de sobreviver em períodos de chuva escassa (WARHURST *et al*., 1996).

Figura 1. *Moringa oleifera*, aspectos gerais.

Dependendo do local onde é cultivada, *M. oleifera* recebe nomes específicos e poucos conhecidos que determinam sua variedade como é o caso da Mbololo (Kenya), Jaffna (Sri Lanka), e Periyakulam 1(Índia) (TSAKNIS *et al*., 1998*).* A planta apresenta longas vagens, sementes aladas, folhas grandes e flores brancas perfumadas.

Nas Filipinas, as folhas, flores e vagens são utilizadas na dieta. No Brasil há um esforço no sentido de difundi-la como hortaliça rica em vitamina A. As folhas, com cerca de 23.000 UI

de vitamina A, sobressaem-se entre olerícolas consagradas como brócolis, cenoura, couve, espinafre e alface, que possuem, respectivamente, 5.000; 3.700; 2.200; 1.900; 1.000 UI de vitamina A (SILVA & KERR, 1999; KERR *et al*., 1998; AMAYA *et al*., 1992).

As folhas contêm todos os aminoácidos essenciais incluindo aqueles que possuem enxofre na composição, numa concentração maior que a recomendada como padrão pela FAO/WHO/UNO e os extratos etanólico das folhas não indicaram presença de taninos, lectinas, inibidores de tripsina e saponinas (MORTON, 1991; MAKKAR & BECKER, 1996). O elevado conteúdo protéico e o potencial de digestão intestinal das proteínas de folhas da moringa sugerem que estas são boas fontes de suplemento protéico na alimentação de ruminantes (MAKKAR & BECKER, 1996).

Os frutos, que são chamados de vagens e usados como vegetais na dieta e as raízes da planta, contêm proteínas, lipídeos, carboidratos, minerais, fibras, vitamina A, ácido β-nicotínico, ácido ascórbico, tocoferol e β-sitosterol (CHAWLA *et al*., 1988; VERMA *et al.,* 1976; CSIR, 1962).

O uso medicinal de moringa é amplo e desta forma, várias partes da planta, como as folhas, raízes, sementes, caule, flores e frutos, têm sido estudadas quanto à ação na cicatrização de ferimentos (UDUPA *et al*., 1994, 1998), e quanto às atividades antitumoral (GUEVARA *et al*., 1999), anti-hepatotóxica (RUCKMANI *et al*., 1998), anti-fertilidade (PRAKASH *et al*., 1988), analgésica (RAO *et al*., 2003) e anti-malárica (CÁCERES, 1991). O uso das raízes da planta é descrito para o tratamento de asma, gota, reumatismo e inflamações internas (VAIDYARATNAM, 1994; NADKARNI & NADKARNI, 1982; BASU & KIRTIKAR, 1980). Estudos vêm sendo feitos visando o isolamento de compostos bioativos de várias partes da planta (GUEVARA *et al.,* 1999).

Extratos aquosos das folhas de *M. oleifera* foram estudados quanto ao efeito na regulação hormonal da tireóide, revelando que a administração de uma dose 175mg/kg durante 10 dias, leva a diminuição dos níveis plasmáticos da triiodotironina (T_3) e a um aumento concomitante da tiroxina (T_4) sérica em camundongos Swiss fêmeas, enquanto que nos machos não houve diferenças significativas em relação ao grupo controle. Estes resultados sugerem que o extrato aquoso das folhas de moringa age inibindo a conversão periférica de T_4 em T_3, o que pode levar ao uso destes extratos na regulação do hipertireoidismo (TAHILIANI & KAR, 1999).

1.3 Sementes de *M. oleifera* e suas aplicações

As sementes (Figura 2) são globulares, aladas e apresentam um peso médio de aproximadamente 3-4 g, e de 1-1,7 cm de diâmetro (GOH, 2005).

Figura 2. Vagem e sementes de *Moringa oleifera*.

O extrato etanólico de sementes de moringa apresentou atividade hipotensiva numa dose equivalente a 30mg/kg. Neste estudo os glicosídeos tiocarbamato e isotiocianato foram isolados e identificados como os princípios hipotensivos de *M. oleifera* (FAIZI *et a.l.*, 1994, 1998).

Atividade antimicrobiana das sementes foi detectada através do método de difusão em disco. O extrato aquoso inibiu o crescimento de bactérias gram-negativa (*Pseudomonas aeruginosa*) e gram-positiva (*Staphylococcus aureus*) (CACERES *et al*., 1991b).

Óleos comestíveis podem ser extraídos das sementes e os resíduos sólidos podem servir como alimento animal e fertilizante. O óleo das sementes da variedade "Periyakulum 1" é tido como excelente fonte de óleo comestível para o consumo humano, o qual é aceitável como substituto nas dietas dos óleos altamente monoinsaturados como o óleo de oliva, pois possui propriedades físicas e químicas equivalentes às deste; contendo todos os ácidos graxos do óleo de oliva, exceto linoleíco e uma elevada quantidade de tocoferóis (LALAS & TSAKNIS, 2002; MORTON, 1991; TSAKNIS et al., 1999).

A casca das sementes, que geralmente é descartada, pode ser convertida em carvão ativado de alta qualidade, através de um processo de etapa única (carbonização – ativação) de pirólise a vapor, funcionando como um adsorvente de impurezas (GHEBREMICHAEL *et al.,* 2005; WARHURST *et al*., 1996). A produção de carvão ativado a partir da casca das sementes de moringa em diferentes condições de temperatura e tempo foi avaliada e os produtos resultantes comparados a carvões ativados de uso comercial, quanto à capacidade de adsorção do fenol, 4-nitrofenol e azul de metileno; mostrando que os dois primeiros foram rapidamente adsorvidos por todos os carvões de moringa nos primeiros trinta minutos enquanto que o azul de metileno teve uma adsorção mais lenta. As características adsortivas dos carvões ativados derivados das cascas de moringa os tornam competitivos com carvões de uso comercial (WARHURST *et al.,* 1997).

Um composto mutagênico também foi isolado das sementes tostadas de *M. oleifera*. Análise espectral revelou o mesmo como sendo o 4(α-L-ramnosiloxi) fenilacetonitrila (VILLASENOR *et al.,* 1989).

Em 1999 Guevara *et al.,* isolaram do extrato etanólico das sementes, oito compostos bioativos os quais foram avaliados quanto à atividade antitumoral, através do efeito inibidor sob EBV-EA (Epstein-Barr Vírus – early antigen) em linfócitos B ativados, todos os compostos testados mostraram efeitos inibitórios na indução da ativação do EBV-EA

Gupta *et al.* (2005) concluíram que o pó das sementes de *M. oleifera* não só tem uma potente atividade antioxidante como também apresenta propriedade quelante na toxicidade por arsênico induzida em ratos. Os autores verificaram que a administração oral de uma dose de 500 mg do pó das sementes/kg durante 4 meses reduziu os níveis sangüíneos de radicais livres, conferindo significante proteção aos efeitos tóxicos e ao estresse oxidativo induzido pela exposição ao arsênico nos animais.

