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**TESE DE DOUTORADO**

# ATIVIDADE INSETICIDA DE TORTA DE SEMENTES DE *Moringa oleifera*

**ANA PATRÍCIA SILVA DE OLIVEIRA**

**ORIENTADORA: Profª Dra. PATRÍCIA MARIA GUEDES PAIVA**
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**ANA PATRÍCIA SILVA DE OLIVEIRA**

# ATIVIDADE INSETICIDA DE TORTA DE SEMENTES DE *Moringa oleifera*

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

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*Aos meus pais pelos exemplos de honestidade*
*e por todo amor que recebo.*

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*“Um passo a frente e você não está mais no mesmo lugar”*
*(Chico Science)*

## RESUMO

Sementes de *Moringa oleifera* contêm lectina solúvel em água (WSMoL) que possui atividades larvicida, ovicida e estimulante de oviposição sobre *Aedes aegypti* e atividade termiticida contra *Nasutitermes corniger*. Esta tese descreve a purificação e caracterização da lectina $WSMoL_C$ a partir de extrato aquoso de torta de moringa, co-produto obtido após extração do óleo, seus efeitos na sobrevivência de larvas, ovos e na oviposição de *A. aegypti*, a atividade inseticida sobre *N. corniger* e os efeitos de extratos, WSMoL e $WSMoL_C$ na sobrevivência e nutrição de insetos *Sitophilus zeamais*. $WSMoL_C$ foi extraída em água destilada, fracionamento com sulfato de amônio e cromatografia em coluna de quitina. A lectina foi avaliada quanto à especificidade a carboidratos, por SDS-PAGE seguida de espectrometria de massas e estabilidade estrutural. Atividade larvicida contra *A. aegypti* foi determinada incubando larvas L4 por 24 h com o extrato ou $WSMoL_C$. A resistência da lectina à degradação por proteases digestivas foi determinada. Atividade ovicida de $WSMoL_C$ foi avaliada incubando ovos com a lectina por 72 h e o efeito na oviposição foi realizado com fêmeas grávidas postas por 14 h em gaiolas contendo recipientes com água destilada e outro com lectina. Atividade termiticida foi avaliada em placas de Petri contendo discos de papel de filtro impregnados com $WSMoL_C$. Para avaliar a atividade inseticida contra *S. zeamais*, os insetos foram incubados por 7 dias em placas de Petri contendo dieta suplementada com extrato de sementes íntegras, extrato da torta, WSMoL ou $WSMoL_C$. A sobrevivência e parâmetros nutricionais foram avaliados. A interação de $WSMoL_C$ com enzimas digestivas dos insetos também foi avaliada. $WSMoL_C$ é uma proteína formada por α-hélices e apresentou duas bandas polipeptídicas (15 e 20 kDa) em SDS-PAGE. Análise por espectrometria de massas revelou similaridades com outras proteínas de sementes de moringa. A atividade hemaglutinante de $WSMoL_C$ foi inibida por frutose, glicose, manose, *N*-acetilglucosamina e galactose. A lectina manteve sua estrutura secundária e terciária quando incubada em até 4 M de uréia, aquecida até 65ºC ou submetida a pressões menores que 2,7 kbar. Com relação aos efeitos sobre *A. aegypti*, a lectina apresentou atividades larvicida ($CL_{50}$ de 0,89 mg/mL) e ovicida ($CE_{50}$ de 0,13 mg/mL) e efeito estimulante de oviposição (0,1 mg/mL). $WSMoL_C$ mostrou-se resistente à degradação por proteases larvais e teve efeito estimulatório nas atividades de α-amilase e proteases totais e inibitório na atividade de tripsina. A lectina apresentou atividade termiticida para soldados ($CL_{50}$ de 0,629 mg/mL, 7 dias) e operários ($CL_{50}$ de 0,361 mg/mL, 6 dias) de *N. corniger*, e inibiu a atividade de endoglucanase, exoglucanase e α-amilase em extratos de intestino dos cupins. O extrato da torta não causou mortalidade significativa de *S. zeamais*, diferentemente do extrato obtido a partir das sementes íntegras ($CL_{50}$ de 214,6 mg/g) que apresentou ação deterrente moderada a forte. WSMoL e $WSMoL_C$ causaram mortalidade significativa de *S. zeamais*, porém apenas $WSMoL_C$ foi deterrente. Ambas lectinas apresentaram efeito estimulatório sobre a atividade de tripsina. Como conclusão, lectina com valor biotecnológico como agente inseticida pode ser recuperada a partir da torta de moringa.



**Palavras-chaves:** Lectina. Semente oleaginosa. Aproveitamento de resíduos. *Aedes aegypti*. *Sitophilus zeamais*. *Nasutitermes corniger*.

## ABSTRACT

*Moringa oleifera* seeds contain a water-soluble lectin (WSMoL) which showed larvicidal, ovicidal and oviposition-stimulant activities on *Aedes aegypti* and termiticidal activity against *Nasutitermes corniger*. This thesis describes the purification and characterization of lectin $WSMoL_C$ from aqueous extract of moringa seed cake, by-product resulting after oil extraction, your effects on the survival of larvae, eggs and on the oviposition of *A. aegypti*, the insecticidal activity on *N. corniger* and the effects of extracts, WSMoL and $WSMoL_C$ on survival and nutrition of *Sitophilus zeamais* adults. $WSMoL_C$ was extracted in distilled water, fractionation with ammonium sulphate and chitin chromatography column. The lectin was evaluated for carbohydrate specificity, by SDS-PAGE followed by mass spectrometry, and structural stability. Larvicidal activity against *A. aegypti* was determined incubating L4 larvae for 24 h with extract or $WSMoL_C$. The resistance of the lectin to degradation by digestive proteases was determined. $WSMoL_C$ ovicidal activity was evaluated incubating eggs with lectin for 72 h and the effect on oviposition was performed with females were put in cages for 14 h with a vessel containing distilled water and other with lectin. Termiticidal activity was evaluated in Petri plates containing filter paper discs impregnated with $WSMoL_C$. To assess insecticidal activity against *S. zeamais*, the insects were incubated for 7 days in Petri dishes containing diet supplemented with extract from whole seeds, cake extract, WSMoL or $WSMoL_C$. Survival rates and nutritional parameters were evaluated. The interaction of $WSMoL_C$ with digestive enzymes of the insects was also evaluated. $WSMoL_C$ is a protein formed by α-helices and showed two polypeptide bands (15 and 20kDa) on SDS-PAGE. Analysis by mass spectrometry revealed similarities with other proteins from moringa seeds. The hemagglutinating activity of $WSMoL_C$ was inhibited by fructose, glucose, mannose, N-acetylglucosamine and galactose. The lectin maintained its secondary and tertiary structures when incubated up to 4 M of urea, heated to 65°C or subjected to pressures lower than 2.7 kbar. With respect to effects on *A. aegypti*, the lectin presented larvicidal ($LC_{50}$ of 0.89 mg/mL) and ovicidal ($EC_{50}$ of 0.13 mg/mL) activities and oviposition-stimulant effect (0.1 mg/mL). $WSMoL_C$ was resistant to degradation by larval proteases and had a stimulatory effect on α-amylase and total protease activities larvae and inhibitory on trypsin activity. The lectin presented termiticidal activity on soldiers ($LC_{50}$ of 0.629 mg/mL, 7 days) and workers ($LC_{50}$ of 0.361 mg/mL, 6 days) of *N. corniger* and inhibited endoglucanase, exoglucanase and α-amylase activities from termite gut extracts. The extract from seed cake did not cause significant mortality of *S. zeamais*, unlike the extract obtained from the whole seeds ($LC_{50}$ 214.6 mg/g) that showed moderate to strong deterrent action. WSMoL and $WSMoL_C$ caused significant mortality of *S. zeamais*, only $WSMoL_C$ exerted deterrent action. Both lectins showed stimulatory effect on trypsin activity. In conclusion, a lectin with biotechnological value as an insecticide agent can be recovered from the moringa seed cake.



**Keywords:** Lectin. Oleaginous seed. Waste recovery. *Aedes aegypti*. *Sitophilus zeamais*. *Nasutitermes corniger*.

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### ARTIGO 1



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## LISTA DE ABREVIATURAS

AE Extrato aquoso de sementes de *Moringa oleifera*

$AE_C$ Extrato aquoso de torta de sementes de *Moringa oleifera*

AH (HA) Atividade hemaglutinante

AHE (SHA) Atividade hemaglutinante específica

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

bis-ANS bis-(8-anilinonaftaleno-1-sulfonato)

CD Dicroísmo circular (do inglês: circular dichroism)

$CE_{50}$ ($EC_{50}$) Concentração letal necessária para impdir a eclosão de 50% dos ovos dos insetos

$CE_{90}$ ($EC_{90}$) Concentração letal necessária para impdir a eclosão de 90% dos ovos dos insetos

$CE_{99}$ ($EC_{99}$) Concentração letal necessária para impdir a eclosão de 99% dos ovos dos insetos

$CL_{50}$ ($LC_{50}$) Concentração letal necessária para matar 50% dos insetos

$CL_{90}$ ($LC_{90}$) Concentração letal necessária para matar 90% dos insetos

$CL_{99}$ ($LC_{99}$) Concentração letal necessária para matar 99% dos insetos

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

DLS Espalhamento de luz dinâmico (do inglês: dynamic light scattering)

DNS Ácido 3,5-dinitrosalicílico (do inglês 3,5-dinitrosalycilic acid)

FDI Índice de deterrência alimentar (do inglês feeding-deterrence index)

MoL Lectina de *M. oleifera* (do inglês: *Moringa oleifera* lectin)

MO2.1 Proteína floculante de *Moringa oleifera*

Mo-cbp3 Proteína ligadora de quitina de *Moringa oleifera*

PAGE Eletroforese em gel de poliacrilamida (do inglês polyacrylamide gel electrophoresis)

Rh Raio hidrodinâmico

SDS Sulfato sódico de dodecila (do inglês dodecyl sodium sulphate)

Tris Trishidroximetilaminometano

WSMoL Lectina solúvel em água de *Moringa oleifera* (do inglês: Water soluble *Moringa oleifera* lectin)

$WSMoL_C$ Lectina solúvel em água de torta de *Moringa oleifera* (do inglês: Water soluble *Moringa oleifera* lectin cake)

**SUMÁRIO**

# 1 INTRODUÇÃO

Insetos constituem uma classe diversificada e amplamente distribuída de animais invertebrados. Alguns insetos são importantes vetores de doenças em todo o mundo como, por exemplo, o mosquito *Aedes aegypti*, conhecido por transmitir os vírus causadores de doenças como a dengue, zika e chikungunya. Outras espécies de insetos apresentam importância econômica, pois são considerados pragas e podem trazer prejuízos ao ambiente em que se instalam. São exemplos os cupins *Nasutitermes corniger*, importante praga urbana e o *Sitophilus zeamais*, conhecido como uma praga agrícola (COSTA-LEONARDO, 2002; BETA & ISAAK, 2016; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2016a).

Inseticidas sintéticos têm sido extensivamente usados no controle de insetos-praga ou no controle de vetores de importância sanitária. Contudo, os insetos possuem capacidade de se adaptar às mudanças das condições do ambiente em que vivem e, quando expostos às adversidades, alguns indivíduos de uma população podem desenvolver mecanismos adaptativos que permitem sua sobrevivência. O uso contínuo de inseticidas químicos tem provocado o aparecimento de populações resistentes de diferentes espécies (LUMJUAN *et al.*, 2011; RAJENDRAN & SINGH, 2016). Devido ao surgimento dessas populações e aos efeitos deletérios no ambiente e na saúde humana causados por inseticidas sintéticos, a busca por inseticidas naturais, tais como os produtos de origem vegetal, tem atraído cada vez mais atenção dos pesquisadores, uma vez que eles são mais seletivos e biodegradáveis (PAIVA *et al.*, 2011a; ANTWI & REDDY, 2015; LOZOWICKA *et al.*, 2016).

Lectinas são proteínas que se ligam específica e reversivelmente a carboidratos. Lectinas vegetais podem ser encontradas em diferentes tecidos como folhas, flores e sementes. A atividade inseticida descrita para lectinas deve-se ao fato das mesmas apresentarem resistência a proteólise pelas enzimas digestivas. Dessa forma, quando ingerida

pelos insetos, a lectina pode se ligar aos gliconjugados e quitina presentes na matriz peritrófica bem como às enzimas presentes no trato intestinal dos insetos interferindo na atividade catalítica e desencadeando a perturbação da organização intestinal dos insetos induzindo-os à morte (PAIVA *et al*., 2011b; NAPOLEÃO *et al*., 2013; PAIVA *et al*., 2013; LIRA *et al*., 2015; AGRA-NETO *et al*, 2014).

*Moringa oleifera* é uma árvore que contêm diversos compostos bioativos e suas sementes contêm as lectinas WSMoL (do inglês: water-soluble *M. oleifera* lectin), cMoL (do inglês: coagulant *M. oleifera* lectin) e MoL (do inglês: *M. oleifera* lectin) (KATRE *et al*., 2008; COELHO *et al*., 2009; SANTOS *et al*., 2009; ROLIM *et al*., 2011). WSMoL foi tóxica contra *N. corniger* na concentração de 1,5 mg/mL (PAIVA *et al*., 2011b), bem como matou larvas de *A. aegypti* em estágio L4 (0,197 mg/mL), impediu a eclosão de ovos estocados ($EC_{50}$ de 0,1 mg/mL) e apresentou efeito estimulante sobre a oviposição pelas fêmeas dessa espécie de mosquito (COELHO *et al*., 2009, SANTOS *et al*., 2012; SANTOS *et al*., 2014). Ainda, WSMoL estimulou a atividade de enzimas digestivas (protease, tripsina e α-amilase) e inibiu a atividade da enzima detoxificadora β-esterase em larvas de *A. aegypti* (COELHO *et al*., 2009, AGRA-NETO *et al*., 2014).

Além de proteínas, sementes de moringa apresentam elevado teor de óleo, o qual é de grande valor para a indústria alimentícia, de cosméticos e produção de biocombustível. A torta de sementes é o co-produto obtido após a remoção do óleo e é rica em componentes que podem ser isolados e aproveitados (TORRES *et al*., 2012; RAWDKUEN *et al*., 2016).

Pouco se conhece a respeito da recuperação de compostos bioativos a partir de torta de sementes de *M. oleifera*. Esse trabalho teve como objetivo verificar a possibilidade de recuperação de uma lectina, denominada $WSMoL_C$ (do inglês: *water-soluble M. oleifera lectin from cake*) a partir desse co-produto e avaliar então preparações contendo essa lectina quanto à atividade inseticida contra *A. aegypti*, *N. corniger* e *S. zeamais.* A exploração da

torta de moringa para recuperação de lectina inseticida agrega valor biotecnológico à torta e a toda cadeia de produção do óleo de moringa.

# 2 FUNDAMENTAÇÃO TEÓRICA

## 2.1 *Aedes aegypti*

O *Aedes aegypti* (Ordem Diptera, Família Culicidae) é um mosquito originário da África, sendo considerado hoje cosmopolita, com maior ocorrência em regiões tropicais e subtropicais (CONSOLI & OLIVEIRA, 1994; FERNÁNDEZ-SALAS *et al*., 2015). Durante o seu ciclo de vida, o *A. aegypti* passa por quatro distintas fases: ovo, larva, pupa e adulto (Figura 1). As três primeiras fases são chamadas de estágios imaturos e ocorrem na água. Seus criadouros preferenciais são os recipientes artificiais, tanto os abandonados pelo homem a céu aberto e preenchidos pelas águas das chuvas, como aqueles utilizados para armazenar água para uso doméstico (CONSOLI & OLIVEIRA, 1994; SIMOY *et al*., 2015).

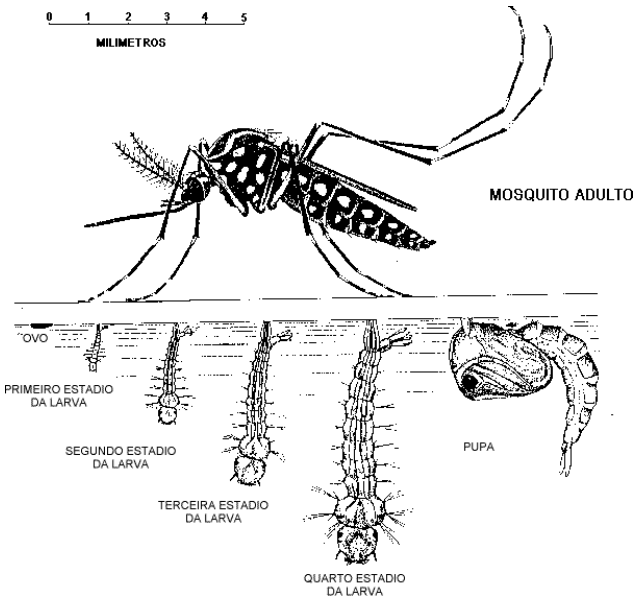


**Figura 1 -** O ciclo biológico do *Aedes aegypti*

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

Os ovos do *A. aegypti* são alongados com leve curvatura dorso-ventral e afilados nas extremidades, apresentando cor pálida no momento da oviposição e tornando-se, logo após,

escuros e brilhantes (Figura 2A). Apresentam alta resistência a condições de desidratação, por períodos de até 1 ano sem perder a viabilidade, podendo ser transportados para outros locais. Sua eclosão é estimulada pelo contato com a água, o que ocorre geralmente no local da oviposição. O tempo entre a oviposição e a eclosão dos ovos dura, em média, de 2 a 5 dias dependendo das condições do ambiente (FORATTINI, 1962; SIMOY *et al*., 2015).

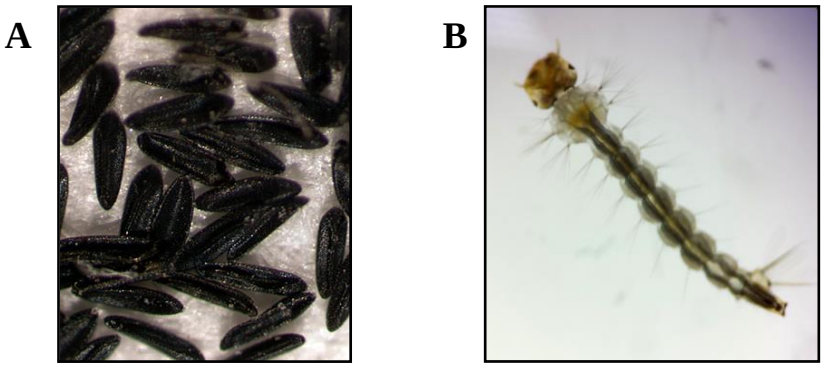


**Figura 2 -** Aspecto dos ovos após a oviposição (A) e larva de *Aedes aegypti* no estágio L4 (B).

Fotos: (A) http://www.ioc.fiocruz.br/dengue/galeria/galeria.html,
(B) a autora

As larvas são compostas por cabeça, tórax e abdômen e possuem quatro estágios evolutivos, L1, L2, L3 e L4, sendo o último destes (Figura 2B) o mais longo. Embora aquáticas, as larvas necessitam do oxigênio do ar, emergindo para a superfície da água para respirar, onde ficam em posição quase vertical. A alimentação é essencialmente composta da matéria orgânica tais como algas, bactérias e esporos de fungos presentes em seus habitats (FORATTINI, 1962; CONSOLI & OLIVEIRA, 1994). A duração da fase larval depende da temperatura, da disponibilidade de alimento e da densidade das larvas no criadouro. Em condições ótimas, o período entre a eclosão e a pupação não excede cinco dias. Contudo, com alterações de temperatura e escassez de alimento, o 4º estágio larval pode prolongar-se por

várias semanas, antes de sua transformação em pupa (MOHAMMED & CHADEE, 2011; BARRERA, 2016).