Segundo Janh (1988, 1986) as sementes de *M. oleifera* possuem propriedades coagulantes efetivas sendo usadas no processo de purificação da água e não são tóxicas a humanos e animais (GRABOW *et al*., 1985; BERGER *et al*., 1984) adquirindo então, grande importância no tratamento de água para o consumo humano (WARHURST; MCCONNACHIE; POLLARD, 1996). Nas zonas rurais do Nordeste brasileiro a utilização das sementes de moringa no tratamento da água para o consumo humano tem sido prática freqüente (ABAS, 1999; GERDES, 1997) dada à escassez de água potável nesta região.

Gassenschimidt *et al.* (1995) utilizaram as sementes trituradas e desengorduradas e obtiveram a partir de extrato em tampão fosfato, seguido de cromatografia de troca iônica, três frações contendo atividade floculante. Uma proteína de massa molecular aproximada de 6,5 kDa, com ponto isoelétrico em torno de 10, foi isolada de uma das frações (MO 2.1); análise da composição de aminoácidos revelou um elevado conteúdo de glutamina, arginina e prolina em um total de 60 resíduos. A comparação da sua estrutura primária com seqüências de proteínas

conhecidas do banco de dados do Laboratório Europeu de Biologia Molecular (EMBL/Heidelberg) não revelou significante homologia. A proteína presente na fração floculante MO 2.1 é um coagulante primário altamente específico e seu efeito foi comparado a um polímero catiônico a base de poliacrilamida, chamado 554K.

Compostos ativos isolados do extrato aquoso das sementes foram descritos como sendo proteínas catiônicas diméricas, que possuíam peso molecular em torno de 13 kDa e ponto isoelétrico com valor entre 10 e 11 (NDABIGENGESERE *et al*., 1995). Estes autores ainda sugeriram que o mecanismo de coagulação envolvendo a proteína de *M. oleifera* parece consistir de adsorção e neutralização das cargas coloidais.

Comparando-se a eficiência do processo de coagulação e a qualidade da água obtida após tratamento com sementes de moringa ou sulfato de alumino, foi revelado que a planta age como coagulante sem requerer ajustes de pH e sem mudanças na condutividade da água. O uso do sulfato de alumínio requer aditivos químicos para correção do pH e a condutividade é consideravelmente elevada (de 150 para 842 $\mu ohm^{-1}. cm^{-1}$) devido à presença dos íons sulfato remanescentes (NDABIGENGESERE *et al*., 1998). A quantidade de sedimento produzida por ambos os tratamentos também foi avaliada neste trabalho, estando o tratamento químico mais uma vez em desvantagem pela produção de um volume 4 a 5 vezes maior que o produzido pelas sementes, o que significa um maior impacto ambiental devido à dificuldade de remoção dos precipitados químicos formados (AWWA, 1990). Uma vantagem adicional é que o sedimento produzido por moringa no tratamento da água é um produto orgânico biodegradável, que pode ser utilizado como fertilizante (NDABIGENGESERE *et al*., 1998).

No sentido de desenvolver melhorias nos métodos de extração da proteína coagulante de *M.oleifera*, OKUDA *et al.* (1999) conduziram experimentos testando a eficiência da extração

com NaCl, KCl, KNO_3 e $NaNO_3$. Foi observado que o coagulante obtido da extração salina (MOC-SC) foi mais eficaz na remoção da turbidez da água, em dose 7,4 vezes menor que a do coagulante isolado do extrato aquoso (MOC-DW), tido como método convencional.

O coagulante ativo extraído por Okuda *et al.* (1999) com NaCl 1M foi isolado e purificado em uma seqüência de processos incluindo diálise, delipidação e cromatografia de troca iônica. Análises quantitativas e qualitativas para determinação de proteínas, lipídeos e carboidratos revelou que o composto ativo MOC-SC é um polieletrólito com peso molecular em torno de 3 kDa (OKUDA *et al.,* 2001). O estudo mostrou que a extração aquosa e salina resulta em diferentes compostos com atividade coagulante.

Santos *et al.* (2005) isolou de extratos aquosos das sementes uma lectina, WSMoL, e uma atividade antioxidante, WSMoAC. WSMoL é uma proteína ácida com massa molecular de aproximadamente 20 kDa que tem atividade hemaglutinante para eritrócitos de coelho. A presença de inibidor de tripsina e taninos foi também investigada e não foram detectados.

1.4 Proteínas bioativas de plantas: lectinas e inibidores de tripsina.

1.4.1 Lectina

Lectinas são (glico) proteínas que se ligam reversivelmente e especificamente a mono ou oligossacarídeos (PEUMANS; VAN DAME, 1998). O sítio de ligação para carboidratos tende a ser na superfície da molécula protéica e a seletividade da ligação é obtida através de pontes de hidrogênio, interações de Van der Walls e hidrofóbicas (ALGAVISH; SHAANAM, 1997; SUROLIA; SHARON SCHWARZ, 1996**).**

As lectinas podem detectadas por sua habilidade em aglutinar eritrócitos, em certos casos com alta especificidade (ASKAR, 1986; LIS & SHARON, 1973). Algumas lectinas são específicas em suas reações com grupos sangüíneos humanos ABO (SHARON & LIS, 1972). Esta propriedade deve-se a habilidade das lectinas de se ligarem a açúcares específicos na superfície celular (DESHPANDE & DAMODARAN, 1990). Além de hemaglutinação, as lectinas podem promover estimulação mitogênica de linfócitos e aglutinação de células cancerosas (LIENER, 1981; LIS & SHARON, 1973).

Embora muitas lectinas reconheçam e se liguem a açúcares simples tais como glicose, manose, galactose, N-acetilgalactosamina, N-acetilglucosamina ou fucose, algumas apresentam maior afinidade para com os constituintes de glicoproteínas, ácido siálico e N-acetilgalactosamina, encontrados em animais e seres humanos (PEUMANS & VAN DAMME, 1996; NICOLSON, 1974).

Muitas lectinas de plantas são tóxicas para células animais. Lectinas consumidas na dieta podem ser inofensivas desde que elas sejam desnaturadas pelo cozimento e proteoliticamente digeridas no consumo. Contudo, lectinas frescas podem ter efeitos deletérios. Por exemplo, a lectina de feijão de soja e a aglutinina de gérmen de trigo induzem a liberação de colecistocinina, sugerindo que esses tipos de lectinas podem ter efeitos diretos na função gastrintestinal e crescimento (AJIT, 2002).

Em animais experimentais, efeitos tóxicos de lectinas após ingestão oral, podem ser devido à habilidade destas substâncias em ligar-se a sítios receptores específicos na superfície das células intestinais, acarretando interferência não específica na absorção de nutrientes (LIENER, 1981). As alterações da função fisiológica causada por lectinas no intestino, parecem que são

produtos da sua estabilidade aos processos digestivos e a especificidade pelas células da mucosa intestinal em diferentes regiões (NAKATA & KIMURA, 1985; BRADY *et al.*, 1978).

A atividade inseticida de algumas lectinas de plantas contra algumas classes de insetos (Coleópteros, Dípteros, Homópteros, Lepdópteros) é descrita por Vasconcelos & Oliveira (2004). Lectinas ligadoras de manose das plantas monocotiledôneas, têm sido relatadas como efetivas no combate a insetos causadores de pragas agrícolas; o provável mecanismo de ação é a ligação específica da lectina a carboidratos de membrana da superfície celular do trato digestivo do inseto (BANDYOPADHYAY, *et al.,* 2001; SAUVION *et al.,* 1996).

Uma aglutinina com potente atividade inseticida para as larvas de Besouro do Colorado (*Leptinotarsa decemlineata*) foi isolada das folhas de *Glechoma hederacea.* A lectina, chamada Gleheda, exibiu uma atividade hemaglutinante para eritrócitos humanos que carregam o antígeno do Tn (GalNAcα1- Ser/Thr) (WANG *et al.,* 2003 a,b).

Mesmo com poucos relatos sobre os prováveis mecanismos de ação destas lectinas entomotóxicas, o que se supõe é que a resistência à degradação proteolítica pelas enzimas digestivas dos insetos faz com que possam se ligar às células do intestino destes. No caso das lectinas ligadoras de quitina, a ligação ocorre na membrana peritrófica dos insetos (PEUMANS & VAN DAMME, 1995; CHRISPEELS & RAIKHEL, 1991). O epitélio intestinal destes insetos é recoberto por glicoproteínas, fornecendo vários sítios de ligação para lectinas (MAJUMDER *et al.,* 2004).

1.4.2 Inibidor de tripsina

Os inibidores de proteases são proteínas de ampla distribuição no reino vegetal, capazes de reduzir ou abolir a atividade de enzimas como a tripsina, quimotripsina, amilase e

carboxipeptidase (BENDER, 1987; XAVIER-FILHO & CAMPOS, 1989). São polipeptídeos que ao formarem complexos estáveis com a enzima obstruem os seus sítios de ligação inibindo a atividade catalítica (UDEDIBIE *et al.,* 1998). Geralmente são denominados como inibidores da primeira enzima contra a qual foi testado e na maioria das pesquisas foi investigada a tripsina (SGARBIERI & WHITAKER, 1982).

Principalmente os inibidores de tripsina encontrados nas sementes de leguminosas, mais especificamente na soja, foram supostamente responsabilizados pelo baixo valor nutritivo de leguminosas cruas (XAVIER-FILHO & CAMPOS, 1989). De acordo com Proll *et al.* (1998) as leguminosas de maneira geral podem conter fatores antinutricionais e outras substâncias nocivas à saúde, desta forma, grãos não convencionais com potencial de uso na alimentação, devem ser testados em dietas animais antes da utilização em dietas humanas.

Inibidores de tripsina são antinutrientes que interferem com o processo fisiológico de digestão e absorção em não ruminantes. Esses inibidores são destruídos no rúmen e por isso não constituem um problema para os animais ruminantes. Em espécies monogástricas inibem o funcionamento normal das enzimas proteolíticas pancreáticas (HOSSAIN *et al.,* 2002). Inibidores de tripsina têm sido implicados na redução da digestão protéica e na hipertrofia do pâncreas (LIENER *et al.,* 1976; HANBURY *et al.,* 2000).

O papel fisiológico creditado a inibidores inclui a regulação de proteases endógenas, mobilização de proteínas de reserva, proteína de reserva e proteção contra enzimas proteolíticas de parasitas e insetos (Haq *et al*., 2004**).**

Bioensaios realizados revelaram a atividade inseticida de inibidores de tripsina isolados do feijão de corda (*Vigna unguiculata*), soja (*Glycine Max*), batata (*Solanum tuberosum*), batata doce (*Ipomea batatus*) e tomate (*Lycospersicum esculentum*). A presença de inibidores leva a

uma taxa de crescimento reduzida (Carlini *et al.*, 2002) e sugere-se que o sítio primário de ação de inibidores de proteases é o sistema digestivo da larva do inseto (Haq *et al*., 2004).

Seqüências de DNA que codificam inibidores de serino ou cisteino proteases que apresentam atividade entomotóxica têm sido incorporadas ao genoma de plantas economicamente importante tais como, cereais, tabaco e batata visando aumentar a resistência das culturas a pestes (Haq *et al*., 2004; Carlini *et al*., 2002). Estudos vêm sendo realizados visando avaliar, em plantas transgênicas que carregam genes de inibidor, as vantagens quanto à resistência a pragas e os potenciais danos ambientais que podem resultar desse processo.

1.5 Importância epidemiológica do *Aedes aegypti*

A dengue é dentre as doenças virais de transmissão vetorial, a que mais causa impacto em termos de morbidade e mortalidade na população mundial em anos recentes e também exige esforços e investimentos cada vez mais intensos dos serviços de saúde pública (GUBLER, 2002; WHO, 1997). A dengue é causada por qualquer um dos quatro sorotipos do vírus da dengue, denominados DEN-1, DEN-2, DEN-3 e DEN-4, e tem como principal vetor urbano mosquitos da espécie *Aedes aegypti* (TAUIL, 2002), que podem causar a dengue clássica e a febre hemorrágica da dengue.

No Brasil, o primeiro registro de casos de dengue ocorreu na década de 1920 e durante seis décadas seguintes, não foram relatados casos no país. O *A. aegypti* foi erradicado do Brasil e de mais 17 países das Américas nas décadas de 1950 e 1960 (TAUIL, 2002; NOGUEIRA *et al.,* 1999). A reinfestação do país pelo vetor ocasionou epidemias em Boa Vista, Roraima, em 1981/1982, e no Estado do Rio de Janeiro, em 1986, causadas pelo sorotipo 1 do vírus.

Em 1990/1991, durante nova epidemia, com a inclusão do sorotipo 2, notificaram-se 1.952 casos de dengue hemorrágica, com 24 mortes (NOGUEIRA *et al.,* 1999). Ainda no ano

2000 foi isolado no Rio de Janeiro o sorotipo 3 (DEN-3), tido como o mais virulento dentre os demais (SCHATZMAYR, 2000), seguindo-se entre os anos 2001 e 2002 uma epidemia com elevados níveis de incidência (FUNASA 2002).