As pupas sucedem o último estágio larval, têm aspecto de vírgula e estão quase sempre paradas em contato com a superfície da água. Diferentemente das larvas, as pupas não se alimentam nem excretam seus metabólitos na água, podendo esse estágio durar até mais de uma semana dependendo das condições de temperatura. Nesse estágio, começam a se distinguir as diferenças entre os sexos, sendo as fêmeas maiores que os machos (GRECH *et al*., 2015; SIMOY *et al*., 2015).

Os mosquitos (Figura 3) possuem seu corpo dividido em cabeça, tórax e abdômen e representam a fase reprodutiva da espécie. A distinção entre os sexos pode ser facilmente observada, pois os machos são menores que as fêmeas e apresentam antena com flagelos mais longos e numerosos dando aspecto plumoso característico. Ambos os sexos se alimentam de carboidratos das plantas, mas somente as fêmeas possuem hábitos hematófagos, uma vez que elas precisam das proteínas do sangue para completar a ovogênese. As fêmeas mostram preferência pelo homem para realizar o repasto sanguíneo (BRIEGEL, 1990; CONSOLI & OLIVEIRA, 1994; SIMOY *et al*., 2015). A seleção do local de oviposição é influenciada pela intensidade luminosa, coloração apresentada pelo criadouro em potencial, temperatura, grau de salinidade, presença de vegetais e microrganismos (ou os seus produtos metabólicos) e substâncias liberadas por formas imaturas do mosquito (FORATTINI, 1962). O mosquito *A. aegypti* é considerado o principal vetor responsável pela transmissão do vírus causadores da dengue, da febre chikungunya e da febre zika (BLACK *et al*., 2002; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2015).

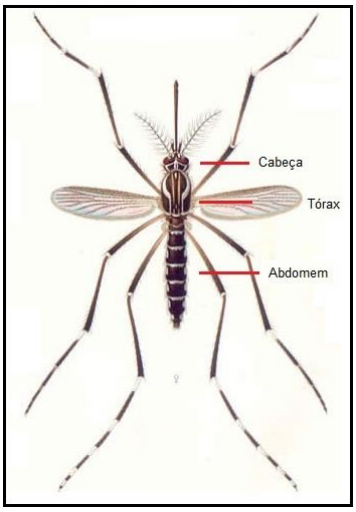


**Figura 3 -** Mosquito fêmea de *Aedes aegypti*.

Fonte: http://www.combateadengue.com.br/mosquito-da-dengue/

A dengue é uma arbovirose transmitida aos seres humanos através da picada da fêmea infectada com o vírus. Após um repasto de sangue infectado, o mosquito está apto a transmitir o vírus, depois de 8 a 12 dias de incubação extrínseca. Uma vez infectado, o mosquito é capaz de transmitir o vírus pelo resto de sua vida (CONSOLI & OLIVEIRA, 1994). A dengue é considerada uma das mais importantes arboviroses e estima-se que, a cada ano, cerca de 390 milhões de indivíduos são infectados em todo o mundo. O vírus da dengue é constiuído por quatro sorotipos diferentes (DENV-1, DENV-2, DENV-3 e DENV-4) e a doença pode se apresentar desde a forma assintomática até formas graves e letais como a febre hemorrágica da dengue e a síndrome de choque da dengue (BHATT *et al*., 2013; ANGEL *et al*., 2015; BENELLI & MELHORN, 2016).

O vírus zika (ZIKV) é um arbovírus causador de grandes epidemias já identificadas em países da África, Ásia, Oceania e Pacífico e, mais recentemente, no Brasil. A sintomatologia da febre do zika é semelhante àquela da dengue e inclui febre baixa, erupções cutâneas, artralgia e hiperemia conjuntival. A picada pelo mosquito infectado é a principal forma de transmissão da doença. A zika é considerada atualmente um problema de saúde

pública e requer grande atenção, especialmente pela associação da infecção em gestantes com surgimento de alterações do sistema nervoso dos fetos, tais como microcefalia (IOOS *et al*., 2014; MINISTÉRIO DA SAÚDE, 2016; MLAKAR *et al*., 2016; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2016b).

Também transmitido pelo *A. aegypti*, o vírus chikungunya (CHIKV) possui ampla distribuição mundial. O vírus já foi identificado em mais de 60 países da Ásia, África, Europa e nas Américas. A doença se apresenta com sintomas de febre alta e dor nas articulações que podem persistir por longos períodos (semanas, meses ou anos) (MADARIAGA *et al*., 2015; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2016a).

A atual situação epidemiológica da dengue, zika e chikungunya em todo o mundo tem levado a estudos que visam o desenvolvimento de diferentes estratégias de controle por diversos setores públicos. Existem várias pesquisas direcionadas para o desenvolvimento de vacinas eficientes e técnicas de diagnóstico específico e precoce dessas viroses (IOOS *et al*., 2014; VILLAR *et al*., 2015; SINAWANG *et al*., 2016; VARGHESE, *et al*., 2016). No entanto, intervenções neste nível ainda esbarram em diferentes aspectos como a rápida disseminação concomitante desses vírus e seu potencial epidêmico, bem como o alto custo e demora na produção de vacinas. Tudo isso direciona a prevenção e o controle das três viroses para medidas de controle do vetor *A. aegypti* (PINTO JÚNIOR *et al*., 2015; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2015, 2016a, 2016b, 2016c). O controle do *A. aegypti* pode ser direcionado para ovos, larvas ou insetos adultos (SANTOS *et al*., 2012; PAIVA *et al*., 2013).

O combate ao *A. aegypti* se faz através da remoção dos seus criadouros bem como aplicação de inseticidas apropriados em recipientes de armazenamento de água e aplicação de inseticidas no espaço por pulverização durante surtos (ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2015). Os inseticidas químicos organofosforados, piretróides, carbamatos e

organoclorados constituem as quatro classes mais comuns de inseticidas usados no controle do mosquito adulto. Os organofosforados são utilizados também como larvicidas. Porém, esses inseticidas sintéticos são não-seletivos e podem ser prejudiciais para outros organismos no ambiente além de desencadear, a longo prazo, o surgimento de populações resistentes (BRAGA & VALLE, 2007; PAIVA *et al*., 2011a; ORGANIZAÇÃO MUNDIAL DE SAÚDE, 2012; CONDE *et al*., 2015; SMITH *et al.*, 2016). Como recomendação para diminuir os riscos do agravamento da resistência nas populações de vetores, tem sido feita a utilização rotativa dos inseticidas bem como a integração com medidas de controle do vetor através de controle biológico e manipulação genética (CONSOLI & OLIVEIRA, 1994; TAHIR *et al*., 2015; ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2016c).

## 2.2 Cupins do gênero *Nasutitermes*

Cupins ou térmitas pertencem à ordem Isoptera e constituem um grupo de insetos eusociais que vivem em colônias e, em sua maioria, em ambientes naturais atuando como decompositores de matéria orgânica (CONSTANTINO & SCHLEMMERMEYER, 2000). As colônias são compostas por três castas morfologicamente distintas responsáveis por diferentes tarefas no ninho (Figura 4). Soldados e operários são os representantes da casta estéril, ou seja, possuem sistema reprodutor reduzido e sem função. Operários são os responsáveis pela busca de alimento, construção do ninho, cuidado como os indivíduos imaturos e alimentação das outras castas. Os soldados desempenham a função de defesa e recebem a alimentação dos operários. A casta fértil compreende o rei e a rainha, responsáveis pela reprodução na colônia e também recebem o alimento estomacal dos operários (CONSTANTINO & SCHLEMMERMEYER, 2000; GALLO *et al*., 2002).

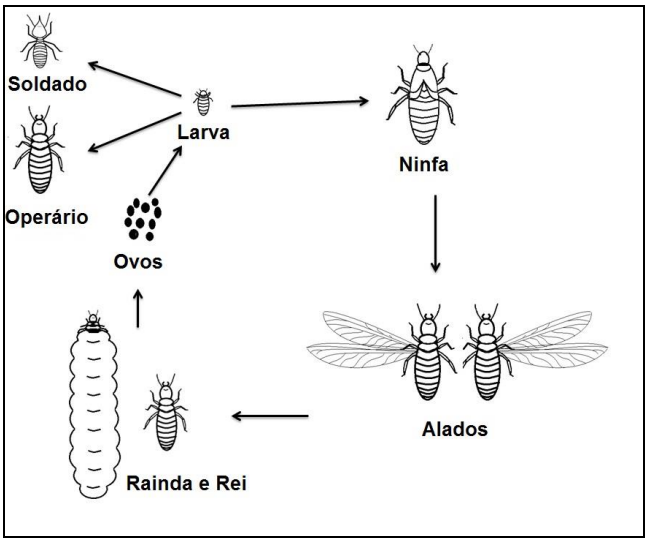


**Figura 4** - Ciclo de vida e distintas castas dos térmitas.

Fonte: Lima *et al.*, 2015.

Os cupins se alimentam de celulose, sendo a fonte dessa celulose variável. A maioria das espécies podem se alimentar de madeira em diferentes estágios de decomposição bem como de uma grande variedade de matéria orgânica como insetos, gramíneas, fungos e líquens. Apesar de se alimentarem de alimentos considerados de baixo valor nutricional, os cupins conseguem extrair grande parte dos nutrientes devido a mecanismos digestivos que conservam e reciclam esses nutrientes (LIMA & COSTA-LEONARDO, 2007; FERREIRA *et al*., 2011).

As diferentes espécies já descritas de cupins estão distribuídas em sete famílias: Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Serritermitidae, Rhinotermitidae e Termitidae. Os cupins podem ser divididos em dois grupos de acordo com a dependência de protozoários simbiônticos que auxiliam na degradação da celulose. Cupins pertencentes à família Termitidae compreendem mais de 70% de todas as espécies de Isoptera e são denominados cupins superiores, pois não dependem de protozoários simbiônticos e as demais famílias são classificadas como cupins inferiores. Os ninhos dos cupins superiores possuem colônias bem populosas com castas bem definidas. A degradação da celulose, no

caso dos cupins superiores, ocorre por ação de enzimas e bactérias simbiontes (GRASSÉ, 1986; COSTA-LEONARDO, 2002; GALLO *et al*., 2002).

Apesar da maior parte dos cupins desempenharem um papel importante no ecossitema, participando na decomposição e ciclagem de nutrientes e na humificação e mineralização de recursos celulósicos, algumas espécies são consideradas pragas e causam prejuízos econômicos em zonas rurais e urbanas, danificando estruturas feitas pelo homem e produtos agrícolas (CONSTANTINO & SCHLEMMERMEYER, 2000; COSTA-LEONARDO, 2002; HU *et al*., 2015).

Entre os cupins tidos como pragas, inserem-se os cupins do gênero *Nasutitermes* (Isoptera: Termitidae), dominantes das Américas Central e do Sul e caracterizados pela presença de soldados com cabeça modificada para defesa com um longo tubo frontal (“nasuto”) que ejeta substância tóxica ou repelente. Os ninhos dos insetos do gênero *Nasutitermes* podem ser encontrados sobre as árvores, mas também dentro das residências, em edículas e em pontos altos das edificações como forros e sótãos, coabitando com o homem (CONSTANTINO & SCHLEMMERMEYER, 2000).

Apesar de serem poucas as espécies de cupins consideradas pragas, estas provocam enormes prejuízos na agricultura e no ambiente urbano. Infestações por cupins do gênero *Nasutitermes* em áreas urbanas destacam-se por comprometerem a integridade da construção e sustentação de telhados. Espécies como *N. corniger* já foram reportadas em áreas urbanas, especialmente em madeiras de edificações (BANDEIRA *et al*., 1989; ZORZENON, 2002; ALBUQUERQUE *et al*., 2012).

Estratégias de prevenção e controle de cupins se baseiam tradicionalmente na utilização de inseticidas organofosforados, carbamatos e piretroides. No entanto, o uso de inseticidas químicos tem alto poder residual podendo causar danos à saúde do homem e ao

ambiente, o que tem levado à procura por novos produtos mais seguros (BERTI FILHO *et al*., 1993; WANG *et al*., 2012; ZHANG et al., 2015).

## 2.3 *Sitophilus zeamais*

A espécie *S. zeamais* (Ordem Coleoptera, Família Curculionidae) corresponde a um inseto conhecido popularmente como gorgulho do milho e apresenta grande importância econômica em regiões tropicais e subtropicais, pois infesta uma grande variedade de cultivos como milho, arroz, maçã, pêssegos e uvas (BOTTON *et al*., 2005; NOOMHORM *et al*., 2013; SULEIMAN *et al*., 2015).

O inseto *S. zeamais* apresenta elevado potencial de multiplicação. A postura dos ovos pela fêmea é feita no grão onde a larva completa o seu desenvolvimento, passa ao estágio de pupa até a emergência do adulto no seu interior (Figura 5A). As fêmeas vivem em média 140 dias dos quais 104 dias correspondem ao período de oviposição. O número médio de ovos por fêmea é de 282. O período de incubação oscila entre 3 e 6 dias e o ciclo de ovo até a emergência de adultos é de 34 dias. As larvas apresentam coloração amarelo-clara e cabeça marrom-escura, e as pupas são brancas. Adultos de *S. zeamais* (Figura 5B) possuem de 2 a 3,5 mm de comprimento, cor castanho-escura e manchas mais claras nas asas anteriores (élitros). Sua cabeça é projetada para frente e o rostro curvado. Insetos machos têm o rostro mais curto e grosso enquanto nas fêmeas é mais longo e afilado (BOTTON *et al*., 2005; LORINI *et al*., 2010).

O gorgulho do milho apresenta infestação cruzada, ou seja, podem infestar sementes no campo e também no armazenamento, onde penetram profundamente na massa de grãos. Na busca de alimentos que garantam energia para a oviposição, os insetos infestam grãos ricos em carboidratos tais como trigo, arroz, cevada e aveia. Uma vez dentro dos grãos, os insetos

fazem a postura dos ovos que se desenvolvem em pupa e se transformam em novos adultos (BOTTON *et al*., 2005; LORINI *et al*., 2010).

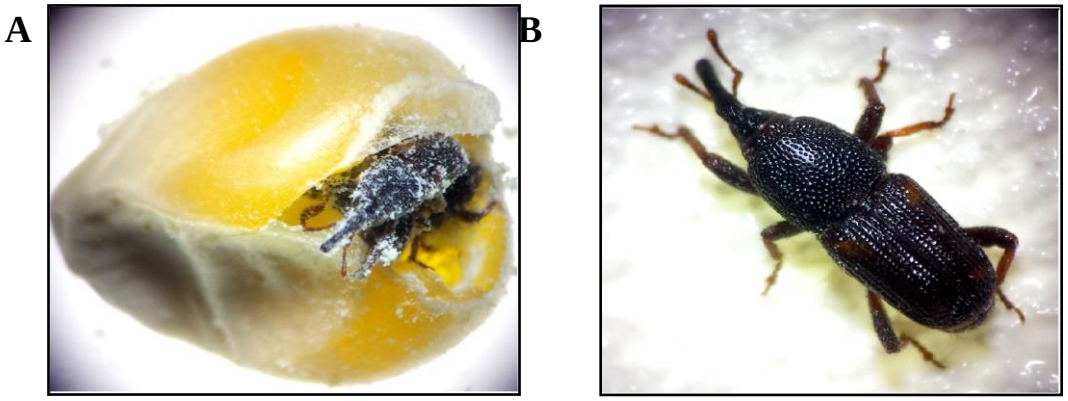


**Figura 5** - (A) Inseto se desenvolvendo dentro do grão de milho e (B) inseto adulto de *Sitophilus zeamais.*

Fonte: A autora

Classificados como uma praga primária, os *S. zeamais* são considerados um dos insetos mais prejudiciais pelo fato de perfurarem e penetrarem os grãos para completar o seu desenvolvimento e assim provocar infestações generalizadas (LORINI, 2014). A praga de gorgulho do milho tem sido cada vez mais comum, devido à expansão de produção de grãos no setor de agrícola e aumento da sua estocagem (NEUMANN *et al*., 2010; BETA & ISAAK, 2016; FLEURAT-LESSARD, 2016).

O *S. zeamais* infesta diversas variedades de milho e como conseqüências ao ataque existem perda de peso do grão, perda do poder germinativo, perda do valor nutritivo, redução do padrão comercial e contaminação da massa do grão de milho (SANTOS, 2006; SULEIMAN *et al*., 2015). O controle de *S. zeamais* é realizado através do uso de inseticidas químicos por meio de técnicas de fumigação e pulverização. No entanto, o uso prolongado e excessivo desses compostos favorece a proliferação de indivíduos resistentes (MONDAL & KHALEQUZZAMAN 2010; NAPOLEÃO *et al*., 2015; LIANG *et al*., 2016)

## 2.4 Bioinseticidas

Inseticidas químicos são tradicionalmente usados no controle de vetores e de insetos praga. Porém, estudos apontam que muitos desses inseticidas não são eficazes o suficiente para erradicar a população de insetos alvo, sendo necessário o uso de grandes quantidades e concentrações que podem trazer prejuízos para o ambiente, para a saúde humana e ainda desencadear o surgimento de espécies de insetos resistentes (LUMJUAN *et al.*, 2011; PAIVA *et al.*, 2013; YU *et al*., 2015; ZHANG et al., 2015).

Na busca por estratégias que visem à diminuição do desenvolvimento de resistência pelos insetos, produtos inseticidas naturais são preferíveis para o controle, uma vez que, em geral, são biodegradáveis e não exercem ou possuem menores efeitos deletérios sobre organismos não-alvo (SANTOS *et al*., 2012; PAIVA *et al*., 2013; TENNYSON *et al*., 2015; REDDY & ANTWI, 2016).

Produtos de origem vegetal com ação inseticida têm sido pesquisados em muitas partes do mundo principalmente em decorrência do aumento no número de casos de doenças transmitidas por insetos vetores. Com relação ao *A. aegypti*, extrato metanólico de casca de *Millettia usaramensis* foi larvicida ($CL_{50}$ de 50,8 µg/mL) após 48 horas (BOSIRE *et al.*, 2014) e inibidor de protease isolado de sementes de *Adenanthera pavonina* reduziu a sobrevivência, peso e atividade de proteinases de intestino de larvas (SASAKI *et al*., 2015).