O *A. aegypti* tem um ciclo de vida curto e é facilmente mantido e manipulado no laboratório. O ciclo de vida completo de *A. aegypti* dura entre 8 e 15 dias sob condições laboratoriais controladas tais como, temperatura de 25°C, 75-80% de umidade relativa e fotoperíodo 12/12–claro/escuro (CONSOLI & LOURENÇO-DE-OLIVEIRA, 1994; MUNSTERMAN & WASMUTH, 1985). Duas vantagens adicionais do uso de *A. aegypti* em bioensasios são que; (1) os ovos podem ser armazenados por mais de 12 meses antes de eclodidos (MUNSTERMAN & WASMUTH, 1985) e (2) informações substanciais da fisiologia, biologia molecular e genética, já são conhecidas e disponíveis para esta espécie de mosquito (BEATY & MARQUARDT, 1996).

O desenvolvimento do mosquito ocorre por metamorfose completa (Figura 3), passando pelas seguintes fases: ovo, quatro estádios larvais (L_1, L_2, L_3 e L_4), pupa e adulto (GUBLER, 1998). As pupas permanecem ativas no ambiente aquático até o momento da eclosão, quando mosquitos adultos emergem. Um dia após a eclosão o mosquito fêmea está apto a acasalar, e então de 2 a 3 dias após ingesta sangüínea, as fêmeas irão por ovos férteis.

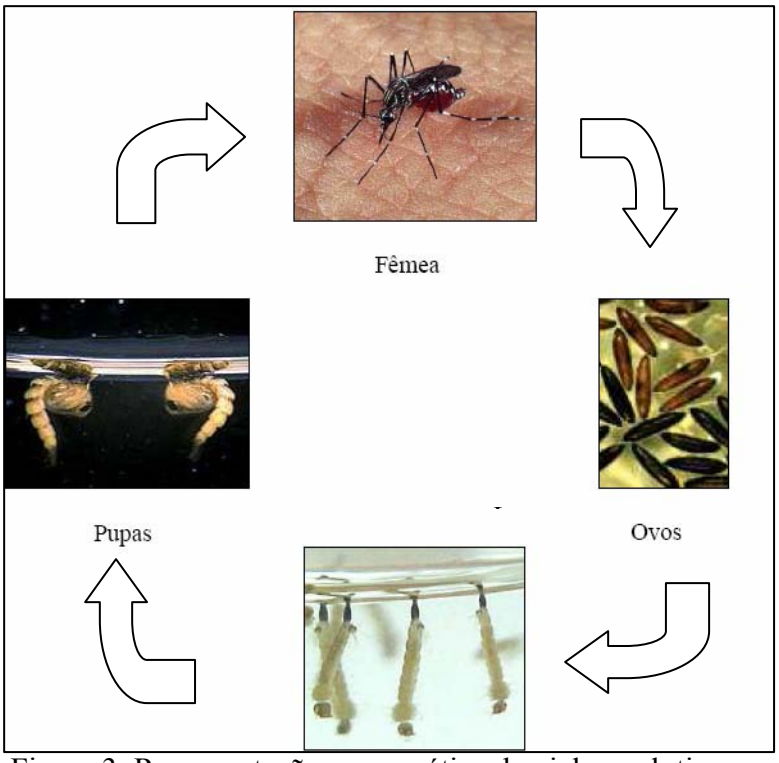

Figura 3. Representação esquemática do ciclo evolutivo do *Aedes aegypti.*

O *A. aegypti* apresenta hábitos antropofílicos e as fêmeas realizam a hematofagia em período diurno, com maior pico no período entre 16 e 18 h (SILVA *et al*., 2002). Após realizarem o repasto sangüíneo necessário à maturação dos seus ovos, repousam e ao final da digestão procuram sítios para ovoposição em pequenas coleções de água limpa e parada, localizadas nas proximidades das casas. Os ovos são depositados isoladamente nas paredes internas de recipientes como tonéis, caixas d'água, descartáveis, vasos, pneus ou outros contendo água que servirão de criadouro para o desenvolvimento de suas formas imaturas (CONSOLI & LOURENÇO-DE-OLIVEIRA, 1994).

A prevenção de epidemias de dengue depende fundamentalmente da redução populacional do vetor da doença no domicílio e peridomicílio principais locais nos quais ocorre transmissão (TAUIL, 2002; TEIXEIRA *et* al., 1999; WHO, 1997). Gubler (2002) ressalta que a

eliminação dos recipientes que acumulam água e servem de habitat para as larvas do *A. aegypti* constitui-se no mais efetivo modo de reduzir sua reprodução e dispersão nos centros urbanos.

A opção para combater a dengue continua sendo o controle do *A. aegypti* por meio de inseticidas químicos sintéticos usados nas campanhas nacionais. Contudo, têm surgido problemas significativos como o aparecimento de resistência devido ao uso freqüente, o que compromete o controle e favorece a transmissão de doenças (CARVALHO et al., 2004; LUNA et al., 2004; FUNASA, 2002; CAMPOS & ANDRADE, 2001; OPAS, 1997; WHO, 1992).

No Brasil, até o ano 2000, O Programa Nacional de Controle da Dengue (PNCD) utilizava exclusivamente inseticidas químicos para combater o inseto vetor (FUNASA 2002) destacando-se os organofosforados e piretróides que requerem monitoramento constante (LUNA *et al*., 2004).

O Ministério da Saúde divulgou em 2000, que entre 69 municípios investigados, em 19 deles foi confirmada a resistência de populações naturais de *A. aegypti* ao temephos. Outros 16 municípios apresentaram populações com indícios de resistência em desenvolvimento, incluindo Recife e Jaboatão dos Guararapes, os quais passaram ao status de resistentes no ano seguinte (FUNASA, 2000). A Secretaria de Vigilância em Saúde (SVS) / Ministério da Saúde registrou até a Semana Epidemiológica nº42 (até 21/10/2006), 280.511 casos de Dengue (SVS, 2006).

Formas alternativas de controle de vetores vêm sendo avaliadas, dentre estas merece destaque o controle biológico com *Bacillus thuringiensis israelensis* (*Bti*), com possibilidade de uso integrado com os produtos sintéticos (PRAÇA *et al.,* 2004; POLANCZYK *et al.,* 2003; ANDRADE & MODOLO, 1991). Produtos importados a base de *Bti* foram incorporados ao PNCD em 2001, visando garantir sustentabilidade das ações de combate ao *A. aegypti* nos

municípios onde a resistência ao temephos foi confirmada (BRAGA *et al.,* 2004; VILARINHOS; MONNERAT, 2004).