Os insetos-praga também são alvos de componentes originados de preparações de plantas ou seus componentes isolados. Por exemplo, β-asarona identificada em rizoma de *Acorus calamus* foi tóxica contra cupins *Coptotermes curvignathus* (ADFA *et al*., 2015). Mortalidade de mais de 53% de *S. zeamais* foi observada após uma hora de contato com óleo de mamona (*Ricinus communis* L.) (WALE & ASSEGIE, 2015). Napoleão *et al*. (2013)

concluíram que dieta contendo extrato ou lectina de folhas de *Myracrodruon urundeuva* causam alteração nos parâmetros nutricionais de adultos de *S. zeamais*. Perda de biomassa e mortalidade foram detectados para adultos do gorgulho do milho submetidos à dieta com ar-turmerona isolada do óleo essencial do rizoma *Curcuma longa* (Zingiberaceae) (TAVARES *et al*., 2013). Lira *et al*. (2015) verificaram que o óleo essencial de inflorescências de *Alpinia purpurata* foi tóxico por fumigação para adultos de *S. zeamais* e, quando ingerido, reduziu o ganho de biomassa e a eficiência de conversão do alimento.

## 2.5 Lectinas

Lectinas são proteínas com a capacidade de reconhecer e de se ligar a carboidratos de forma específica e reversível, por pelo menos um domínio (PAIVA *et al*., 2011a). Elas podem ser encontradas em diversas formas de vida tais como plantas, animais e fungos e estão aassociadas a uma variedade de processos fisiológicos e patológicos que envolvem a ligação específica entre carboidrato e lectina (FU *et al*., 2011; JOHN *et al*., 2013; VARROT *et al*., 2013; SUN *et al*., 2015). Em plantas, as lectinas estão presentes em diferentes tecidos tais como folhas, flores e sementes (LORIS *et al*., 1998; VANDENBORRE *et al*., 2011; BOSE et al., 2016).

As lectinas representam um diversificado grupo de proteínas no que diz respeito ao tamanho, composição e estrutura. Elas podem se apresentar em múltiplas formas moleculares e podem ser obtidas com alto rendimento e grau de pureza através de técnicas convencionais de purificação de proteínas (KENNEDY *et al.*, 1995; PAIVA *et al.*, 2011a).

A detecção da presença das lectinas pode ser realizada através de um ensaio de atividade hemaglutinante (Figura 6A) que consiste na realização de diluição seriada da lectina seguida de incubação com eritrócitos. Já a sua especificidade se dá por ensaio da inibição da

atividade hemaglutinante (Figura 6B), utilizando diferentes monossacarídeos, oligossacarídeos ou glicoproteínas (KENNEDY *et al*., 1995; JOHN *et al*., 2013).

As lectinas exibem uma variedade de propriedades biológicas devido a sua especificidade de ligação a carboidratos. Lectinas de plantas são utilizadas em estudos de eventos de reconhecimento a glicoconjugados importantes em vários processos biológicos, tais como infecções, bem como exibem atividades antiviral, antibacteriana, antifúngica e inseticida (SANTOS *et al*., 2014; SILVA *et al*., 2014; CARVALHO *et al*., 2015; GUO *et al*., 2015). A caracterização físico-química é importante para o entendimento da relação estrutura-função das lectinas e envolve a definição da integridade e estabilidade da conformação secundária e terciária frente a diferentes agentes desnaturantes. Para os estudos de estabilidade conformacional das lectinas existem as técnicas espectroscópicas de dicroísmo cicular e fluorescência (KHAN *et al.*, 2013; GROSSI *et al.*, 2016).

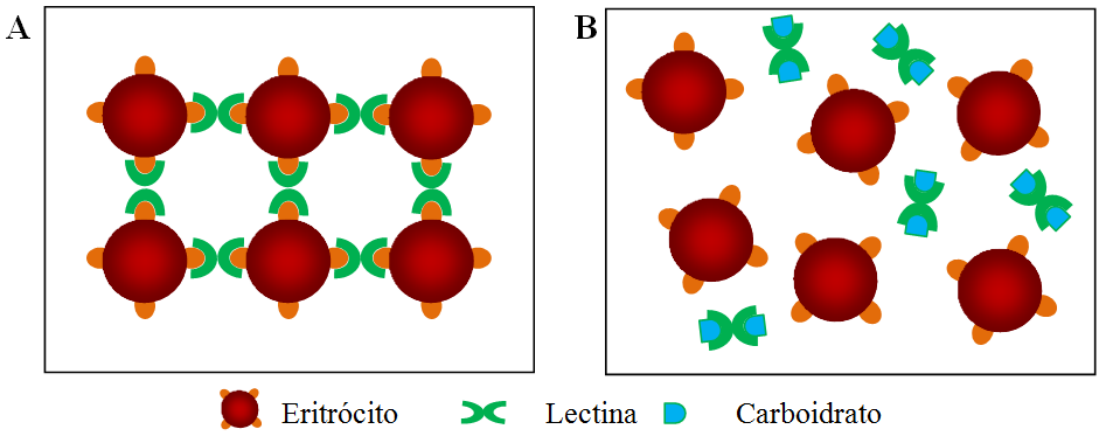


**Figura 6 -** (A) Representação esquemática da aglutinação de eritrócitos por lectinas; (B) Representação esquemática da inibição de aglutinação de eritrócitos por lectinas em presença de carboidrato.

Fonte: A autora

A atividade inseticida atribuída às lectinas envolve a sua ligação a estruturas presentes no intestino do inseto, tais como a glicoconjugados na superfície das células epiteliais e à quitina encontrada na matriz peritrófica. A toxicidade das lectinas para insetos pode estar

envolvida com a resistência à hidrólise por proteases digestivas do intestino do inseto e com perturbações da organização do trato intestinal, morte de microrganismos simbiontes presentes no trato digestivo dos insetos e, ainda, ligação a proteínas glicosiladas presentes no intestino, tais como enzimas, interferindo na atividade catalítica (MACEDO *et al*., 2007; COELHO *et al*., 2009; CACCIA *et al*., 2012; NAPOLEÃO *et al.*, 2013; PAIVA *et al.*, 2013; AGRA-NETO *et al*, 2014).

## 2.6 *Moringa oleifera* Lam.

*Moringa oleifera* (Família Moringaceae) é uma planta nativa da Índia e amplamente cultivada nos trópicos de todo o mundo (AMAGLO *et al.*, 2010). Árvore de crescimento rápido, já era utilizada no mundo antigo por romanos, gregos e egípcios com os mais diversos fins (PRABHU *et al.*, 2011). A excelente capacidade em se adaptar a solos pobres e climas áridos torna a moringa uma alternativa ao consumo como fonte de proteínas de alta qualidade, de óleo e de compostos antioxidantes (FERREIRA *et al.*, 2008; NOUMAN *et al*., 2016).

As sementes (Figura 7) de *M. oleifera* são amplamente utilizadas como coagulante natural para tratamento de água, principalmente em países em desenvolvimento onde os recursos hídricos adequados não estão disponíveis. As sementes contêm proteínas coagulantes que podem ser facilmente extraídas em água e promovem a precipitação de partículas em suspensão, levando à diminuição da turbidez da água (GHEBREMICHAEL *et al*., 2005; BHUPTAWAT *et al*., 2007; SANTOS *et al.*, 2009; BAPTISTA *et al*., 2015; DEZFOOLI *et al*., 2016).

**Figura 7 -** Sementes de *M. oleifera*.

Fonte: Autora

As sementes possuem alto teor de óleo, o qual é altamente resistente à oxidação e amplamente utilizado para o consumo humano, na indústria de cosméticos, como biolubrificantes e como matéria-prima para produção de biodiesel (FERREIRA *et al.*, 2008; RASHID *et al*., 2008; MOFIJUR *et al*., 2014; SILVA *et al*., 2015).

Preparações contendo moringa foram descritas como bioativas contra diversos insetos. Extrato aquoso de sementes de moringa apresentou atividades larvicida e ovicida contra *A. aegypti* (COELHO *et al.*, 2009; SANTOS *et al.*, 2012). Larvas e pupas de *Anopheles stephensis*, vetor da malária, morreram após exposição a extrato metanólico das sementes de moringa (PRABHU *et al.*, 2011). Além das sementes, outros tecidos da moringa também constituem potenciais fontes de inseticidas, tendo o extrato da casca apresentado ação larvicida e adulticida contra os mosquitos vetores da filariose *Culex gelidus* e *Culex quinquefasciatus* (KAMARAJ & RAHUMAN, 2010). Extrato aquoso de flores de moringa contendo inibidor de protease induziu a mortalidade de larvas de *A. aegypti* no primeiro (L1), segundo (L2), terceiro (L3) e quarto (L4) estágios, sendo capaz de inibir a atividade de tripsina de larvas L4 em 98,6% após 5 horas de exposição (PONTUAL *et al.*, 2012).

### *2.6.1 Lectinas de sementes M. oleifera*

Três lectinas foram isoladas a partir das sementes de *M. oleifera*, denominadas WSMoL (do inglês water-soluble *M. oleifera* lectin), cMoL (do inglês coagulant *M. oleifera* lectin) e MoL (do inglês *M. oleifera* lectin), as quais diferem quanto a características estruturais e físico-químicas, bem como propriedades biológicas. WSMoL é uma proteína acídica ligadora de quitina enquanto cMoL e MoL são proteínas básicas isoladas por cromatografia em gel de guar e DEAE-celulose/Sephadex, respectivamente (KATRE *et al.*, 2008; COELHO *et al.*, 2009; SANTOS *et al.*, 2009; ROLIM *et al.*, 2011).

As três lectinas diferem quanto à massa molecular e às atividades biológicas. A cMoL possui propriedades coagulantes e apresenta peso molecular de aproximadamente 26,5 kDa (SANTOS *et al*., 2009). WSMoL possui elevada solubilidade em água e pode adquirir formas multiméricas de 60 kDa (SANTOS *et al*., 2005; MOURA *et al.*, 2016). MoL é uma proteína dimérica com peso molecular de 14 kDa com atividade hemaglutinante e especificidade de ligação a açúcares complexos (KATRE *et al.*, 2008).

A cMoL causou retardo no desenvolvimento de larvas de *Anagasta kuehniella* (OLIVEIRA *et al*., 2011) e exerceu fraca atividade termiticida contra *N. corniger*, promovendo mortalidade somente em altas concentrações (PAIVA *et al.*, 2011b). Essa lectina foi capaz de afetar parâmetros homeostáticos, prolongando o tempo de coagulação sanguínea, o tempo de tromboplastina parcial ativada e o tempo de protrombina (LUZ et al., 2013).

WSMoL foi termiticida contra *N. corniger* na concentração de 1,5 mg/mL (PAIVA *et al.*, 2011b). A elevada solubilidade em água de WSMoL estimulou a investigação do seu efeito sobre as larvas de *A. aegypti*. WSMoL matou larvas L4 de *A. aegypti* ($CL_{50}$ de 0,197 mg/mL) e impediu a eclosão de ovos estocados ($EC_{50}$ de 0,1 mg/mL) por promover a morte do embrião ainda dentro do ovo. Adicionalmente, não foram visualizados embriões dentro dos

ovos depositados pelas fêmeas na solução de WSMoL, indicando que a lectina prejudicou o desenvolvimento embrionário. WSMoL apresentou atividade estimulante de oviposição pelas fêmeas do mosquito o que, provavelmente, está associado à percepção da presença da lectina na água através de sensores gustatórios (COELHO *et al*., 2009; SANTOS *et al*., 2012; SANTOS *et al*., 2014). Estudo do efeito de WSMoL sobre a atividade de enzimas de larvas L4 revelou que a lectina é capaz de estimular a atividade de enzimas digestivas (protease, tripsina e α-amilase) e inibir a atividade da enzima detoxificadora β-esterase (COELHO *et al*., 2009; AGRA-NETO *et al*., 2014). A toxicidade de WSMoL sobre *A. aegypti* e *N. corniger*, dois insetos pertencentes a ordens diferentes, torna essa lectina um importante composto com potencial bioinseticida.

## 2.7 Torta de sementes

Durante o processo de extração do óleo de sementes obtém-se um co-produto denominado de torta. As tortas de diversas oleaginosas, incluindo a moringa, têm sido utilizadas na alimentação animal, sendo também ricas em componentes minoritários que podem ser fracionados e aproveitados (KOOTSTRA *et al*., 2011; TORRES *et al*., 2012; RAWDKUEN *et al*., 2016). Contudo, ainda existem poucos estudos a respeito da recuperação de compostos bioativos de sementes a partir da torta.

Triterpenos e saponinas obtidos a partir de torta de *Camellia oleifera* e *Camelina sativa* apresentaram atividade antitumoral (ZONG *et al*., 2015) e compostos fenólicos possuem atividade antioxidante (TERPINC *et al*., 2012). Torta de moringa foi eficiente na remoção da turbidez da água (SANTOS *et al*., 2011) e carvão ativado preparado a partir da torta de *Jatropha curcas* L. foi eficiente na remoção de azul de metileno em soluções aquosas (KURNIAWAN & ISMADJI, 2011).

As sementes de *M. oleifera* são ricas em proteínas, incluindo lectinas com diversas atividades biológicas e potenciais biotecnológicos. A exploração da torta de moringa para isolamento de uma lectina agrega valor biotecnológico a cadeia de produção do óleo de moringa.

# 3 OBJETIVOS

## 3.1 GERAL

Purificar e caracterizar uma lectina a partir da torta de sementes de *M. oleifera* (denominada $WSMoL_C$), bem como investigar o potencial inseticida de preparações contendo $WSMoL_C$ contra *A. aegypti, N. corniger* e *S. zeamais*.

## 3.2 ESPECÍFICOS

### 3.2.1 Purificação e caracterização da lectina

- Isolar $WSMoL_C$ a partir de extrato aquoso de torta de sementes de *M. oleifera* obtido após remoção do óleo com solvente orgânico.
- Caracterizar $WSMoL_C$ quanto à especificidade de ligação a carboidratos.
- Determinar massa molecular, composição em subunidades e similaridade de $WSMoL_C$ com proteínas presentes em bancos de dados.
- Caracterizar $WSMoL_C$ quanto ao tipo de estrutura secundária predominante.
- Investigar a estabilidade conformacional de $WSMoL_C$ frente à agentes desnaturantes (uréia, temperatura e pressão).

### 3.2.2 Atividade inseticida contra *A. aegypti*

- Avaliar a atividade larvicida de extrato de torta e de $WSMoL_C$.
- Investigar a lectina quanto às atividades ovicida e efeito sobre a oviposição.

- Investigar a resistência de $WSMoL_C$ à digestão pelas proteases do intestino das larvas.
- Obter extratos de intestino de larvas e determinar as atividades de proteases, tripsina e α-amilase.
- Determinar o efeito *in vitro* de $WSMoL_C$ sobre as atividades enzimáticas detectadas.
- Investigar expressão de proteases em larvas tratadas com $WSMoL_C$.

3.2.3 Atividade inseticida contra *N. corniger*

- Avaliar o efeito termiticida de $WSMoL_C$ sobre operários e soldados de *N. corniger*.
- Obter extratos de intestino de soldados e operários e determinar as atividades de proteases, tripsina, α-amilase e celulases (endoglucanase e exoglucanase).
- Determinar o efeito *in vitro* de $WSMoL_C$ sobre as atividades enzimáticas detectadas.

3.2.4 Atividade inseticida contra *S. zeamais*

- Investigar a toxicidade por ingestão de extrato de sementes íntegras, extrato de torta, WSMoL e $WSMoL_C$ sobre *Sitophilus zeamais.*
- Determinar o índice de deterrência alimentar de extratos e lectinas e seus efeitos nos parâmetros nutricionais: taxa de crescimento relativo, taxa de consumo relativo e eficiência de conversão do alimento ingerido.
- Obter extratos de intestino de *S. zeamais* e determinar as atividades de tripsina, α-amilase e endoglucanase.
- Determinar os efeitos *in vitro* de WSMoL e $WSMoL_C$ sobre as atividades enzimáticas detectadas.

**4 ARTIGO 1**

# Biotechnological value of *Moringa oleifera* seed cake as source of insecticidal lectin against *Aedes aegypti*

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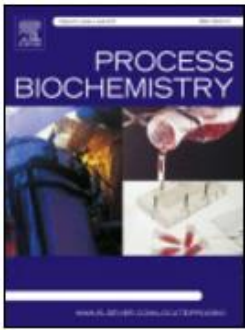


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# Biotechnological value of *Moringa oleifera* seed cake as source of insecticidal lectin against *Aedes aegypti*

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ABSTRACT

*Moringa oleifera* seed cake, which is the coproduct of oil extraction, may be a source of lectin. This study reports the purification of a lectin from *M. oleifera* cake ($WSMoL_C$) and the determination of its effect on larvae, eggs and oviposition of *Aedes aegypti*. $WSMoL_C$ was isolated (purification fold: 3573) by treatment of water extract with ammonium sulphate (60%) and chromatography of lectin-rich fraction on chitin column. In gel trypsin, digestion followed by mass spectrometry revealed the similarity of $WSMoL_C$ peptides with other *M. oleifera* seed proteins. $WSMoL_C$ exerted deleterious effects on larvae ($LC_{50}$: 0.89 mg/mL) and eggs ($EC_{50}$: 0.14 mg/mL) and served as an oviposition-stimulant at the concentration of 0.1 mg/mL. Larvicidal activity may be linked to lectin resistance to digestion and stimulatory effect on the activities of protease and α-amylase at larvae gut. Furthermore, the larvae did not start to express different enzymes in response to lectin effect. This study adds value to *M. oleifera* seed cake as a source of insecticidal lectin with high potential to be used in control of *A. aegypti* because, at the same time that it stimulates the oviposition by females, it could kill the eggs or larvae that may arise from them.



## 1. Introduction

Three lectins (carbohydrate-binding proteins) were isolated from *Moringa oleifera* seeds: WSMoL (water-soluble *M. oleifera* lectin), MoL (*M. oleifera* lectin) and cMoL (coagulant *M. oleifera* lectin) [1–3]. WSMoL is an anionic protein isolated by chromatography on chitin column, while cMoL and MoL are cationic proteins isolated by guar gel and DEAE-cellulose/Sephadex chromatographies, respectively. These lectins differ in molecular mass, structural arrangement and biological properties [1–5].

*Aedes aegypti* is the vector of dengue, chikungunya and zika, which are arboviral infections considered important public health problems in the world, particularly in tropical and temperate countries. The incidence of dengue is estimated in a mean of 390 million infections per year, although this value is probably underestimated; in addition, the cases of more severe forms of the disease, such as dengue hemorrhagic fever and dengue shock syndrome, have increased [6,7]. Chikungunya fever is characterized by abrupt fever accompanied by joint pain that may persist for weeks, months, or even years. This disease has been reported in Africa, Asia, Europe and Americas and has been associated with severe and chronic morbidity and cases of death [8]. Outbreaks of zika virus infection have been recently reported in South America, Central America and the Caribbean [7,9,10]. In Brazil, the *A. aegypti* is the main vector and zika virus infections during pregnancy have been associated with fetal microcephaly [7,11]. Vaccines against the four serotypes of dengue virus are being developed [12,13] but currently there are no vaccines for the control of these three diseases, which makes the reduction of vector population as the main strategy for minimizing their incidence.

Chemical control of mosquito populations has been performed using synthetic insecticides such as organochlorides, organophosphates, pyrethroids, and carbamates. However, these compounds are usually unselective and persistent in the environment as well as their indiscriminate use have resulted in the emergence of resistant mosquito populations [14]. In this scenario, plant compounds stand out as biodegradable and more selective agents active against mosquito larvae, eggs, pupae, and adults as well as are considered as important alternatives for use in rotation programs aiming to prevent the emergence of resistant insects [14–17].

WSMoL was insecticidal for *A. aegypti* by killing fourth-stage larvae ($LC_{50}$ of 0.197 mg/mL), impairing the hatching of mature eggs ($EC_{50}$ of 0.1 mg/mL) and preventing the development of embryos in eggs laid by females directly on solution containing it [2,18]. In addition, WSMoL showed oviposition-stimulant activity under semi-field conditions [19].

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*M. oleifera* seeds possess a high content of nonvolatile oil (27–40%), which is used for human consumption and in cosmetic industry, as well as considered an acceptable raw material for biodiesel production [20,21]. Seed cakes correspond to the residual product generated after oil extraction. The recovery of bioactive compounds from *Camellia oleifera* and *Camelina sativa* seed cakes, such as triterpenes and saponins, with antitumor activity [22] and phenolic compounds with antioxidant activity [23] has been reported. *Azadirachta indica* seed cake has been reported as source of insecticidal compounds against mosquito species [24]. In spite of this, the potential of seed cakes in yielding molecules of biotechnological value has not received great attention. The richness of *M. oleifera* seeds in bioactive compounds makes the cake obtained from them a potential source of components that can be extracted, fractionated and used with biotechnological purposes.

The exploitation of cakes is important for the oleaginous market, because aggregates value for this coproduct and the recovery of lectin from *M. oleifera* seed cake constitutes an interesting strategy for utilization of this material. In this sense, this study reports the isolation and characterization of a water-soluble lectin from *M. oleifera* seed cake (the so-called $WSMoL_C$). In addition, $WSMoL_C$ was evaluated for insecticidal properties against *A. aegypti*.

## 2. Materials and methods

### 2.1. Plant material

*M. oleifera* seeds were collected at the campus of the *Universidade Federal de Pernambuco*, Recife City, State of Pernambuco, Brazil, with authorization (38690) of the *Instituto Chico Mendes de Conservação da Biodiversidade* from the Brazilian Ministry of Environment. A voucher specimen (number 73,345) was archived at the herbarium Dárdano de Andrade Lima (*Instituto Agronômico de Pernambuco*, Recife, Brazil). The seeds were powdered using a blender and stored at −20 °C. The seed powder (100 g) was put on a filter paper cartridge and placed in a Soxhlet extractor. The solvent, *n*-hexane, was added to remove oil after 6 h and the resulting material corresponded to the *M. oleifera* seed cake.

### 2.2. Protein concentration and haemagglutinating activity

Protein concentration was determined according to Lowry et al. [25] using bovine serum albumin (31.25–500 μg/mL; Sigma-Aldrich, USA) as standard. The haemagglutinating activity was assessed using microtitre plates (Kartell S.P.A., Italy). The sample (50 μL) was serially twofold diluted in 0.15 M NaCl before incubation with a suspension (2.5% v/v) of rabbit erythrocytes fixed with glutaraldehyde. The collection of rabbit erythrocytes was approved by Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* (process 23076.033782/2015-70). The number of haemagglutinating activity units (HAUs) was determined as the reciprocal of the highest dilution of sample promoting full erythrocyte agglutination. Specific haemagglutinating activity (HAU/mg) was defined as the ratio of the titre to protein amount (mg).

### 2.3. Isolation of water-soluble *M. oleifera* lectin from cake ($WSMoL_C$)

Seed cake was suspended (10%, w/v) in distilled water or 0.15 M NaCl and homogenized for 16 h at 25 °C using a magnetic stirrer. After filtration through gauze and centrifugation (9000*g*, 15 min, 4 °C), the collected supernatants corresponded to the water and saline extracts, which were evaluated for protein concentration and haemagglutinating activity. Water extract, with specific haemagglutinating activity (525 HAU/mg) higher than that of saline extract (290 HAU/mg), was selected for $WSMoL_C$ isolation.

Soluble proteins in water extract were precipitated with ammonium sulphate at a saturation of 60% according to Green and Hughes [26]. After centrifugation (9000*g*, 15 min, 4 °C), the precipitate was collected and dialysed (3.5-kDa cutoff membrane) against distilled water (4 h) and 0.15 M NaCl (4 h) at 25 °C. The dialysed fraction with haemagglutinating activity (lectin-rich fraction) was loaded (52 mg of proteins) onto a chitin column (7.5 × 1.5 cm) equilibrated (flow rate 0.3 mL/min) with 0.15 M NaCl. After washing with the equilibrating solution, the proteins adsorbed on the column were eluted with 1.0 M acetic acid and the fractions with haemagglutinating activity were pooled ($WSMoL_C$) and dialysed (3.5-kDa cutoff membrane) against distilled water (4 h) at 25 °C for eluent elimination.

Haemagglutinating inhibitory activity assays were performed by previous incubation (45 min) of $WSMoL_C$ with monosaccharide solutions (200 mM) D-(−)-fructose, D-(+)-galactose, D-(+)-glucose, D-(+)-mannose and *N*-acetyl-D-glucosamine).

### 2.4. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of $WSMoL_C$ (heated for 15 min) was performed on 12% (w/v) gel in the presence of sodium dodecyl sulphate (SDS-PAGE) according to the method proposed by Laemmli [27]. Polypeptides and molecular mass markers (bovine serum albumin, 66,000 Da; carbonic anhydrase, 29,000 Da; trypsin inhibitor, 20,000 Da; cytochrome c, 12,500 Da; aprotinin, 6500 Da; from Sigma-Aldrich, USA) were stained with Coomassie Brilliant Blue (0.02%, v/v) in 10% (v/v) acetic acid. SDS-PAGE of WSMoL, isolated as described by Coelho et al. [2] was also performed at the same conditions.

### 2.5. Mass spectrometry

Polypeptide bands of $WSMoL_C$ detected in SDS-PAGE were cut from the gel and separately washed thrice with 25 mM ammonium bicarbonate/acetonitrile 1:1 (v/v). Then, the material was covered with 100% acetonitrile, which was removed after a few seconds and replaced by 25 mM ammonium bicarbonate. The material was incubated for 45 min at 56 °C. Then, a digestion buffer containing 10 mg/mL trypsin in 25 mM ammonium bicarbonate was added to the gel, incubated for 45 min, and then replaced by 50 mM ammonium bicarbonate without the enzyme. The samples were then incubated at 37 °C overnight. Then, the peptides were extracted from the gel pieces by incubation in 5% trifluoroacetic acid/acetonitrile 1:1 (v/v) for 30 min and the remaining supernatant was removed and stored. The extraction step was repeated twice and all fractions were pooled and lyophilized to a final volume of 10 μL. The peptides were analysed by electrospray ionization–quadrupole–time of flight (ESI-QUAD-TOF). The spectra of peptides were compared with others present in the NCBInr database using MASCOT software (http://www.matrixscience.com).

### 2.6. Larvicidal assay

The insect colony that was used in the experiment has been maintained in the *Laboratório de Ecologia Química* at the *Universidade Federal de Pernambuco* since 2003. The rearing room was kept at 25–27 °C and 75–80% humidity, with a 12:12 light–dark photope-

riod. *A. aegypti* eggs were hatched in distilled water at a temperature range of 25–27 °C and cat food (Whiskas®) was offered to larvae. When reaching the early fourth stage (L4), larvae were collected and used in the bioassays. The larvicidal activity was evaluated using an adaptation of the World Health Organization [28] method described by Navarro et al. [29]. Stock solutions of water extract (19.5 mg/mL of protein), lectin-rich fraction (25.9 mg/mL of protein) and $WSMoL_C$ (1.4 mg/mL of protein) were diluted in distilled water to provide test solutions in the protein concentration ranges of 1.5–8.0, 0.2–2.0 and 0.2–1.0 mg/mL, respectively. The final volume of each larvicidal assay was 20 mL of test solution or negative control (distilled water or 0.15 M NaCl) and contained 20–25 larvae in the early fourth stage. Mortality rate (%) was determined after 24 h of incubation at 25–27 °C and 12:12 (light–dark) photoperiod. Larvae that were unable to reach the surface solution or did not respond to mechanical stimulus were considered dead [28]. The experiment was performed in triplicate.

### 2.7. Preparation of larval gut extracts

Groups of 50 *A. aegypti* L4 were immobilized at 4 °C for 10 min and their guts were removed using an 8-mm-long, 0.3-mm-thick needle (BD Ultra-Fine II from Becton, Dickinson and Company, NJ, USA) under a stereomicroscope. The dissected guts were homogenized with 1 mL of acetate buffer (0.1 M sodium acetate pH 5.5 containing 0.02 M $CaCl_2$) or Tris buffer (0.1 M Tris-HCl pH 8.0) using a 2-mL tissue grinder. After centrifugation at 9000*g* and 4 °C for 15 min, the supernatants (gut extracts) were collected and evaluated for protein concentration.

### 2.8. Evaluation of $WSMoL_C$ resistance to digestion by gut extract

$WSMoL_C$ (1 mL; 300 μg) was incubated at 37 °C with larval gut extract in Tris buffer (1 mL; 3 mg of protein) for 1, 12, 24, 48 and 72 h. After the end of each incubation period, the digestion was stopped by immersing the tubes in boiling water for 2 min. Aliquots (20 μL) of each mixture were submitted to SDS-PAGE using a 12.5% (w/v) gel. Controls containing only the lectin (replacing the gut extract by Tris buffer) or gut extract (replacing the lectin by distilled water) were also performed. The polypeptide bands were stained with 0.02% (v/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid.

### 2.9. Effects of lectin on larval digestive enzymes

The effects of $WSMoL_C$ on enzyme activities were evaluated by incubation (30 min at 28 °C) of the gut extract with the lectin before the determination of enzyme activities. The results were compared with those from assays performed without lectin (100% activity control). Control assays were also performed with only $WSMoL_C$.

α-Amylase activity was determined according to the method proposed by Bernfeld [30]. The gut extract in acetate buffer (50 μL; 80 μg of protein) plus 50 μL of $WSMoL_C$ (12.5–50 μg) or distilled water (100% activity control) were incubated at 50 °C for 10 min with 400 μL of a 1% (w/v) soluble starch suspension prepared in acetate buffer. The reaction was stopped by adding 500 μL of dinitrosalicylic acid. Then, the assays were heated at 100 °C in boiling water for 6 min, immediately cooled on ice for 15 min, and evaluated for absorbance at 540 nm. The amount of reducing sugars was determined using a standard curve of glucose reaction with DNS ($Y = 0.4153X - 0.0026$, where Y is the absorbance at 540 nm and X is the glucose concentration in mg/mL). One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of glucose per minute.

In order to determine protease activity, the sample containing a mixture of the gut extract in Tris buffer (100 μL; 780 μg of protein) and 100 μL of $WSMoL_C$ (12.5–100 μg) or distilled water (100% activity control) was mixed with 300 μL of 0.1 M sodium phosphate (pH 7.5) containing 50 μL of 0.6% (w/v) azocasein. Next, 100 μL of Triton X-100 (0.1%, v/v) was added and the mixture was incubated at 37 °C for 3 h. The reaction was stopped by adding 200 μL of 10% (v/v) trichloroacetic acid, and the assay was incubated at 4 °C for 30 min. The reaction mixture was then centrifuged (9000*g* for 10 min) and the absorbance of the supernatant at 366 nm was read. One unit of protease activity was defined as the amount of enzyme required to increase absorbance by 0.01 [31].

The trypsin activity was determined by incubating (30 min, 37 °C) the gut extract in Tris buffer (15 μL; 78 μg of protein) plus 15 μL of $WSMoL_C$ (12.5–200 μg) or distilled water (100% activity control) with 8 mM *N*-benzoyl-DL-arginyl-*p*-nitroanilide (BApNA; 5 μL) in 0.1 M Tris-HCl (pH 8.0, 165 μL). Trypsin activity was followed by the measurement of absorbance at 405 nm. One unit of trypsin activity is defined as the amount of enzyme that hydrolyses 1 μmol of BApNA per minute.

### 2.10. Zymography for proteases

Larvicidal assay was performed as described in Section 2.6 using $WSMoL_C$ at the $LC_{50}$ and distilled water as negative control. After 24 h, 20 live larvae from each treatment were collected and gut extracts in Tris buffer were prepared as described in Section 2.7. Protease activity from the gut extracts of larvae from control and $WSMoL_C$ treatments was evaluated in gel according to the method described by Garcia-Carreño et al. [32]. The gut extract sample (100 μg of protein) was submitted to polaycrylamide gel electrophoresis at 4 °C, using a 12% (w/v) SDS-PAGE gel. After run, the gel was immersed in 2.5% (v/v) Triton X-100 in 0.1 M Tris-HCl (pH 8.0) to remove SDS and incubated with 3% (w/v) casein in 0.1 M Tris-HCl (pH 8.0) for 30 min at 4 °C and then for 90 min at 37 °C to allow the casein digestion by peptides with protease activity. Finally, the gel was stained for 16 h using 0.02% (v/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid and then distained with 40% ethanol, 10% acetic acid and 50% distilled water. Light bands against the blue background indicated protease activity.

### 2.11. Egg-hatching assay

This assay was performed according to Santos et al. [18]. *A. aegypti* eggs stored on filter papers at 25–27 °C were selected by considering their integrity using a stereomicroscope (Leica M80). $WSMoL_C$ was diluted in filtered tap water to provide a test concentration range of 0.08–0.2 mg/mL. The final volume of the assay was 20 mL of test solution containing 50 eggs. Controls contained distilled water (volume equivalent to that of sample used to achieve each concentration) and filtered tap water (volume enough to complete 20 mL). The number of hatched larvae was determined after 72 h of incubation at 25–27 °C. The experiment was performed in triplicate.

### 2.12. Oviposition assay

Oviposition assay was performed according to Navarro et al. [29]. A total of 25 *A. aegypti* gravid females (3 days after blood feeding) were transferred to a cage containing two plastic vessels (diameter

10 cm), each containing 20 mL of distilled water and placed diagonally at opposite corners of the cage. $WSMoL_C$ (1 mL) was added to one of the vessels, resulting in a final lectin concentration of 0.1 mg/mL. The same volume of distilled water was added to the control vessel. A piece of filter paper was placed in 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 deposited in each vessel were manually counted with the aid of the stereomicroscope. Three independent experiments were performed, each with eight replicates. The oviposition response was expressed as follows: % oviposition = 100 × [(number of eggs in $WSMoL_C$ vessel)/(number of eggs in sample and control vessel)]. Oviposition active index (OAI) was also calculated as follows: (number of eggs in $WSMoL_C$ vessel − number of eggs in control vessel)/(number of eggs in $WSMoL_C$ vessel + number of eggs in control vessel). OAI value higher than +0.3 indicates attractant effect while OAI lower than −0.3 are indicates repellent effect [33].

### 2.13. Statistical analysis

Standard deviations (SDs) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) and data were expressed as mean of replicates ± SD. One-way fixed-effects ANOVA (significance at $p < 0.05$) was conducted using the IBM® SPSS® Statistics version 24 (IBM Corp.). The linear regressions and the concentrations required to kill 50%, 90% and 99% of larvae ($LC_{50}$, $LC_{90}$, $LC_{99}$) in 24 h or reduce egg hatching in 50%, 90% and 99% ($EC_{50}$, $EC_{90}$ and $EC_{99}$) in 72 h were established by probit analysis with a reliability interval of 95% also using the IBM® SPSS® Statistics software.

**Table 1**
Purification of water-soluble *M. oleifera* lectin from seed cake ($WSMoL_C$).

| Sample | Protein (mg/mL) | HA (HAU) | SHA (HAU/mg) | Purification (times) |
|---|---|---|---|---|
| Water extract | 19.5 | 512 | 525 | 1 |
| Lectin-rich fraction | 25.9 | 1024 | 790 | 1.5 |
| $WSMoL_C$ | 1.4 | 131,072 | 1,872,440 | 3567 |

The number of hemagglutinating activity units (HAU) was defined as the reciprocal of the highest dilution of sample promoting full erythrocyte agglutination. Specific HA (SHA) corresponded to the ratio between HA and protein amount (mg). Purification was measured as the ratio between the SHA in the stage and SHA of water extract.

## 3. Results and discussion

*M. oleifera* seed oil has a high biotechnological potential because of its rich content of unsaturated fatty acids. It is a source of cosmetic ingredients and antioxidant additives, can be used as fuel, and is used in general culinary [34]. The extraction of oil results in generation of cake, which may be rich in valuable components, and is usually discarded or underused after oil extraction. This study determined the potential use of *M. oleifera* seed cake as source of lectin with insecticidal activity.

High values of both protein concentration (19.5 mg/mL) and specific haemagglutinating activity (525 HAU/mg) were detected in the water extract (Table 1), which reveals that the carbohydrate-binding property of *M. oleifera* seed lectins resisted to oil extraction using *n*-hexane. Oil extraction from seeds using organic solvents can cause protein denaturation by exposing the hydrophobic groups of proteins and reducing their solubility [35]. The specific haemagglutinating activity and protein concentration in water extract from cake were higher than those reported by Santos et al. [4] for water extract prepared using powder from whole seeds, which showed protein concentration of 4.1 mg/mL and specific haemagglutinating activity of 300 HAU/mg. This result can be related to the elimination of seed oil, which makes the cake mass more concentrated in protein than an equivalent mass of whole seed powder. Sereewatthanawut et al. [36] reported that the protein content of rice bran increased after oil extraction. Furthermore, the decrease in the interference of oil in solubilization of lectins in water could have been a reason for the highest haemagglutinating activity in water extract from cake. According to Boatright and Hettiarachchy [37] the presence of lipids in soya protein isolates caused the decrease of protein solubility. Indeed, Liang [38] showed that the interaction between soya bean oil and soya proteins resulted in decrease of water solubility of proteins, and Labuckas et al. [35] obtained higher solubility of proteins from walnut flour after delipidation.