A atividade larvicida do *Bti* está relacionada com cristais protéicos, geralmente em forma bipiramidal, que incluem 4 principais pró-toxinas de pesos moleculares variados. Quando ingeridos pelas larvas suscetíveis, estes cristais são solubilizados no lúmen intestinal, em pH alcalino, liberando as pró-toxinas, que são clivadas por enzimas proteolíticas em fragmentos menores, tornando-se toxinas ativas. As toxinas interagem especificamente com receptores da membrana apical do intestino médio causando sérios danos ao epitélio, que culminam com a morte da larva (GILL, COWLES; PIETRANTONIO, 1992).

Estudos morfológicos que esclarecem os efeitos tóxicos de extratos de plantas sobre larvas de *A. aegypti*, auxiliam na compreensão das diversas formas de ação desses produtos (ARRUDA *et al.,* 2003a, b; GUSMÃO *et al*., 2002). A demonstração do local de atuação e da forma de ação tem grande importância para a potencialização de seus efeitos e para o desenvolvimento do produto inseticida.

2. Objetivos

2.1. Geral

- Avaliação de atividade hemaglutinante, inibidora de tripsina e larvicida sobre *Aedes aegypti* na água tratada com sementes de *M. oleifera* (MoW).

2.2. Específicos

- Determinação quantitativa de proteína em MoW obtida em quatro diferentes condições.
- Avaliação da atividade hemaglutinante de MoW utilizando eritrócitos de coelho.
- Avaliação da atividade inibidora de tripsina de MoW utilizando o substrato sintético benzoilarginina p-nitranilida.
- Determinação do efeito de MoW sobre o desenvolvimento larval de *A. aegypti*.
- Avaliação da mortalidade larval.
- Comparação morfológica entre as larvas tratadas com MoW e o grupo controle (água destilada).

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4. Artigo a ser submetido ao periódico Journal of Ethnopharmacology

Seeds of *Moringa oleifera* have larvicidal activity on *Aedes aegypti*

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Abstract

Moringa oleifera seeds have been frequently used as coagulant for water treatment in Brazilian Northeast, place with high rates of incidence of dengue fever. This paper reports hemagglutinating activity (HA) in water treated with *M. oleifera* and the possible advantage of the *M. oleifera* water for the dengue vector control. Water (1 L) was treated with 1, 3, 6 and 15 seeds of *M. oleifera* (MoW). Lectin assay used rabbit erythrocytes. MoW was also evaluated by trypsin inhibitory activity using N-α-benzoyl-DL-arginyl-ρ-nitroanilide (BAPNA). Larvicidal bioassay was performed using dengue vector *Aedes aegypti*. Development, mortality and morphological aspects of larvae were analyzed. MoW contains HA and was not able to inhibit trypsin. Reduction of larvae development was observed after 24, 48 and 72 h of incubation with MoW. Fourth instar larvae (L4) were detected in control, MoW_1 and MoW_3 only. Significant ($p<0.0001$) larval mortality was detected in the more active lectin preparation, MoW_{15} (45%). Morphological changes in L4 incubated with MoW1 were demonstrated at light microscope level. The results obtained suggest the application of *M. oleifera* seeds for the control of the dengue vector. Also, the presence of HA in MoW preparations active on *A. aegypti* larvae can be suggest the involvement of lectin in the MoW larvicidal effect.

Key-words: *Aedes aegypti*; larvicidal activity; lectin; *Moringa oleifera*.

1. Introduction

In Brazil the incidence of classical and hemorrhagic dengue is around 500,000 cases annually and, with a mortality index of 5%, the disease causes 24,000 deaths per year (Teixeira *et al*., 1999). *Aedes aegypti* acts as vector and promotes the spreading of four serotypes (DEN-1, DEN-2, DEN-3 e DEN-4) of the dengue virus. Insecticides have been used for vector control, mainly organic compounds such as organochlorides, organophosphates, carbamates and pyrethroids. This method of control has proved to be ineffective and undesirable with development of insect resistance and environmental pollution due to continued accumulation of slowly degradable toxic compounds (Palchick, 1996).

The diversity of plants in Brazil stimulates studies with plant extracts aiming isolation of compounds with insecticide properties. Compounds were evaluated for *A. aegypti* larvicidal (Kiran *et al*., 2006), repellent (Murugan *et al*., 2007) and oviposition-deterrent (Prajapati *et al*., 2005) activities. Larvicidal bioassays were made using *A. aegypti* 3rd instar (Kiran *et al*., 2006), 4th instar (Luna *et al*., 2005) only or comparing the larval development (Murugan *et al*., 2007).

Fifty-one ethanolic extracts of leaf, root, seed and stem from Brazilian medicinal vegetal species were evaluated for effect on *A. aegypti* fourth stage larvae (L4) and six, showed significant larvicidal activity (Omena *et al*., in press). Transmission electron microscopy showed histological alterations in the midgut of *A. aegypti* larvae treated with an aqueous extract of *Derris urucu* and was observed that larval mortality was associated to peritrophic matrix damage (Gusmão *et al.,* 2002). This matrix, constituted of proteins, glycoproteins and proteoglycans (Miller and Lehane, 1993) has important roles in facilitating the digestive process in insect gut (Terra, 2001).

Plant proteins as lectins and trypsin inhibitors with insecticidal activity have been described (Carlini and Grossi-de-Sá, 2002) and studies have been made to evaluate protein potentialities as bioinsecticides as well as to elucidate the action mechanism evolved in this property.

It has been suggested that insecticidal activity of lectin is due to interference on nutrient digestion and absorption by their binding to glycosylated receptors at the surface of stomach epithelial cells (Sauvion *et al*., 2004), to brush border digestive enzymes of larvae causing catalysis inhibition (Fitches and Gatehouse, 1998) or to peritrophic matrix (Harper *et al*., 1998). Lack of correlation between insecticidal effect and lectin monosaccharide specificity has been demonstrated (Carlini and Grossi-de-Sá, 2002).

Plant serine proteinase inhibitors with entomotoxic activity on Coleoptera, Hemiptera and Lepdoptera have been described. The trypsin inhibitor reduce insect growth rate (Carlini and Grossi-de-Sá, 2002) and has been suggested the primary site of inhibitor action is the digestive system of insect. Bees fed with trypsin inhibitor had reduced midgut proteolytic enzyme activity (Sagili *et al*., 2005). It has also been suggested that the interference on larvae development by trypsin inhibition can be due to impair of biomolecule synthesis such as neuropeptides (Haq *et al*., 2004).

The seeds of *Moringa oleifera* (Moringaceae family) contain lectin and antioxidant component (Santos *et al*., 2005). The seeds have been shown to be one of the most effective primary coagulants for water treatment (Katayon *et al*., 2005). In the Brazilian Northeast an emulsion of *M. oleifera* seeds is used for water turbidity removal in regions of difficult access to potable water. The water treated with *Moringa* seed flour is commonly maintained in recipients and drank by people. The aim of this work was to evaluate hemagglutinating **and trypsin**

inhibitor activities (HA) as well as the effect on *A. aegypti* larval development of water treated with *M. oleifera* seeds (MoW).

2. Material and Methods

2.1 Plant collection

Moringa oleifera seeds were collected in Recife city, State of Pernambuco, Brazil Northeast and stored at -20º C with coat. Taxonomic identification was performed and voucher specimens were deposited under number 73,345 (IPA – Instituto de Pesquisas Agropecuárias de Pernambuco).

2.2 *Moringa oleifera* preparations

The water treatment was made according to standard protocol of AS-PTA (Assessoria e Serviços a Projetos em Agricultura Alternativa). The moringa emulsions were obtained by mixture of distilled water (1 L) and macerated seeds (one, three, six or fifteen) without coats by 5 min under agitation. *M. oleifera* preparations were obtained for mixture of each moringa emulsion (1 L) with distilled water (1 L) followed by rapid mixing (1 min), slow mixing (5 min) and sedimentation (15 min, 27°C). The resultant preparations were named *M. oleifera* water (MoW_{1}, MoW_{3}, MoW_{6} and MoW_{15}). The numeric index corresponded to initial quantity of seeds used.

2.3 Determination of protein

The protein concentration was estimated in all samples according to Lowry *et al*. (1951) using bovine serum albumin (31-500μg ml^{-1}) as standard; absorbance at 280 nm was also measured.

2.4 Hemagglutinating activity (HA)

MoW lectin activities were evaluated according to Correia and Coelho (1995) using glutaraldehyde-treated rabbit erythrocytes. The hemagglutinating activity (HA) was obtained by mixing a twofold serial dilution of MoW (50 µl) in 0.15 M NaCl followed by the addition of a 2.5% (v/v) suspension of erythrocytes (50 µl), in microtiter plates (Kartell S. P. A., Italy). Titer was defined as the lowest sample concentration after 45 min which showed hemagglutination.

2.5 Trypsin inhibitory activity

The assay (Kakade *et al*., 1969) was made with bovine trypsin (10 µg ml^{-1} in 0.1 M Tris-HCl, pH 8.0; 20 µl); pre-incubation (37°C, 10 min) of enzyme and MoW preparations (100 µl) was followed by addition (30 µl) of 4 mM N-α-benzoyl-DL-arginyl-ρ-nitroanilide (BAPA) dissolved in dimethyl sulfoxide and diluted with tris buffer. After 30 min at 37°C, 10% (v/v) acetic acid was added (300 µl). The substrate hydrolysis was followed by measurement of absorbance at 405 nm. The inhibitory activity evaluated the remaining hydrolytic activity towards BAPNA.

2.6 *Aedes aegypti* bioassay

Bioassays were conducted employing *A. aegypti* eggs maintained for this purpose at Centro de Vigilância Ambiental, Recife-Pernambuco, Brasil. Larvicidal activity was performed as recommended by the World Health Organization (1996). Bioassay was performed with 1st instar (L1) larvae of *A. aegypti*. Five larvae were placed into disposable plastic cups containing the test MoW (20 ml) or distilled water (20 ml) and incubation by 24, 48 and 72 h at 27°C was

performed. The larval development was observed at the start of experiment (t_0) and 24, 48 and 72 h thereafter. Anatomic-morphologic and morphometric aspects of the abdomen and staining of the previous region (head) of each larval group were compared with a standard larvae of each instar fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate solution, pH 7.2. To every interval of 24 h the number of dead larvae in each sample was counted and the mortality rate was established. Larvae were considered dead when they were unable to reach the surface solution and did not respond to stimulus when the cups were shaken (World Health Organization, 1981). Four replicates for each MoW sample were made using 5 larvae (each).

2.7 Morphologic analysis

Morphological effects of MoW preparations on *A. aegypti* larvae were evaluated. After 72 h of treatment, larvae from control and MoW treated groups were successively fixed with alcohol and xylol in different concentrations (70-100%), for 1 h at 27°C and mounted on glass slides with entelan. Every larval group was examined and photographed using a Leica DM *IL* inverted microscope at a magnification of 100x and Camera Leica DFC 280, respectively, under identical conditions. Leica system software was employed to display the image on the monitor.

2. Results and discussion

Seeds of *M. oleifera* have been used as an alternative purpose to obtain consumption water to people with difficult access to potable water. This paper evaluated if plant defense proteins like lectin and trypsin inhibitor were present in MoW and if the preparations showed insecticidal activity on *A. aegypti*. In Brazil it is high the incidence of dengue fever and the distribution and abundance of dengue vector *A. aegypti* is strongly influenced by the presence of man and level of population poverty (Forattini *et al.,* 1993). The biological cycle of vector occurs in water and since a dengue vaccine is still under development, vector control is the only practical measure to control a dengue epidemic. *M. oleifera* coagulants have been described (Okuda *et al*., 1995; Ghebremichael *et al*., 2006).

MoW did agglutinate rabbit erythrocytes (Table 1). MoW_{15} had the best lectin activity (SHA of 127.4) and highest protein concentration (0.5 mg/ml). Santos *et al.* (2005) described the presence of a water soluble *M. oleifera* lectin (WSMoL) in water obtained after soaking *M. oleifera* intact seeds (7.0 g/l). The authors obtained extracts with high protein concentration (71 to 208 mg/ml) and highest SHA of 0.90 with rabbit erythrocytes. MoW_1 was obtained as recommended (AS-PTA) for water treatment to people consumption. The other preparations were obtained with a higher number of seeds as protein source. In the present paper conditions used promoted higher WSMoL solubilization in comparison to Santos *et al*. (2005); higher SHA was detected in MoW_3 (104), MoW_6 (95), and MoW_{15} (127.4).

Enzyme inhibition assay revealed absence of trypsin inhibitors in moringa preparations. The substrate BAPA was similarly hydrolyzed after incubation of commercial trypsin with Tris-HCl buffer (A405 nm = 0.245) or MOW (A405 nm = 0.244). Thus, trypsin inhibitor was not solubilized from intact seeds (Santos *et al*., 2005).

The bioassay using *A. aegypti* larvae evaluated the development of L1 until L4 by 24, 48 and 72 h of incubation with *M. oleifera* preparations or distilled water (control). Parameters such as anatomic-morphologic aspects, mobility and number of surviving larvae were evaluated. MoW retarded larval development (Table 2). L1 was mainly detected after 24 h with MoW while 50% of evolution for 2nd instar was observed in control group. After 48 h, L3 was mainly detected in control and in lower number in MoW_1 and MoW_3.and not detected in MoW_6 and MoW_{15}. Incubation by 72 h resulted in evolution for 4th instar in control and MoW_1. Larval development was practically stopped in the 3rd instar in the presence of MoW_3, MoW_6 and MoW_{15}.