Table 1 shows a summary of $WSMoL_C$ purification. The treatment of water extract with 60% ammonium sulphate resulted in concentration of lectin in precipitated fraction (lectin-rich fraction) as evidenced by increase of specific haemagglutinating activity. Chitin column chromatography was efficient to bind $WSMoL_C$, which was eluted with 1.0 M acetic acid as a single peak (Fig. 1A) with specific haemagglutinating activity of 1,872,440 HAU/mg, higher than that of chromatographed lectin-rich fraction. The purification factor obtained

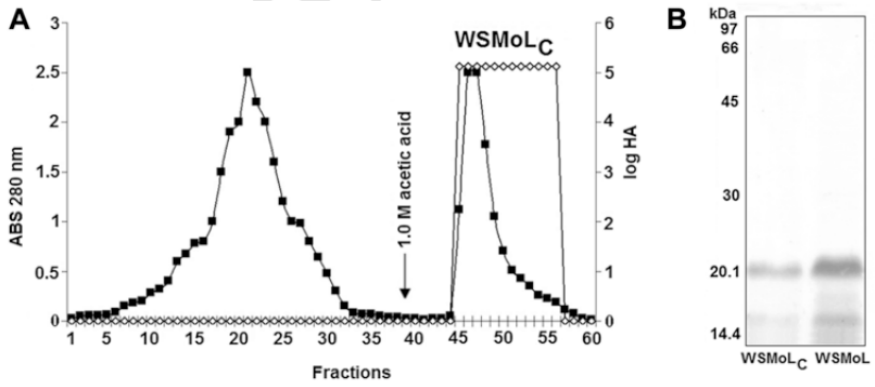


**Fig. 1.** Purification of water-soluble *M. oleifera* lectin from seed cake ($WSMoL_C$). (A) Chromatography of lectin-rich fraction on chitin column. The washing step used 0.15 M NaCl; arrow represents the addition of the eluent. Fractions (2.0 mL) were collected and evaluated for absorbance at 280 nm (■) and haemagglutinating activity (HA, ◊). (B) SDS-PAGE (12%, w/v) of $WSMoL_C$ (100 μg) and WSMoL (100 μg). The gel was stained with Coomassie Brilliant Blue.

for $WSMoL_C$ (3567) was very higher than that reported for WSMoL (137) isolated from *M. oleifera* whole seeds [2,39]. It is possible that the oil extraction with hexane increased not only the extraction of proteins from seeds but also the ability of lectin molecules in agglutinate rabbit erythrocytes.

SDS-PAGE revealed two polypeptide bands for $WSMoL_C$ heated for 15 min, with molecular masses of 15 and 20 kDa (Fig. 1B). The SDS-PAGE profile of $WSMoL_C$ was similar to that obtained for WSMoL under denaturing and nonreducing conditions (Fig. 1B), indicating that these lectins assume oligomeric forms. The profile was distinct from those reported for MoL (two polypeptide bands with 13.6 and 27.1 kDa) and cMoL (a single polypeptide band with 26.5 kDa) [1,3]. SDS-PAGE profile of $WSMoL_C$ was also different from that of a chitin-binding protein ($Mo\text{-}CBP_3$) isolated from *M. oleifera* seeds, which showed a single polypeptide band with 18 kDa [40].

Peptides derived from digestion of both polypeptide bands from $WSMoL_C$ showed similar sequence excerpts to the coagulant protein MO2.1 (gi|12721; score 71) and $Mo\text{-}CBP_3$ (2 S albumin precursor; gi|575764275; score 101) from *M. oleifera* seeds [41,42]. The excerpt of MO2.1 sequence (QAVQLTHQQQGQVGPQQVR) with homology to $WSMoL_C$ was also identified in peptide derived from WSMoL digestion and is also present in the sequence of cMoL [2,5]. $WSMoL_C$ showed homology with two excerpts (QAVQSAQQQQGQVGPQQVGHMYR and IPAICNLQPMR) of $Mo\text{-}CBP_3$ sequence reported by Freire et al. [42]. The sequence of cMoL was also similar to $Mo\text{-}CBP_3$ (75.7–94.2%) [5].

Compilation of these data shows that *M. oleifera* seed proteins share regions of high similarity in their primary structures. However, these proteins differ in their molecular mass and structural organization, which are certainly associated with the different number and types of amino acids in their entire chain. These structural differences result in the distinct characteristics of the lectins present in the seeds.

Specific haemagglutinating activity of $WSMoL_C$ (1,872,440) was reduced in presence of fructose, glucose, mannose, *N*-acetylglucosamine and galactose to 91.4, 365.7, 365.7, 731.4, and 1,462.9, respectively. The effect of monosaccharides on $WSMoL_C$ haemagglutinating activity is shown in Table 2 besides data previously reported for WSMoL, cMoL and MoL. The results showed that the profile of inhibition of $WSMoL_C$ haemagglutinating activity was similar to that of WSMoL and distinct of cMoL and MoL. Together with the solubility in water, chitin-binding property and SDS-PAGE profile, these data provide evidence that the lectin recovered from *M. oleifera* seed cake resembles WSMoL. This stimulated us to investigate whether $WSMoL_C$ would have larvicidal, ovicidal and oviposition-stimulant effects on *A. aegypti*, similarly to WSMoL.

The results from larvicidal assay revealed that water extract, lectin-rich fraction and $WSMoL_C$ were toxic for *A. aegypti* larvae after 24-h incubation (Fig. 2). The decrease of $LC_{50}$ value after each purification step (Table 3) shows that $WSMoL_C$ is the larvicidal principle. However, WSMoL from whole seeds showed a $LC_{50}$ value of 0.197 mg/mL against *A. aegypti* larvae [2], which is a value 4.5 times

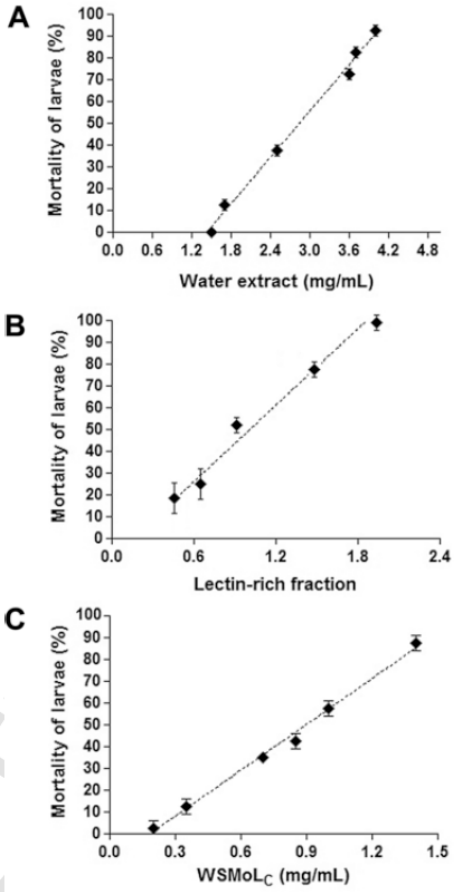


**Fig. 2.** Mortality of *Aedes aegypti* L4 incubated with water extract (A), lectin-rich fraction (B) and isolated $WSMoL_C$ (C) from *Moringa oleifera* seed cake.

**Table 2**
Inhibition of hemagglutinating activity of *M. oleifera* lectins by monosaccharides.

| Monosaccharide (0.2 M) | $WSMoL_C$ | WSMoL[a] | cMoL[b] | MoL[c] |
|---|---|---|---|---|
| Fructose | + | + | – | – |
| Glucose | + | + | + | – |
| Mannose | + | + | – | – |
| *N*-Acetylglucosamine | + | + | ND | ND |
| Galactose | + | + | + | – |

[a] According to Santos et al. [4], Coelho et al. [3] and Rolim et al. [39].
[b] According to Santos et al. [3].
[c] According to Katre et al. [1]. Inhibited (+) and not inhibited (−). ND: not described.

**Table 3**
Larvicidal activity of lectin preparations from *M. oleifera* seed cake against *A. aegypti*.

| Sample | Protein concentration (mg/mL) | | | Regression equation ($R^2$) | F-statistics |
|---|---|---|---|---|---|
| | $LC_{50}$ | $LC_{90}$ | $LC_{99}$ | | |
| Water extract | 2.92 [2.60–3.21] | 4.06 [3.70–4.67] | 4.99 [4.45–5.99] | $Y = 34.333X–48.587$ (0.993) | 729.11 |
| Lectin-rich fraction | 0.99 [0.92–1.07] | 1.75 [1.62–1.91] | 2.35 [2.16–2.63] | $Y = 52.263X–4.489$ (0.972) | 104.56 |
| $WSMoL_C$ | 0.89 [0.84–0.94] | 1.42 [1.33–1.54] | 1.86 [1.71–2.05] | $Y = 72.459X–14.344$ (0.994) | 641.24 |

Lethal concentrations required to 50% ($LC_{50}$), 90% ($LC_{90}$) and 99% ($LC_{99}$) of larvae in 24 h were calculated by probit analysis with a reliability interval of 95%. In regression equations, $Y$ is the mortality rate (%) and $X$ is the $WSMoL_C$ concentration in mg/mL. F-statistics values were obtained by one-way ANOVA.

lower than that found for $WSMoL_C$. As discussed earlier, the high specific haemagglutinating activity of $WSMoL_C$ in comparison with WSMoL is probably due to the fact that the treatment with *n*-hexane resulted in change in the ability of interaction of lectin with erythrocyte carbohydrates. In the same way, these changes may also be linked to the differences between the larvicidal potential. Larvicidal activity against the mosquitoes *Culex quinquefasciatus*, *Aedes albopictus* and *A. aegypti* have been reported for powder or preparations of *A. indica* seed cake [24].

The ingestion of lectins may cause insect death by different mechanisms, but, in order to exert these effects, these proteins usually need to be resistant to hydrolysis by proteases from insect gut [43]. Fig. 3A shows SDS-PAGE of the mixture containing $WSMoL_C$ and *A. aegypti* L4 gut extracts incubated for different periods. It can be observed that $WSMoL_C$ polypeptide band was observed even after incubation for 48 h; initial disappearing of the band was observed only in the sample incubated for 72 h. This result evidences a remarkable resistance of this lectin to digestion by larval gut proteases.

Once lectin molecules have been able to remain active at the insect gut, they may bind to epithelial cells, peritrophic matrix and glycosylated molecules (*e.g.* enzymes) present in the gut lumen [43]. Particularly for WSMoL, it was previously reported that this lectin had a stimulatory effect on protease, trypsin-like and α-amylase activities from gut of *A. aegypti* larvae, suggesting that larvicidal effect involves deregulation of digestive process [44]. These authors also reported that cMoL, which did not have larvicidal activity, exerted inhibitory effects on the same activities. When $WSMoL_C$ was evaluated for its effects on larval digestive enzymes, stimulatory effects were detected on amylase (Fig. 3B) and protease (Fig. 3C) activities, similarly to those reported for WSMoL; however, $WSMoL_C$ exerted inhibitory effect on trypsin-like activity (Fig. 3D) differently from WSMoL. This difference in the effects on digestive enzyme activities may be linked to the lower larvicidal activity of $WSMoL_C$ in comparison with WSMoL.

Insects may physiologically adapt to an imbalance caused by plant toxic proteins on their enzymatic apparatus by increasing the expression of an enzyme or expressing other enzymes that are distinct from that affected by the insecticidal agent [43]. In this sense, it was evaluated whether the protease profile of gut extracts from *A. aegypti* larvae that survived to treatment with $WSMoL_C$ would be different from that of control larvae. The zymography (Fig. 3E) indicated increased expression of four polypeptide bands with protease activity by larvae exposed to the lectin (solid arrows) in comparison with control. On the contrary, one polypeptide band was observed only in control (dotted arrow). These results are in accordance with those obtained after *in vitro* incubation of lectin with larvae extract, which revealed an increase of total protease activity and inhibition of a specific protease (trypsin). It can also be noted that no polypeptide band with protease activity appeared only in zymography of gut extract from larvae treated with $WSMoL_C$, indicating no expression of different enzymes after 24-h incubation with the lectin.

$WSMoL_C$ reduced the egg-hatching rate in a dose-dependent manner (Fig. 4A) and the $EC_{50}$, $EC_{90}$ and $EC_{99}$ determined were 0.14 [0.13–0.15], 0.24 [0.22–0.26], and 0.32 [0.30–0.36] mg/mL, respectively. Santos et al. [18] reported similar result for WSMoL ($EC_{50}$ of 0.1 mg/mL) and showed that the ovicidal activity of lectin involves blocking of the hatching process by killing the embryo inside the egg. Furthermore, they suggested that the *A. aegypti* eggshells, which contain a serosal cuticle constituted by chitin, may act as a binding target for WSMoL and allow the lectin to adhere to the eggs and then penetrate inside them. The chitin-binding property of $WSMoL_C$ may also be involved in its ovicidal activity.

The oviposition assay used $WSMoL_C$ at the same concentration at which WSMoL showed oviposition-stimulant activity [18]. Similarly, most of the eggs were deposited in solutions containing $WSMoL_C$ (Fig. 4B), with an OAI of 0.42. Adult females choose their oviposition sites through olfactory receptors or gustatory sensilla organs that detect volatile and nonvolatile signals, respectively. The mechanism involves multigene families of chemoreceptors [45,46]. Santos et al. [18,19] reported evidence that the oviposition-stimulant action of WSMoL involves gustatory stimuli. The *A. indica* seed cake was also explored as source of compounds able to affect oviposition behaviour of mosquitoes: hexane, methanol and ethyl acetate extracts exerted strong oviposition deterrent effect (above 73%) on *A. albopictus* females [47].

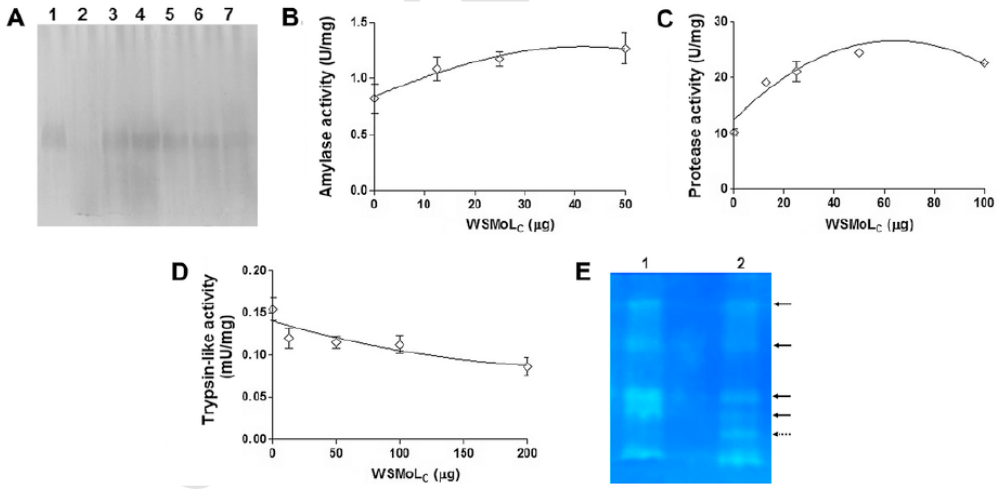


**Fig. 3.** Investigation of mechanisms involved in $WSMoL_C$ larvicidal activity. (A) SDS-PAGE of $WSMoL_C$ after incubation with larval gut extract. 1: control with only lectin; 2: control with only gut extract; 3, 4, 5, 6 and 7: mixtures containing lectin and gut extract incubated at 37 °C for 1, 12, 24, 48 and 72 h, respectively. (B, C and D) *In vitro* effects of $WSMoL_C$ on α-amylase, protease and trypsin-like activities, respectively, from larval gut extracts. (E) Zymography for proteases of gut extracts obtained from live larvae after incubation for 24 h with WSMoL at the $LC_{50}$ (lane 1) or distilled water (control, lane 2).

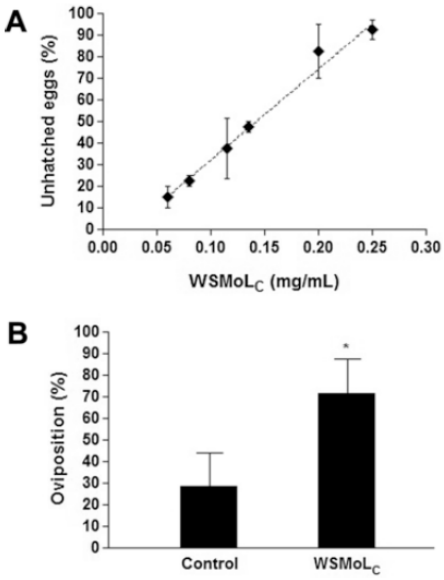


**Fig. 4.** Effects of water-soluble *M. oleifera* lectin from seed cake (WSMoL$_C$) on egg hatching and oviposition of *Aedes aegypti*. (A) Percentage of unhatched eggs after incubation with WSMoL$_C$ for 72 h. The regression equation is $Y = 72.459X–14.344$ ($R^2$: 0.992; F-statistics: 641.24), where $Y$ is the percent of unhatched eggs and $X$ is the WSMoL$_C$ concentration in mg/mL. (B) Mean of eggs laid by gravid females in distilled water (control) and WSMoL$_C$ solution (0.1 mg/mL). (*) indicates significant differences ($p < 0.05$) between control and test groups.

In summary, the WSMoL$_C$ larvicidal concentration was higher than that of WSMoL, while the ovicidal and oviposition-stimulant activities were similar to that of lectin from whole seeds. It is important to highlight that WSMoL$_C$ has a high potential to be used in control of *A. aegypti*, because it can be used as an oviposition-stimulant to increase the capture of eggs using ovitraps at the same time that it could be able to kill the eggs or larvae that may arise from them, similarly to WSMoL. Thus, the *M. oleifera* seed cake is an interesting source of lectin that can be used in control of *A. aegypti* populations, which aggregates value to all production chain of moringa oil, as other products can be also exploited from the cake.

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**5 ARTIGO 2**

# Evaluation of structural stability and termiticidal activity of the lectin from *Moringa oleifera* seed cake

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**Evaluation of structural stability and termiticidal activity of the lectin from *Moringa oleifera* seed cake**

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**Abstract**

*Moringa oleifera* seed cake contains the lectin $WSMoL_C$, which was reported to be an insecticidal agent against *Aedes aegypti*. The conformational stability is important for the biotechnological application of lectins. This work investigated $WSMoL_C$ for structural stability towards denaturation by physical and chemical agents and for termiticidal activity against *Nasutitermes corniger*. Changes in conformation regarding presence of urea, heating and high pressures were monitored by circular dichroism (CD) and fluorescence (using the extrinsic probe bis-ANS) techniques. The termiticidal activity was evaluated by submitting the insect to a diet composed by filter papers impregnated with the lectin. Interference of the lectin on termite digestive enzymes was also evaluated. The conformation of $WSMoL_C$ changed in presence of urea, showing two states of denaturation: the first was marked by a decrease in bis-ANS fluorescence in presence of until 4 M urea and the other was characterized by increase of bis-ANS fluorescence accompanied with expressive loss of secondary structure in treatments with urea at concentration higher than 4 M. Temperature (until 65°C) and pressure (lower than 2.7 kbar) did not promote conformational changes. The lectin was termiticidal agent for *N. corniger* workers ($LC_{50}$ of 0.361 mg/mL, 6 days) and soldiers ($LC_{50}$ of 0.629 mg/mL, 7 days) as well as showed an inhibitory effect on endoglucanase and exoglucanase activities from termite gut extracts. Protease activity from gut extracts was not altered in presence of the lectin. In conclusion, $WSMoL_C$ is a promising termiticidal agent with great structural stability towards denaturing agents.