The protein concentration, HA and larvicidal activities (Table 1) revealed that MoW_{15} was the richest protein preparation, with higher HA than MoW_1, MoW_3 and MoW_6, and significant ($p<0.0001$) mortality rate. The reduction of viable larvae number detected with MoW_3 was not significant ($p=1,000$). MoW_{15} activity in comparison with other Brazilian plants showed potential use to control *A. aegypti*. Ethanolic extracts from *Annona glabra* roots and *Schinus terebinthifolius* stem promoted 100 and 35% larvae mortality, respectively (Mendonça *et al.*, 2005).

MOW results indicated that the larvicidal activity was directly related with amount of solubilized protein. In fact, lectin concentration was associated with effectiveness in reduction of insect oviposition (Sadeghi *et al*., 2006). MoW containing lower HA had no effect on larval viability. Perhaps, a minimal lectin dose was required to cause mortality like in oviposition bioassay. Also, the effect of larvicidal grade associated to larval stage should not be disregarded. *A. aegypti* 1st instar larvae were more susceptible than the other larval instar to plant methanolic extracts (Murugan *et al*., 2007).

It has been suggested that insecticidal activity of lectin can be due to interference on insect nutrient use binding to digestive enzymes as well as to digestive tract. In vitro assay demonstrated that *Bauhinia monandra* leaf lectin influenced the α-amylase activity of insect larvae (Macedo *et al*., 2006). Immunolocalization study (Sauvion *et al*., 2004) revealed that the stomach was the primary target for Con A but at high lectin concentration the whole aphid digestive tract was stained; ConA appeared to cross the intestinal epithelial barrier.

Evaluation using inverted optical microscopy was made with larvae incubated (72 h) with MoW1 (Figure 1A) and control (Figure 1B) groups where L4 was achieved in similar quantity (Table 2). Morphological alterations were detected. MoW1 treated larvae revealed segments with hypertrophic aspects (Figure 1A.1) and increased in midgut volume (Figure 1A.2), aspect not detected in control larvae (Figure 1B.1' and 1B.2'). Light and electron microscopy studies made with Con A insecticidal lectin and *Acyrthosiphon pisum* aphid showed binding of lectin with glycosylated receptors at the surface of digestive tract cells. Con A induced morphological changes, including epithelial cell distention, enlargement and shedding in the midgut region (Sauvion *et al*., 2004).

Plant insecticidal mechanisms involve defense proteins, including serineproteinase inhibitors and lectins (Haq *et al*., 2004; Macedo *et al*., 2006). Trypsin inhibitor was not detected in MoW and the water showed HA. Although the precise mode of insecticidal action of plant lectins is not fully understood it is possible the retarded development and mortality of *A. aegypti* larvae after incubation with MoW of higher SHA. The larval development effect of MoW1, water obtained similarly to people treatment, can be an advantage of *M. oleifera* seed use inducing damage to biological cycle of dengue vector**.**

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FIGURE CAPTIONS

Figure 1 – Photomicrography of anterior midgut region of *A. aegypti* larvae in fourth instar larvae (L4) after 72 h of incubation in MoW1 preparation (A) and distilled water control (B).

Table 1.Characterization of MoW preparations.

Assay	**MoW_1**	**MoW_3**	**MoW_6**	**MoW_{15}**
Protein ($mg.ml^{-1}$)	**-**	**0.077**	**0.168**	**0.500**
Lectin ($titer^{-1}$)	**8**	**8**	**16**	**64**
Larvicide (% of mortality)	**-**	**-**	**-**	**45**

Not detected: (-). Larvicidal value (%) is means ($p<0.0001$) from four replicates determination after 72h.

Table2. Larval instar (%) at different time of incubation with MoW.

Larval instar	**Control**			**MoW_1**			**MoW_3**			**MoW_6**			**MoW_{15}**		
	Incubation time (h)														
	24	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**
L1	50	-	-	65	-	-	75	15	5	75	35	5	80	83	36
L2	50	25	5	35	60	30	25	50	31	25	65	85	20	17	46
L3	-	75	55	-	40	30	-	35	53	-	-	10	-	-	18
L4	-	-	40	-	-	40	-	-	11	-	-	-	-	-	-

Values (%) are means ($p<0.05$) from four replicates determination. Not detected: (-)

5. Conclusões

1. Tratamento da água com sementes de *M. oleifera* (MoW) solubilizou AH. Inibidor de tripsina não foi detectado.
2. A utilização de um maior número de sementes resultou em MoW com maior concentração de proteínas e AH.
3. Incubação de larvas (L1) de *A. aegypti* com MoW revelou que as preparações de moringa interferiram no desenvolvimento larval.
4. Atividade larvicida foi detectada na preparação de maior concentração proteíca e AH (MoW15).
5. Alterações morfológicas foram identificadas por microscopia óptica nas larvas em L4 após 72 horas de incubação em MoW_1.

6. ANEXOS

JOURNAL OF ETHNOPHARMACOLOGY

An Interdisciplinary Journal Devoted to Indigenous Drugs
The Official Journal of the International Society of Ethnopharmacology

Guide for Authors

I. Scope of the journal

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

In recent years the preservation of local knowledge, the promotion of indigenous medical systems in primary health care, and the conservation of biodiversity have become even more of a concern to all scientists working at the interface of social and natural sciences but especially to ethnopharmacologists. Recognizing the sovereign rights of States over their natural resources, ethnopharmacologists are particularly concerned with local people's rights to further use and develop their autochthonous resources.
Accordingly, today's Ethnopharmacological research embraces the multidisciplinary effort in the documentation of indigenous medical knowledge, scientific study of indigenous medicines in order to contribute in the long-run to improved health care in the regions of study, as well as search for pharmacologically unique principles from existing indigenous remedies.

The *Journal of Ethnopharmacology* publishes original articles concerned with the observation and experimental investigation of the biological activities of plant and animal substances used in the traditional medicine of past and present cultures. The journal will particularly welcome interdisciplinary papers with an **ethnopharmacological**, an **ethnobotanical** or an **ethnochemical** approach to the study of indigenous drugs. Reports of **anthropological** and **ethnobotanical** field studies fall within the journal's scope. Studies involving **pharmacological** and **toxicological** mechanisms of action are especially

welcome. **Clinical studies** on efficacy will be considered if contributing to the understanding of specific ethnopharmacological problems.

The journal welcomes review articles in the above mentioned fields especially those highlighting the multi-disciplinary nature of ethnopharmacology. Commentaries are by invitation only. All reviews and commentaries are fully peer-reviewed. Potential authors are strongly encouraged to contact the Reviews Editor jethnopharmacol@pharmacy.ac.uk prior to writing a review. A one-page outline and a short C.V. of the (senior) author should also be included.

THE "RULES OF 5"

The Editors and Editorial Board have developed the "Rules of 5" for publishing in JEP. We have produced five clear criteria that each author needs to think about before submitting a manuscript and setting the whole process of editing and reviewing at work. Click here.

II. Preparation of manuscripts Authors who want to submit a manuscript should consult and peruse carefully recent issues of the journal for format and style. Authors must include the following contact details on the title page of their submitted manuscript: full postal address; fax; e-mail. All manuscripts submitted are subject to peer review. The minimum requirements for a manuscript to qualify for peer review are that it has been prepared by strictly following the format and style of the journal as mentioned, that it is written in good English, and that it is complete. Manuscripts that have not fulfilled these requirements will be returned to the author(s).

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In the covering letter, the author must also declare that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights. See below for further information. The ethnopharmacological importance of the study must also be explained in the cover letter.

Animal and clinical studies - Investigations using experimental animals must state in the Methods section that the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in for example the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or the US guidelines (NIH publication #85-23, revised in 1985). Investigations with human subjects must state in the Methods section that the research followed guidelines of the Declaration

of Helsinki and Tokyo for humans, and was approved by the institutional human experimentation committee or equivalent, and that informed consent was obtained. The Editors will reject papers if there is any doubt about the suitability of the animal or human procedures used.

Biodiversity rights - Each country has its own rights on its biodiversity. Consequently for studying plants one needs to follow the international, national and institutional rules concerning the biodiversity rights.

1. Manuscript types

The *Journal of Ethnopharmacology* will accept the following contributions:

1. Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations
2. Ethnopharmacological communications (formerly Short Communications) - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
3. Letters to the Editors;
4. Reviews - Authors intending to write review articles should consult and send an outline to the Reviews Editor (see inside front cover for contact information) before preparing their manuscripts. The organization and subdivision of review articles can be arranged at the author's discretion. Authors should keep in mind that a good review sets the trend and direction of future research on the subject matter being reviewed. Tables, figures and references are to be arranged in the same way as research articles in the journal. Reviews on topics that address cutting-edge problems are particularly welcome.
5. Book reviews - Books for review should be sent to the Reviews Editor.
6. Commentaries - *invited*, peer-reviewed, critical discussion about crucial aspects of the field but most importantly methodological and conceptual-theoretical developments in the field and should also provide a standard, for example, for pharmacological methods to be used in papers in the *Journal of Ethnopharmacology*. The scientific dialogue differs greatly in the social / cultural and natural sciences, the discussions about the common foundations of the field are ongoing and the papers published should contribute to a transdisciplinary and multidisciplinary discussion. The length should be a maximum of 2-3 printed pages or 2500 words. Please contact the Reviews Editorj.ethnopharmacol@pharmacy.ac.uk with an outline.
7. Conference announcements and news.
8.

2. General procedures

The language of the Journal is English. Manuscripts should be neatly typed, double-spaced throughout, including tables, on pages of uniform size with at least

2,5 cm margins on all sides. Use one font type and size throughout the manuscript. Author(s) should not break or hyphenate words. When using an electronic printer, the right-hand margin should not be justified. Footnotes in text are not permitted. The text of the manuscript must be paginated, the first page being the title page. The manuscript, typed with double spacing and ample margins, should be submitted with a cover letter (containing the declaration that the study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights and a clear explanation of the ethnopharmacological importance of the study) and a completed Author Checklist (click here).

The following format and order of presentation is suggested.

2.1. Title, author(s), address(es)

The title should be no longer than 100 letters, including spaces. Initials or first and middle names followed by last name of the author or authors must be given (**not** last name followed by initials). If there are two or more authors with different addresses, use a superscripted letter (a, b, c etc.), not a number, at the end of the last name of each author to indicate his her corresponding address. The full address of the corresponding author (the way the author wishes to be contacted) should be provided. The corresponding (usually, the senior) author, to whom correspondence and proofs will be sent, must be indicated by an asterisk and footnoted, and in the footnote, his/her the telephone and fax numbers, and e-mail address must be indicated. Address(es) should be underlined or italicised.

2.2. Abstract

The abstract should present a summary of the problem, scientific method, major findings and conclusions, in no more than 200 words and in one paragraph and presented at the beginning of the paper. Unsubstantiated speculation should not be included. Footnotes may not be used. References, if cited, must provide complete publication data.

2.3. Text layout

The text of a research paper should be divided into the following headings: Introduction, Methodology (or Materials and Methods), Results, and Discussion and conclusions. Each heading (and subheading) must be numbered using the convention established in the journal. Acknowledgements should come after Discussion and conclusions and before References; Acknowledgements and References are not to be numbered. Headings must be bold-faced and written in an upper-and-lower case style [not in caps], while subheadings should be underlined or italicised. Tables and figures are to be placed at the end of the text, after References. Authors are required to include: (i) the chemical structure, formula and proprietary name of novel or ill-defined compounds; (ii) the w/w yield of prepared extracts in terms of starting crude material; (iii) complete formulation details of all crude drug mixtures; (iv) the voucher herbarium specimen number of the plant(s) studied in case of less well known plants, cited using the collector and collection

number (e.g., *Doe 123*), and indicating the name of the herbarium institution where it has been deposited. All plant materials must be fully identified as in the following illustration: *Catharanthus roseus* (L.) G. Don f. *albus* Pich. (Apocynaceae) as authenticated by Dr. John Doe, Department of Botany, University of Connecticut.

2.4. Guidelines for Plant and Animal Names

All scientific names (Latin binomials) must be underlined or italicised throughout the text and in the tables and figures. For plant and animal species, full or complete scientific names, genus-species and the correct authority citation, must be used, *when that name appears for the first time in text*. The authority citation may be dropped in subsequent mention of that name throughout the text. The family name must follow the scientific name in parentheses when the name appears for the first time in the text. Full scientific names and the family name of the subject plants/animals must be used in the Abstract. Synonyms must be indicated in parentheses and preceded by the word "syn." followed by a colon. Authors are advised to consult the International Plant Name Index (IPNI) (http://www.ipni.org and W3Tropicos (http://www.mobot.org) web-based databases to determine the correct spelling of full plant scientific names. Generic names may be abbreviated (e.g., C. *roseus* for *Catharanthus roseus*), provided such practice does not lead to confusion; generic names, however, must not be abbreviated when the name appears for the first time in the text. Specific epithets must never be abbreviated; thus, the use of *Catharanthus r*. is not allowed.

2.5. Keywords

Authors are requested to assign 3-6 keywords to the manuscript, preferably taken from Index Medicus or Excerpta Medica Index, for abstracting and indexing purposes. These keywords should be typed at the end of the Abstract. Each keyword should start with a capital letter and be separated from each other by a semi-colon.

2.6. Tables, illustrations and graphs Tables should be on separate sheets, one table per sheet, and should bear a short descriptive title. Footnotes in tables should be indicated by consecutive superscript letters, not numbers.

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