**Keywords:** lectin; *Moringa oleifera;* conformational changes; *Nasutitermes corniger*; denaturation.

## 1. Introduction

Lectins are hemagglutinating proteins that recognize and bind specifically and reversibly to carbohydrates (Paiva et al., 2011a, 2011b). They are found in plants, animals and microorganisms, being involved in a variety of physiological and pathological processes (Fu et al, 2011; John et al., 2013; Varrot et al., 2013; Sun et al., 2015). In plants, many lectins are present in different tissues such as leaves, flowers and especially seeds (Vandenborre et al., 2011, Vasconcelos et al., 2015). Lectins have shown deleterious effects against virus, bacteria, fungi, and insects (Santos et al., 2014; Silva et al., 2014; Carvalho et al., 2015; Guo et al., 2015).

The biological activities of lectins are related with their secondary and tertiary structures. The definition of the conformational stability is important for the biotechnological application of proteins and to understand the structure-function relationship. The physicochemical characterization of lectins involves, besides other characteristics, the determination of structural stability toward denaturant conditions (Varejão et al, 2010; Khan et al, 2013).

*Moringa oleifera* is a tree belonging to the Moringaceae family, widely cultivated throughout India, some parts of Africa and America (Pritchard et al., 2010). The moringa seeds have high oil content and, in the process of oil extraction, a solid residue called “cake” is obtained. This cake have shown potential as a natural coagulant for wastewater treatment (Abdulkarim et al., 2005; Kleiman et al., 2008; Azad et al., 2015; Tie et al., 2015). A lectin ($WSMoL_C$) isolated from the cake showed larvicidal, ovicidal and oviposition-stimulant activities against *A. aegypti* (Oliveira et al., 2016). The authors showed that this lectin shares similar characteristics with the lectin WSMoL (isolated from whole seeds) but is more distinct from other *M. oleifera* lectins, cMoL and MoL.

Termites have important ecological role acting in the nutrient cycling and decomposition of organic material. However, some species are potential urban pests responsible for deterioration of wood structures of buildings (Constantino and Schlemmermeyer, 2000; Costa-Leonardo, 2002; Ferreira et al., 2011). The termite *Nasutitermes corniger* (Isoptera: Termitidae) is known by cause damage in urban properties (Zorzenon, 2002; Albuquerque et al., 2012). The use of insecticides products derived from plants, such as lectins, has been evaluated as an alternative for termite control (Souza et al., 2011; Paiva et al., 2011b; Adfa et al., 2015).

The insecticidal activities of lectins involve the recognition and binding to different targets at the insect body and usually depend on the carbohydrate-binding ability of lectins (Paiva et al, 2011b; Paiva et al., 2013). Thus, it is important to determine the stability of insecticidal lectins under unfavorable conditions. In this context, this paper investigated the structural stability of $WSMoL_C$ when exposed to physical and chemical denaturants as well as its potential as a termiticidal agent against *N. corniger*.

## 2. Materials and methods

### 2.1. Isolation of $WSMoL_C$

*M. oleifera* seeds were collected in Recife city, Pernambuco, Brazil and stored at -20° C. The cake was obtained by extraction of oil with n-hexane solvent using Soxhlet extractor (6 h). The lectin was isolated according to procedure described by Oliveira et al. (2016). The cake was homogenized (10 g) with distilled water (100 mL) for 16 h at 4ºC. The extract obtained after filtration and centrifugation (9,000 *g*, 15 min) was submitted to protein precipitation using ammonium sulfate at saturation of 60% and the precipitate was dialyzed

(3,500 Da cut-off membrane) against distilled water and 0.15 M NaCl (4 h) resulting in the 0-60 fraction. The fraction was loaded (30 mg of proteins) onto a chitin column (7.5 cm x 1.5 cm) previously equilibrated with 0.15 M NaCl (0.3 mL/min flow rate). After washing with equilibration solution, the lectin ($WSMoL_C$) was recovered by elution with 1.0 M acetic acid and dialyzed (3,500 Da cut-off membrane) against distilled water (4 h) for eluent elimination.

2.2. Protein concentration and hemagglutinating activity (HA)

Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin (31.25–500 µg/mL; Sigma-Aldrich, USA) as standard. Hemagglutinating activity of lectin was assessed using rabbit erythrocytes treated with glutaraldehyde (Paiva and Coelho, 1992) in microtiter plates (Kartell S.P.A., Italy). The number of hemagglutinating activity units was defined as the reciprocal of the highest dilution of sample promoting full erythrocyte agglutination. Specific hemagglutinating activity was defined as the ratio between the titer and protein concentration (mg/mL).

2.3. Dynamic light scattering (DLS)

The lectin (2 µM or 20 µM) was centrifuged at 10,000 rpm for 15 min and filtered through 0.22 µm syringe filter and then DLS measurements were performed using a DynaPro-TC-04 equipment (Protein Solutions, Wyatt Technology). Measured size corresponded to the average value of 10.0 runs. All the samples were evaluated for DLS berofe denaturation assays.

2.4. Circular dichroism (CD) and fluorescence measurements

CD spectra were recorded on a JASCO-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) in the wavelength range 190–260 nm. The data were obtained at a scan speed of 100 nm/min with 0.1 nm steps and response time of 4 s. Five scans were recorded for the sample ($WSMoL_C$ at 20 µM ) and their average was used for further analysis.

Fluorescence measurements were performed on a Jasco spectrofluorometer FP-6300 (Jasco Corp., Tokyo, Japan). For extrinsic fluorescence measurement, $WSMoL_C$ (2 µM) was incubated with 50-fold molar excess of bis-ANS (bis(8-anilinonaphthalene-1-sulfonate)) in the dark. The fluorescence spectra of bis-ANS were recorded by setting the emission wavelength from 400 to 600 nm and excitation at 360 nm. Bis-ANS binding was evaluated by area of fluorescence intensity in arbitrary units.

The denaturation curves were performed using a fixed concentration of lectin (2 µM or 20 µM). $WSMoL_C$ was incubated for 20 h with increasing concentrations of urea (0-9 M) at 25ºC or heated for 5 min at different temperatures (25-65ºC) or submitted for 10 min to different pressures (0-2.7 kbar). After each treatment, the samples were analyzed by CD and/or fluorescence measurements.

## 2.5. Termiticidal activity

Colonies of *N. corniger* were collected at the campus of *Universidade Federal Rural de Pernambuco*. Termiticidal activity was evaluated according to the method described by Kang et al. (1990). The experimental units consisted of a Petri plate (90 x 15mm) with the lower plate covered by filter paper. A filter paper disk (4 cm diameter) impregnated with 200 µL of $WSMoL_C$ (0.1–1.0 mg/mL) or 0.15 M NaCl (negative control) was placed on each plate. A total of 20 active termites (4 soldiers and 16 workers) was transferred to each plate

and maintained at 28ºC in dark. Evaluation of insect survival was made daily until death of all insects. Bioassays were performed in quintuplicate for each concentration and survival rates (%) were obtained of each treatment.

2.6. Effects of $WSMoL_C$ on digestive enzymes from gut of soldiers and workers of *N. corniger*

*N. corniger* gut extracts were obtained according to Napoleão et al. (2011). Groups of 100 workers or soldiers were immobilized by placing them at -20°C for 15 min. The gut of each insect was removed by hand and homogenized with 1 mL of Tris buffer (0.1 M Tris-HCl, pH 8.0, containing 0.02 M $CaCl_2$ and 0.15 M NaCl) or acetate buffer (0.1 M sodium acetate pH 5.5, containing 0.02 M $CaCl_2$ and 0.15 M NaCl). The homogenates were centrifuged at 9,000 *g* at 4°C for 15 min. The supernatants were collected, pooled (worker or soldier gut extracts) and evaluated for protein concentration (Lowry et al., 1951).

Endoglucanase, exoglucanase and α-amylase activities were determined according to adaptations of the methods described by Li et al. (2009), Wood and Bhat (1988) and Bernfeld (1955), respectively. The worker gut extract (100 µL; 79 µg of protein) or soldier gut extract (100 µL; 47 µg of protein) was incubated for 10 min at 50ºC with 400 µL of solutions (1%, w/v) of carboxymethylcellulose (for endoglucanase activity) or Avicel (for exoglucanase activity) or soluble starch (for α-amylase activity) in sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 µL of 3,5-dinitrosalicylic acid (DNS) were added and the assay was heated (100ºC, 6 min) and immediately cooled in ice (15 min). Next, the absorbance at 540 nm was measured. The amount of reducing sugars was determined using a standard curve of glucose ($Y$=0.4153$X$–0.0026; $Y$ is the absorbance at 540 nm; $X$ is the glucose concentration

in mg/mL). One unit of enzyme activity was defined as the amount of enzyme that produces 1 µmol of glucose per minute.

To determine protease activity, the worker gut extract (100 µL; 99 µg of protein) or soldier gut extract (100 µL; 65 µg of protein) in Tris buffer were mixed with 300 µL of 0.1 M sodium phosphate (pH 7.5) containing 50 µL of 0.6% (w/v) azocasein. Next, 100 µL of Triton X-100 (0.1%, v/v) was added and the mixture was incubated at 37°C for 3 h. The reaction was stopped by adding 200 µL of 10% (v/v) trichloroacetic acid, and the assay was incubated at 4°C for 30 min. The reaction mixture was then centrifuged (9000 g for 10 min) and the absorbance of the supernatant at 366 nm was read. One unit of protease activity was defined as the amount of enzyme required to increase absorbance by 0.01.

The effects of $WSMoL_C$ on enzyme activities were evaluated by incubation (30 min at 28°C) of the worker or soldier gut extracts with the lectin (at different concentrations) before the determination of enzyme activities. The results were compared with those from assays performed without lectin (100% activity control). Control assays were also performed with only $WSMoL_C$.

## 2.7. Statistical analysis

Significant differences between treatment grups from termiticidal assay were analysed by Student's t-test (significance at $p<0.05$) using Origin 6.0 program. Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA) and data were expressed as mean of replicates ± SD. The lethal concentrations required to kill 50% ($LC_{50}$) of soldiers and works after 6 and 7 days were calculated by probit analysis using the computer software StatPlus®2006 (Analyst Soft, Canada).

## 3. Results and discussion

The interaction of lectins depends on the native conformation required to the correct geometry of the carbohydrate-binding sites (Ghosh and Mandal, 2014; Tang et al, 2015). $WSMoL_C$ is a lectin isolated from *M. oleifera* seed cake, which showed very high specific hemagglutinating activity (93,600), which shows that its ability to bind carbohydrates was preserved after the treatment of seeds with n-hexane. This result stimulated us to study more deeply the stability of this lectin toward denaturing conditions.

The determination of conformational stability through spectroscopic studies requires the presence of the studied molecule in a homogeneous state. The homogeneity of $WSMoL_C$ samples was then verified by DLS measurements, which revealed a single hydrodynamic radius (Rh) of 1.5 nm for folded $WSMoL_C$ (Figure 1). This result confirms the homogeneity regarding lectin forms present in the solutions used in the denaturing assays. Then, it was evaluated the stability of $WSMoL_C$ using temperature, urea and high pressures as denaturants. To study the conformational stability, circular dichroism and fluorescence assays were used to monitor changes in secondary and tertiary structures.

Chemical denaturants such as urea are able to change noncovalents interactions of proteins and therefore can compromise the secondary and tertiary structures of proteins and thus their biological activity (O'Brien et al., 2007; Shao et al., 2014). The bis-ANS is a fluorescent probe that binds to hydrophobic center of proteins and is used to detect unfolded states of proteins (Rosen and Weber, 1969). The bis-ANS fluorescence intensity decreases when hydrophobic patches in protein are exposed to water in the unfolding process. In the native state, fluorescence spectra of $WSMoL_C$ with bis-ANS revealed that the lectin has hydrophobic clusters that were bound by the bis-ANS (Figure 2A). The intensity of bis-ANS fluorescence after treatment of $WSMoL_C$ with different concentrations of urea is also shown

in Figure 2B. The denaturation process shows two states: in the first moment, with addition of urea, the lectin exhibited decrease capacity to bind the probe and the minimum emission of bis-ANS fluorescence occurred at 4 M urea. The reduction of fluorescence at 4 M urea can be justified by the formation of unfolded structure or formation of aggregates without hydrophobic clusters.

Interestingly, the bis-ANS fluorescence emission increased gradually in treatments at concentrations higher than 8 M urea (Figure 2B). This increase may be explained by a conformational rearrangement of $WSMoL_C$, with bis-ANS binding to hydrophobic centers; however, this does not necessarily imply in the presence of defined secondary structures. In this sense, we performed the CD analyses, which are able to reveal changes in the secondary structure of the lectin in function of urea addition. In the absence of urea, $WSMoL_C$ spectrum was characteristic of proteins with α-helix secondary structure, with two negative bands at 222 nm and 209 nm. It was observed that $WSMoL_C$ loses more than 50% of its secondary structure when incubated with 4 and 8 M urea (Figure 3).

Conformational changes induced by urea have also been reported in other studies with proteins. Devaraj et al (2011) reported that ficin undergo conformational changes and is susceptible to unfolding by urea at lower pH. The result of urea denaturation of champedak galactose-binding lectin (CGB) monitored through ANS fluorescence showed two steps characterized by increase of ANS binding and complete denaturation observed at 5 M and 9 M urea, respectively (Kameel et al, 2016). Ghosh and Mandal (2012) described that urea induced unfolding of peanut agglutinin (PNA), resulting in inactive typical β-sheet conformation.

Changes in temperature can also interefere with the conformation of proteins (Wang et al., 2014). Bis-ANS fluorescence spectra associated with $WSMoL_C$ were then obtained after heating of lectin at different temperatures (Figure 4). The lectin did not show significant

structural changes even when submitted to heating at 65°C. This stability is in agreement with the fact that $WSMoL_C$ was recovered active from the cake after the process of oil extraction at 70°C. Other proteins from *M. oleifera* are known for their thermostability. CD analysis revealed that the coagulant *M. oleifera* lectin (cMoL) maintained its conformation after heated at 80ºC for 30 min (Luz et al, 2013). A coagulant protein from *M. oleifera* seeds remained active after 5 h of heat treatment at 95ºC and the secondary structure of MoL (another lectin from *M. oleifera* seeds) was not affected by temperature and this protein showed hemagglutinating activity even after incubation at 85ºC for 30 min at pH 7.2 (Ghebremichael et al., 2005; Katre at al., 2008).

High pressure also results in loss of tertiary and secondary conformation and causes protein denaturation (Foguel and Silva, 2004; Cinar and Czeslik, 2015). $WSMoL_C$ was submitted to high pressures in order to evaluate the moment that starts the process of lectin denaturation. Figure 5A shows the bis-ANS spectral area and there were no significant changes in the lectin structure up to 2.7 kbar pressure. However, there was a decrease in the bis-ANS fluorescence spectra when $WSMoL_C$ was submitted to a pressure of 2.7 kbar (Figure 5B), indicating that the lectin suffered a little loss of the hydrophobic clusters present in the native state. $WSMoL_C$ incubated with 4 M urea and also submitted to different pressures showed no change in the bis-ANS fluorescence (Figure 6), indicating that the pressure did not lead to additional changes in conformation beyond those already resulting from treatment with urea. The conformational resistance of $WSMoL_C$ to high pressures shows its large stability.

The hydrostatic pressure is a tool used to evaluate the conformational transition of proteins. The stability of native conformations changes easily in the presence of high pressures, because the tertiary structure is affected due to reduction of entropic and compressible interactions (Silva et al., 2001; Ishimaru et al., 2002). Myofibrillar soluble

proteins submitted at pressure at 200 MPa lost solubility and native functionality (Grossi et al., 2016).

The reports of insecticidal activity of WSMoL and $WSMoL_C$ (Coelho et al., 2009; Paiva et al., 2011a, 2011b; Oliveira et al., 2016) stimulated the investigation of $WSMoL_C$ effect on survival of *N. corniger*. $WSMoL_C$ was termiticidal agent to workers since survival curves were significantly ($p< 0.05$) different in comparison with control after 7 days (Figure 7A). The mortality rate of soldiers was also significantly different from control in treatments with the lectin (Figure 7B). The $LC_{50}$ values for workers and soldiers were 0.36 mg/mL and 0.63 mg/mL for 6 and 7 days, respectively.

The $LC_{50}$ values found for $WSMoL_C$ were similar to those of the lectins MuBL and MuLL, which were toxic against workers ($LC_{50}$ of 0.974 mg/mL and 0.374 mg/mL, respectively) and soldiers ($LC_{50}$ of 0.787 mg/mL and 0.432 mg/mL, respectively) of *N. corniger* (Napoleão et al., 2011; Paiva et al., 2011b). WSMoL was also previously evaluated for insecticidal activity against *N. corniger* but termiticidal activity was only detected at higher concentration (1.5 mg/mL) (Paiva et al., 2011b). The results obtained in the present work reinforce the evidences raised by Oliveira et al. (2016) that $WSMoL_C$ and WSMoL, although similar in some aspects, have differences in their conformation resulting to different biological properties. In their work, these authors showed that WSMoL was more effective as larvicidal agent against *A. aegypti*, otherwise the results obtained here for *N. corniger*.

The ability of the termiticidal lectin from *Microgramma vacciniifolia* rhizome in modulating the activity of digestive enzymes from *N. corniger* gut has been described (Albuquerque et al., 2012). $WSMoL_C$ was also able to affect the enzyme activities in gut extracts from *N. corniger* soldiers and workers. Endoglucanase and exoglucanase activities of soldiers and workers were significantly ($p<0.05$) reduced in the presence of $WSMoL_C$ in a dose-dependent manner (Figure 8A and 8B). Also, the lectin was able to reduce the α-amylase

activity from workers (Figure 8C), but did not modify the protease activity from gut extracts of soldiers and workers (Figure 8D).

According to Lima et al. (2014) endoglucanases are the main cellulolytic enzymes identified in the gut extracts from *N. corniger* workers and soldiers, followed by exoglucanases. Workers have the role of digesting food for nourishment of the other castes, such as soldiers (Lima et al., 2015). The toxicity of $WSMoL_C$ on workers may be associated to the reduction of cellulase activities and deregulation of the digestive apparatus. The α-amylase is also an important digestive enzyme for the optimal development of insects especially in pests living in grains rich in starch. in termites, it was demonstrated that workers have amylase activity higher that soldiers (Kaur et al., 2014; Lima et al., 2014). Thus, $WSMoL_C$ can interfere with the global digestion process of termites, inhibiting different enzymes present at the digestive tract.

## 4. Conclusions

The data revealed $WSMoL_C$ as a lectin resistant to denaturation caused by chemical and physical agents and with termiticidal activity. The study contributes to the understanding of the folding process of the protein and reports $WSMoL_C$ as a promising insecticide component of great structural stability.

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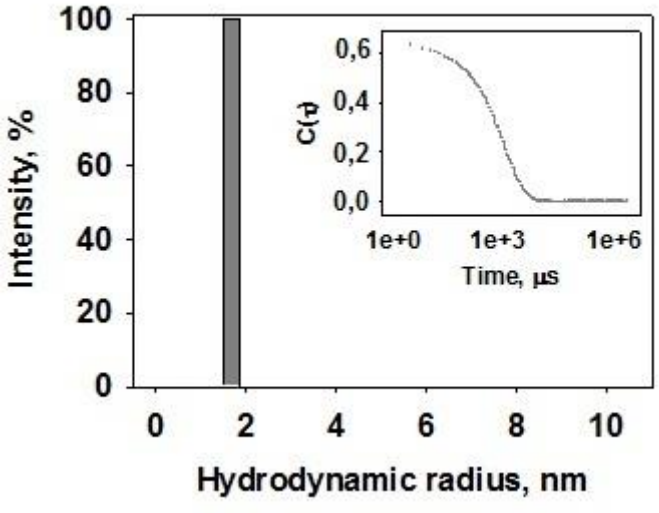


**Figure 1.** Hydrodynamic radius of $WSMoL_C$ visualized by DLS measurement at 20°C.

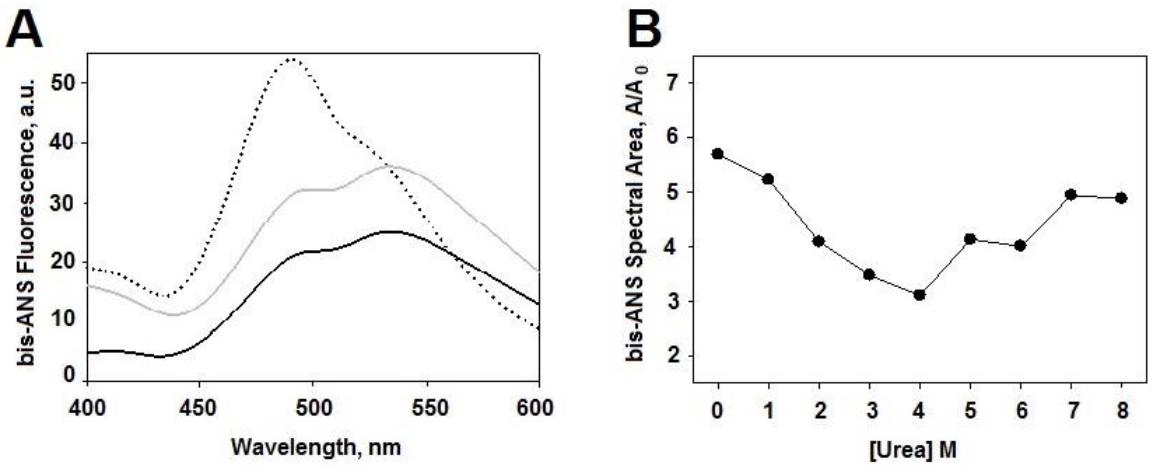


**Figure 2.** Denaturation of $WSMoL_C$ induced by urea monitored by bis-ANS fluorescence. (A) Change in the fluorescence spectra of bis-ANS in the absence of urea (dashed line), or at 4 M (solid line) and 8 M (gray line) urea. (B) Change in bis-ANS spectral area as a function of increase in urea concentration. All the measurements were performed after 20 h incubation of $WSMoL_C$ with urea at 25 °C.

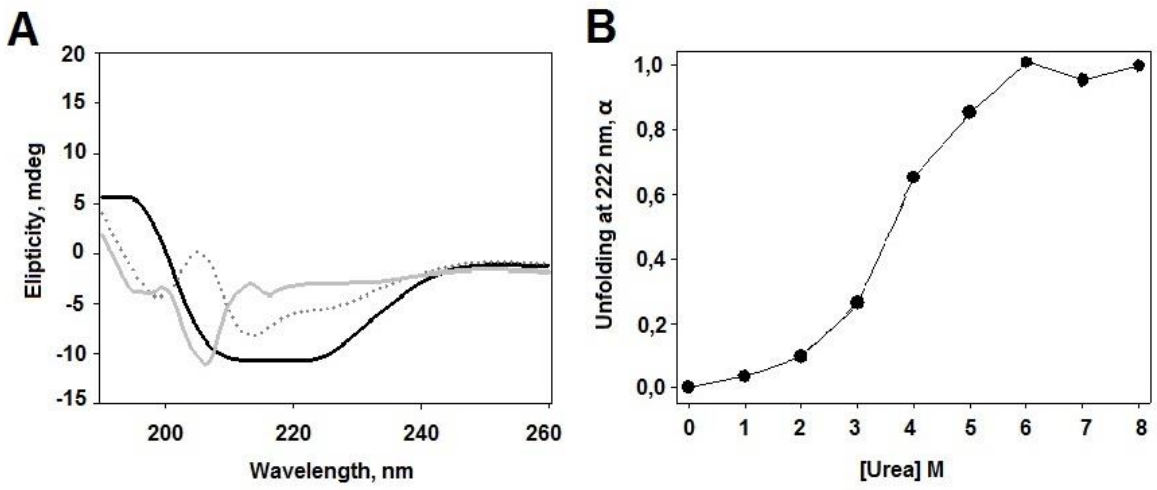


**Figure 3.** Changes in $WSMoL_C$ secondary structure induced by urea. (A) Change in the elipticity in the absence of urea (dashed line), or at 4 M (solid line) and 8 M (gray line) urea. (B) Unfolding at 222 nm as a function of urea increase. All the measurements were performed after 20 h incubation of $WSMoL_C$ with urea at 25°C.

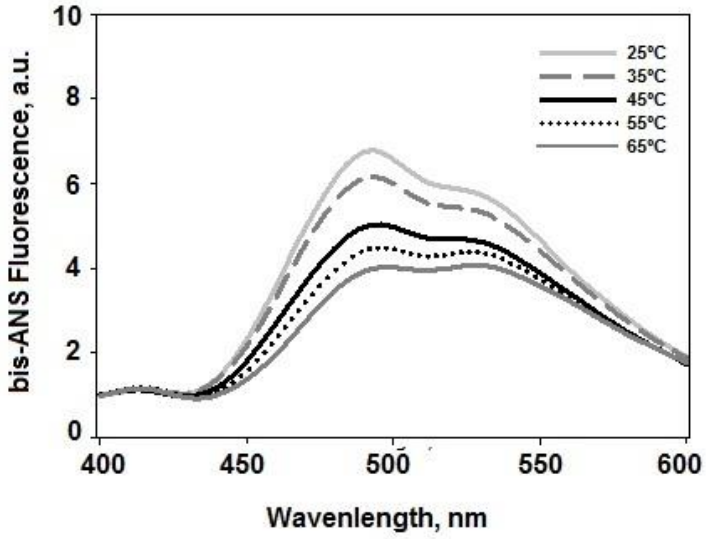


**Figure 4.** Changes in tertiary structure of $WSMoL_C$ induced by temperature and monitored by bis-ANS fluorescence.

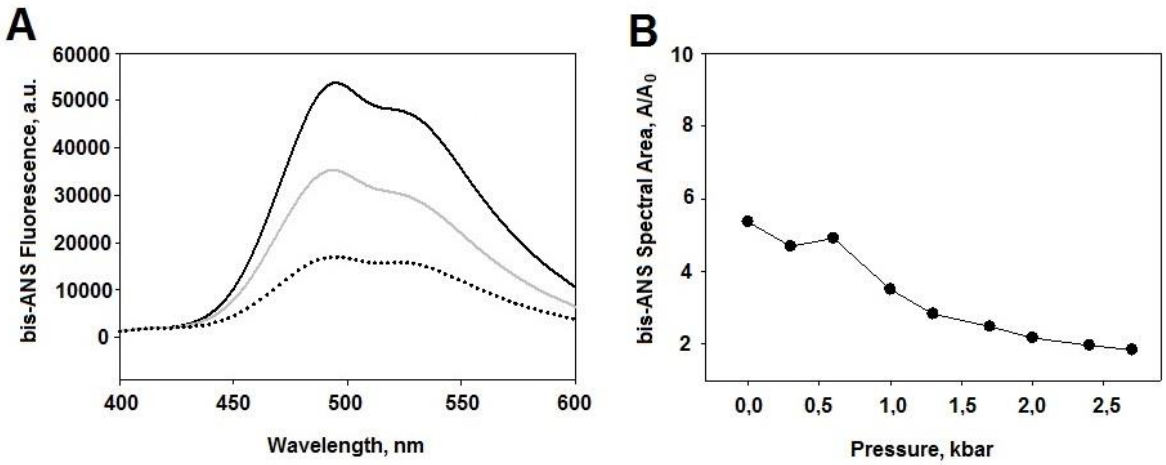


**Figure 5.** Changes in tertiary structure of $WSMoL_C$ induced by pressure and monitored by bis-ANS fluorescence. (A) Fluorescence in the absence of pressure (solid line) or in treatments at 1.0 kbar (gray line) and 2.7 kbar (dashed line). (B) bis-ANS spectral area as a function of pressure increase.

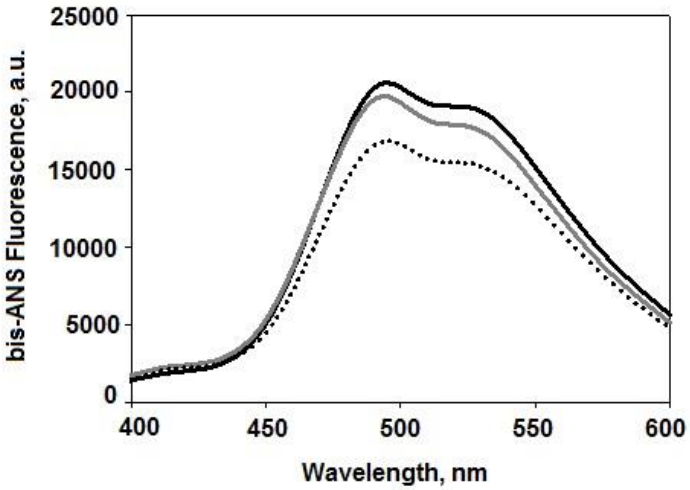


**Figure 6:** Changes in tertiary structure of $WSMoL_C$ induced in presence of urea at 4 M and submitted to pressure treatment. Fluorescence in the absence of pressure (solid line) or in treatments at 1.0 kbar (gray line) and 2.7 kbar (dashed line).

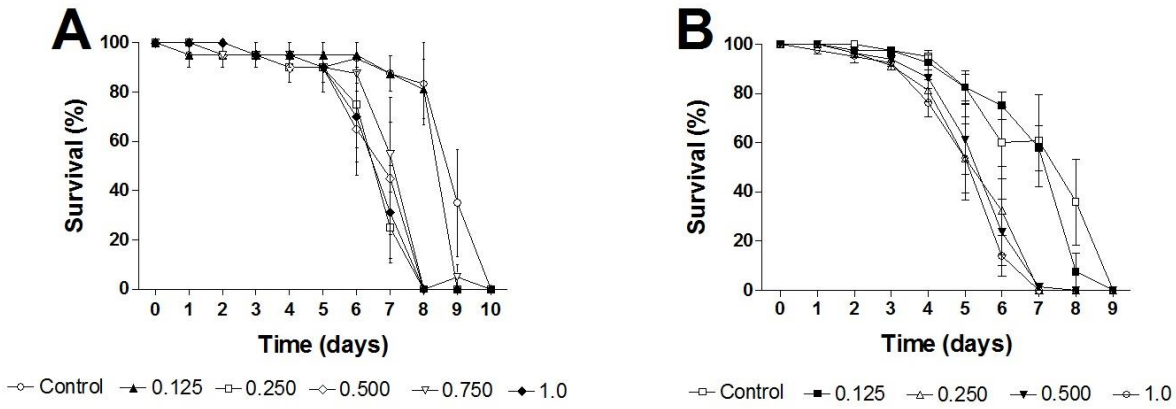


**Figure 7:** Survival rates of *Nasutitermes corniger* workers (A) and soldiers (B) castes treated or not (control) with $WSMoL_C$ (0.125–1.0 mg/mL). Each point represents the mean of five replicates.

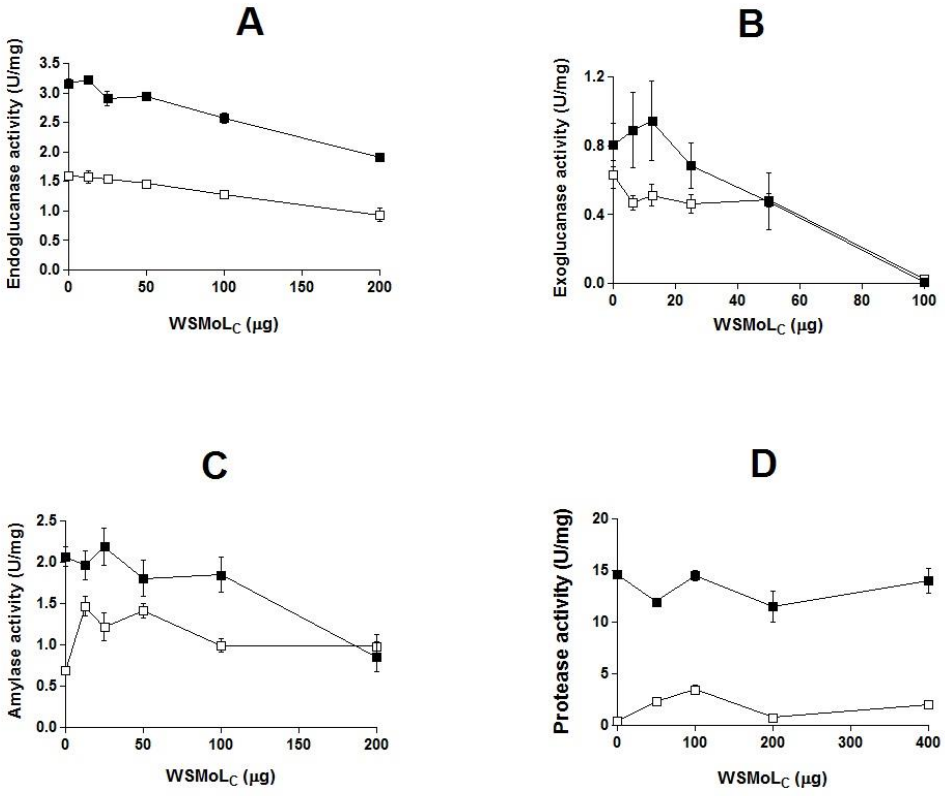


**Figure 8:** Effect of $WSMoL_C$ on endoglucanase (A), exoglucanase (B), α-amylase (C) and protease (D) activity from *N. corniger* workers (-■-) and soldiers (-□-) gut extracts.

**6 ARTIGO 3**

# Insecticidal activity evaluation of aqueous extracts and lectins from *Moringa oleifera* whole seeds and seed cake against *Sitophilus zeamais* Motsch.

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## ABSTRACT

The maize weevil *Sitophilus zeamais* has a strong economic appeal since it causes deterioration of stored grains, processed cereals and industrial food. In this work, aqueous extracts from *Moringa oleifera* whole seeds (AE) and seed cake ($AE_C$) as well water-soluble lectins isolated from them (WSMoL and $WSMoL_C$, respectively) were investigated for effects on survival, feeding and nutritional parameters of *S. zeamais* adults. The assay consisted in maintain the insect in artificial diet corresponding to wheat flour disks supplemented with the sample. Also, the effects of these preparations on trypsin-like, α-amylase and endoglucanase activities from insect gut were investigated. Both extracts contained saponins, phenylpropanoids, alkaloids and reducer sugars; however, AE (58–636 mg/g) was toxic to *S. zeamais* ($LC_{50}$: 214.6 mg/g) while $AE_C$ (123–615 mg/g) did not kill the insects. WSMoL caused slight mortality (12.0±2.7%y) only at 60 mg/g while $WSMoL_C$ was not lethal at any tested concentration (1.25–40 mg/g). The intake of AE exerted moderate to strong deterrent effect while $AE_C$ did not alter nutritional parameters. Both WSMoL and $WSMoL_C$ decreased the efficiency in conversion of ingested food but only WSMoL decreased the relative biomass gain rate while only $WSMoL_C$ exerted deterrent action. The extracts and $WSMoL_C$ stimulated the α-amylase and endoglucanase activity while trypsin-like activity was only modulated by WSMoL and $WSMoL_C$. In conclusion, AE was toxic to *S. zeamais* probably due to its feeding-deterrent activity and the lectins were damaging to the nutritional physiology of insects probably by promoting an imbalance of digestion process due to stimulation of trypsin-like activity.


**Keywords:** *Moringa oleifera*; maize weevil; antinutritional effect; digestive enzymes; lectin.

## Introduction

Cereal grains have a major relevance in the global economy since they are components of the basic diets for human populations worldwide. Intensified land management and introduction of new technologies increased the global grain production. This permited the increase production in grain in different times and regions of year which made storage these products a standard practice among major producers (Neumann et al., 2010). However, storage pests and pathogens can attack stored grains and cause quantitative and qualitative damages such as: weight loss; loss of nutritional value; mycotoxins contamination and off-odors on produce (Baoua et al., 2015; Czembor et al., 2015; Garcia-Lara and Saldivar, 2016).

The beetles of Curculionidae family, popularly known as grain weevils, are considered the major primary pests of cereals. More than 30 species have been recorded in stored food, and among them, the *Sitophilus zeamais* (maize weevil) is the major pest of corn that occurs in tropical regions, infecting healthy grains in the field and during postharvest storage (Ukeh, 2008; Abebe et al., 2009; Napoleão et al., 2015).

The control of *S. zeamais* over the years has been performed through the use of synthetic insecticides. Nevertheless, these compounds are toxic to humans and non-target organisms and their continuous use can generate serious problems such as the emergence of resistant populations (Obeng-Ofori, 2007; Sahaf et al., 2008; Mondal and Khalequzzaman, 2010). Aiming to change this scenario and to contribute with integrated pest management programs, researchers have investigated plant preparations and isolated constituents for insecticidal activity against *S. zeamais*, for example: crude extracts (Ukeh et al., 2012; Thein et al. 2013), essential oils (Suthisura et al., 2011; Zoubiri and Baaliouamer, 2012), isolated secondary metabolites (Yang et al., 2011; Tavares et al., 2013), peptides (Mouhouche et al., 2009), and lectin (Napoleão et al., 2013). Lectins are proteins that recognize and interact

reversibly with carbohydrates and glycoconjugates. These proteins have shown deleterious effects against insects from several orders and at all development stages, interfering with growth, survival, nutrition, digestion and reproduction (Kaur et al., 2013; Paiva et al., 2013; Dang and Van Damme, 2015; Macedo et al., 2015).

*Moringa oleifera* (Moringaceae) is a tree widely distributed in the tropics, resistant to drought and able to grow in poor soils. Its seeds contain a lectin called WSMoL (water-soluble *M. oleifera* lectin), which showed antibacterial activity (Ferreira et al., 2011) and was an insecticidal agent against *Aedes aegypti* eggs and larvae (Coelho et al., 2009; Santos et al., 2012). The seed cake, a co-product of oil extraction from *M. oleifera* seeds, contains $WSMoL_C$ (water-soluble lectin from *M. oleifera* cake) which also showed toxicity against *A. aegypti* larvae and eggs as well as oviposition-stimulant activity (Oliveira et al., 2016).

This work reports the investigation of the effects of aqueous extracts from *M. oleifera* whole seeds (AE) and from the seed cake ($AE_C$) as well as of isolated WSMoL and $WSMoL_C$ on feeding, survival, and nutritional parameters of *S. zeamais* adults. Also it was investigated the effects of the lectins on activities of digestive enzymes from insect gut.

## Material and methods

### Plant material

*Moringa oleifera* seeds were collected in Recife, Pernambuco, Brazil, powdered using a blender and stored at -20 ºC. The voucher specimen is deposited under number 73,345 at the herbarium *Dárdano de Andrade Lima* from the *Instituto Agronômico de Pernambuco*, Recife, Brazil. Plant collection was authorized (number 38690-2) by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from the Brazilian Ministry of Environment.

## Preparation of extracts

Aqueous extract from whole seeds (AE) was prepared by homogenization of seed flour with distilled water, in a proportion of 10% (w/v), for 16 h at 4°C using a magnetic stirrer. The mixture was filtered through gauze and centrifuged (3000 *g*, 15 min). The supernatant corresponded to AE.

The aqueous extract from seed cake ($AE_C$) was prepared following the same methodology described above. In order to obtaining the cake, *M. oleifera* seed powder (100 g) was put in a filter paper cartridge and placed in a Soxhlet extractor. The seed oil was removed by passing n-hexane through the cartridge during 6 h and the resulting material corresponded to the cake.

Protein concentration in the extracts was determined according to Lowry et al. (1951) using a standard curve of bovine serum albumin (31.25–500 µg/mL).

## Isolation of lectins

WSMoL and $WSMoL_C$ were isolated according to the procedures described by Coelho et al. (2009) and Oliveira et al. (2016), respectively. The extracts were treated with ammonium sulphate at 60% saturation (Green and Hughes, 1955) during 4 h at 28°C. Next, the precipitated proteins were collected by centrifugation (3000 *g*, 15 min) and dissolved in distilled water. After dialysis (3.5 kDa cut-off membrane) against distilled water (4 h) and 0.15 M NaCl (4 h), lectin-rich fractions were obtained. The fractions were loaded (10 mg of protein) onto chitin columns (7.5 × 1.5 cm) equilibrated (flow rate of 20 mL/h) with 0.15 M NaCl. After washing with equilibrating solution, WSMoL or $WSMoL_C$ was eluted from the

column with 1.0 M acetic acid and dialyzed (3.5 kDa cut-off membrane) against distilled water (6 h at 4 °C) for eluent elimination.

## Hemagglutinating activity

Hemagglutinating activity was determined in microtiter plates (Kartell S.P.A., Italy) according to Paiva and Coelho (1992). The assay was performed using a suspension (2.5%, v/v) of glutaraldehyde-treated rabbit erythrocytes (Bing et al. 1967) in 0.15 M NaCl. A two-fold serial dilution of sample (50 µL) in 0.15 M NaCl was performed in the microtiter plate and then the erythrocyte suspension (50 µL) was added to each well. The assay was incubated at 28°C for 45 min. The number of hemagglutination units was calculated as the reciprocal value of the highest dilution of sample that promotes full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between titer and protein concentration (mg/mL).

## Phytochemical analysis

The extracts were evaluated for the presence of secondary metabolites by thin-layer chromatography (TLC) on silica plates (60F254, aluminum backed, 200 µm layer thickness, 8.0×5.0 cm, Merck, Darmstadt, Germany). The development systems and revealers were, respectively: EtOAc/HCOOH/AcOH/$H_2O$ (100:11:11:26 v/v) and Dragendoff's reagent for alkaloids; EtOAc/HCOOH/AcOH/$H_2O$ (100:0.5:0.5:0.5 v/v) and Lieberman-Burchard's reagent for triterpene and steroids; EtOAc/HCOOH/AcOH/$H_2O$ (100:11:11:27 v/v) and Neu's reagent for aglycone and flavonoid heterosids as well as cinnamic acid derivatives;

EtOAc/HCOOH/AcOH/$H_2O$ (100:11:11:26 v/v) and vanilin-chloridric acid for proanthocyanidins (Roberts et al., 1957; Wagner and Bladt, 1996; Harborne, 1998).

## Insecticidal assay

*S. zeamais* adults were obtained from the breeding maintained at the *Departamento de Bioquímica* from the *Universidade Federal de Pernambuco* at 28±2°C in glass containers containing maize grains and closed with TNT-type fabric. Breeding is authorized (number 36301-2) by the ICMBio/Brazilian Ministry of Environment.

Insecticidal assay was performed according to an adaptation of Xie et al. (1996) method described by Napoleão et al. (2013). For each bioassay, 5 mL of a sample (AE, $AE_C$, WSMoL or $WSMoL_C$) solution in distilled water was added to 2.0 g of wheat flour and the mixture was stirred for 5 min in order to obtain a suspension. In the control treatment, 5 mL of distilled water was mixed with the wheat flour. Next, five aliquots of 200 µL of the suspension were placed in a Petri plate (90 x 100 mm), which was then incubated at 56°C for 16 h. After this period, 20 insects were transferred to the plate and the bioassay was maintained in dark at 28±2ºC. Each assay was performed in quadruplicate and the weight of flour disks and insects was determined before the starting of test and after 7 days. Mortality rates (%) were evaluated after 7 and 10 days of experiment. The final concentrations of AE, $AE_C$, WSMoL and $WSMoL_C$ in the disks were 58.0-636.0, 123.0-615.0, 0.5-60.0 and 1.25-40 mg/g (mg of protein per g of wheat flour), respectively.

## Feeding-deterrence index (FDI) and nutritional parameters

FDI values were calculated as follows: FDI (%) = 100 × (*A* – *B*)/(*A*), where *A* is the mass of food ingested by insects in the control assay and *B* is the mass of food ingested by insects in the sample test (Isman et al., 1990). According to FDI, the samples were classified as: no-deterrent (FDI < 20%), weakly deterrent (50% > FDI ≥ 20%), moderately deterrent (70% > FDI ≥ 50%) or strongly deterrent (FDI ≥ 70%) (Liu et al., 2007).

The nutritional indices were calculated according to Xie et al. (1996) using the data obtained in the insecticidal assay after 7 days of experiment: (1) relative consumption rate = *C*/(*D* × days), where *C* is the mass (mg) of ingested food and *D* corresponds to the initial insect biomass (mg); (2) relative biomass gain rate = *E*/(*D* × days), where *E* corresponds to the biomass gained (mg) by the insects; (3) efficiency in conversion of ingested food = *E*/(*C* × 100).

Effect of extracts and lectins on digestive enzyme activities from gut of *S. zeamais* adults

Groups of 50 adults were collected and immobilized by placing them at -20 °C for 10 min. The gut of each insect was dissected by hand and immediately homogenized with 1 mL of Tris buffer (0.1 M Tris-HCl, pH 8.0, containing 0.02 M $CaCl_2$ and 0.15 M NaCl) or acetate buffer (0.1 M sodium acetate pH 5.5, , containing 0.02 M $CaCl_2$ and 0.15 M NaCl) using a 3-mL tissue grinder (Pyrex®, Corning Inc., NY, USA). The homogenates were centrifuged at 9,000 × *g* at 4 °C for 15 min. The supernatants were collected, pooled (gut extract) and evaluated for protein concentration (Lowry et al., 1951).

Trypsin activity was determined by incubating (30 min, 37 ºC) gut extract in Tris buffer (50 µl; 200 µg of protein) with 8 mM *N*-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA, 5 µl) in Tris-HCl 0.1 M pH 8.0 (145 µl). Trypsin activity was followed by measurement of

absorbance at 405 nm (Kakade et al., 1969). One unit of trypsin activity was defined as the amount of enzyme that hydrolyzes 1 µmol of BApNA per minute.

Endoglucanase and α-amylase activities were determined according to adaptations of the methods described by Li et al. (2009) and Bernfeld (1955), respectively. The sample (100 µL; 645 µg of protein) was incubated for 10 min at 50ºC with 400 µL of solutions (1%, w/v) of the substrates carboxymethylcellulose or soluble starch in sodium acetate pH 5.5 containing 0.15 M NaCl. After incubation, 500 µL of 3,5-dinitrosalicylic acid (DNS) were added and the assay was heated (100ºC, 6 min) and immediately cooled in ice (15 min). Next, the absorbance at 540 nm was measured. The amount of reducing sugars was determined using a standard curve of glucose ($Y$=0.4153$X$–0.0026; $Y$ is the absorbance at 540 nm; $X$ is the glucose concentration in mg/mL). One unit of enzyme activity was defined as the amount of enzyme that produces 1 µmol of glucose per minute.

The effect of extracts and lectins on enzyme activities was evaluated by incubating (30 min at 37 ºC) the gut extracts in Tris buffer (50 µl; 200 µg of protein) or acetate buffer (100 µL; 645 µg of protein) with different volumes of sample (AE, $AE_C$, WSMoL or $WSMoL_C$) before determination of the enzyme activities as described above. Control assay was performed by submitting only the samples to the same reaction steps.

Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and data were expressed as the mean of replicates ± SD. Significant differences between treatment groups were analyzed using Tukey's test for multiple comparison (significance at $p < 0.05$) with Action 2.4.163.322 softwares.

## Results

Incorporation of AE (specific hemagglutinating activity of 32) in diet resulted in mortality of insects after 7 days (Table 1), with $LC_{50}$ of 214.6 mg/g. There was no interference on the relative biomass gain rate and efficiency in conversion of ingested food in regard to control but the relative consumption rates were lower than that determined for control treatment (Figure 1A). FDI values were 57.2±20.2%, 65.0±5.5%, 70.8±2.8% and 91.5±12.3% for the treatments at 60.0, 115.0, 145.0 and 636.0 mg/g, respectively, indicating a moderate to strong deterrent effect. In addition to hemagglutinating activity (lectin), the extract contained saponins, phenylpropanoids, alkaloids and reducer sugars.

Mortality rates of *S. zeamais* adults that ingested $AE_C$ (specific hemagglutinating activity of 26.2) ranged from 2.5 to 10% after 7 days of experiment (Table 1). The incorporation of the $AE_C$ in diet did not interfere with biomass gain and did not alter the relative consumption rates (Figure 1B). Also, the $AE_C$ did not show deterrent effect. Phytochemical analysis also showed the presence of saponins, phenylpropanoids, alkaloids and reducer sugars in $AE_C$.

The ingestion of WSMoL (specific hemagglutinating activity of 2,240) did not have a remarkable impact on survival of *S. zeamais* adults since the mortality rates (Table 1) were not significantly different ($p > 0.05$) from control, except in treatment at 60 mg/g (12.0±2.7%). In despite of this, WSMoL was damaging to physiology of *S. zeamais* adults since the values of relative biomass gain rate and efficiency in conversion of ingested food were negative in all treatments (Figure 2A). There were no significant alterations in the relative consumption rate for all treatments in comparison with control (Figure 2A) and FDI values could not be determined since there were no significant differences ($p > 0.05$) between the amount of food ingested in treatments with lectin and in control.

$WSMoL_C$ (specific hemagglutinating activity of 93,600) was also incorporated into an artificial diet offered to the insects for 7 days. The ingestion of $WSMoL_C$ did not result in significant increase ($p > 0.05$) in the mortality of *S. zeamais* regarding the control, except in treatment at 15 and 30 mg/g (6.2±2.3% and 13.7±4.9% respectively) (Table 1). $WSMoL_C$ also did not change the relative biomass gain rate (Figure 2B). However, in the treatments with 6.25, 15 and 40 mg/g the efficiency for ingested food was negative and there were significant alterations in the relative consumption rate for all treatments in comparison with control (Figure 2B). $WSMoL_C$ exhibited FDI values of 40.5±9.2%, 74.9±4.5%, 80.4±78.3%, 92.7±75.3 and 94,4±56,7 for the treatments at 1.25, 3.75, 6.25, 15 and 40 respectively, indicating a deterrent effect.

Trypsin-like activity in extract from gut of *S. zeamais* adults was not affected by AE and $AE_C$ but α-amylase and endoglucanase activities increased after incubation with these extracts (Figure 3A and 4A). The incubation with WSMoL (Figure 3B) resulted only in increase of the trypsin-like activity in a dose-dependent manner. Trypsin-like, amylase and endoglucanase activities increased after incubation with $WSMoL_C$ (Figure 4B).

## Discussion

Crude extracts, essential oils, secondary metabolites and other biomolecules from plants have been indicated as alternatives for controlling *S. zeamais* by affecting survival and nutrition (Liu et al., 2007; Restello et al., 2009; Ootani et al., 2011; Almeida et al., 2013; Haddi et al., 2015). Seeds of *M. oleifera* have insecticidal compounds and the seed cake, the co-product generated after extraction of oil, contain compounds that can be recovered and used biotechnologically. The importance of *S. zeamais* as economic pest together with the

presence of actives compounds in the seeds of *M. oleifera* stimulated the investigations described in this work.

Similarly to the results obtained with the AE, the survival rate of *S. zeamais* adults was reduced after ingestion of artificial diet containing the leaf extract from *Myracroduon urundeuva* ($LC_{50}$ = 72.4 mg/g) (Napoleão et al., 2013). The reduction in relative consumption rate in treatments with AE and the strong feeding-deterrent effect may have contributed to the death of insects. Although the adults of *S. zeamais* have ingested a smaller amount of food, the efficiency in conversion of nutrients in biomass remained unchanged, showing that the digestibility was not damaged and that the metabolism of insects was able to adjust their growth to the reduced food intake. In this sense, the deterrent action of the extract seems to be due a pre-ingestion effect.

Since WSMoL and $WSMoL_C$ are reported to be insecticidal agents against *A. aegypti*, we investigate their effects on *S. zeamais*. Differently from *A. aegypti* larvae, the adults of *S. zeamais* were not killed by WSMoL. Similarly to WSMoL, the lectin isolated from *M. urundeuva* (MuLL) did not promote mortality of *S. zeamais* adults (Napoleão et al., 2013). Nevertheless, MuLL exerted a strong feeding-deterrent effect while WSMoL was not able to interfere with the relative consumption rate, showing no deterrent action. Although a wide range of insecticidal lectins have already been published, to our knowledge the reports on the effects these biomolecules on *S. zeamais* are restricted to this work with MuLL.

$WSMoL_C$ did not caused death of insects, similarly to WSMoL reported in this study and MuLL (Napoleão et al. 2013). However, it was observed that ingestion of $WSMoL_C$ interfered in the insect nutrition. Similar to MuLL, $WSMoL_C$ decreased the relative consumption rate showing deterrent action and reduced the efficiency in conversion of ingested food for insects at the concentration 6.25 mg/g.

Only AE induced significant death of *S. zeamais* adults but this was not linked to the lectin WSMoL. This result indicates that others compounds present in the AE, such as saponins, phenylpropanoids, alkaloids and reducer sugars, may be associated with the mortality of the insects adults caused by this extract. Although the extracts from whole seeds and cake contained the same classes of phytochemicals, the $AE_C$ did not promote significant mortality of the insects. These distinct results may be due to: different concentrations of the active principles in the extracts from whole seeds and from the cake; presence of different compounds, although belonging to the same chemical classes; or alteration in the properties of the active principle due to interaction/reaction with the solvent

Other researchers suggest the reduction in the growth and in food consumption and conversion as a triggering factor of increased mortality of *S. zeamais*. These nutritional parameters were affected negatively by the treatment with eugenol, isoeugenol and methyleugenol resulting in mortality ($LC_{50}$ = 30 mg/mg) of *S. zeamais* adults (Huang et al., 2002). Tavares et al. (2013) reported that the coumpound ar-turmerone isolated from *Curcuma longa* rhizome (Zingiberaceae) caused loss of biomass and mortality rate of adults of this species. Essential oil from *Alpinia purpurata* inflorecences reduced the biomass gain rate and the efficiency in conversion if ingested food of *S. zeamais* adults (Lira et al., 2015).

The imbalance in activity of digestive enzymes has been also reported as cause of mortality of insects, and thus we evaluated the effect of AE, $AE_C$, WSMoL and $WSMoL_C$ on trypsin-like, α-amylase and endoglucanase activity from gut of *S. zeamais*. AE and $AE_C$ did not affect the activity of trypsin-like and, indeed, the values of efficiency in conversion of ingested food suggest that the digestibility was not affected as previously discussed. On the other hand, we detected an increase of activity of trypsin-like enzymes from *S. zeamais* gut after incubation with WSMoL and $WSMoL_C$. Similarly to detected here, WSMoL also increased the trypsin like activity from gut of *A. aegypti* Rockefeller larvae and the authors

suggested that the excessive proteolytic activity may lead to imbalance of digestion and consequently result in the harmful effects of this lectin (Agra-Neto et al. 2014). The activity of trypsin-like enzymes from the midgut of *Ephestia kuehniella* larvae was reduced after treatment with the *Annona coriacea* lectin (Coelho *et al.* 2007). Napoleão *et al*. (2013) reported that MuLL was also able to interfere with trypsin-like enzymes from *S. zeamais* gut, but unlike WSMoL, this lectin reduced the enzyme activity. According to Macedo et al. (2007), lectins are able to interfere with the activity of enzymes by binding to the sugar moiety or to domains involved in catalysis in glycosylated or non-glycosylated enzymes, respectively.

In conclusion, the extract from whole seeds from *M. oleifera* contains compounds that induced mortality of *S. zeamais* adults and showed feeding-deterrent effect. Purified WSMoL and $WSMoL_C$ affected the nutritional physiology of the insects probably due to imbalance of digestion process; in addition, $WSMoL_C$ showed deterrent effect.

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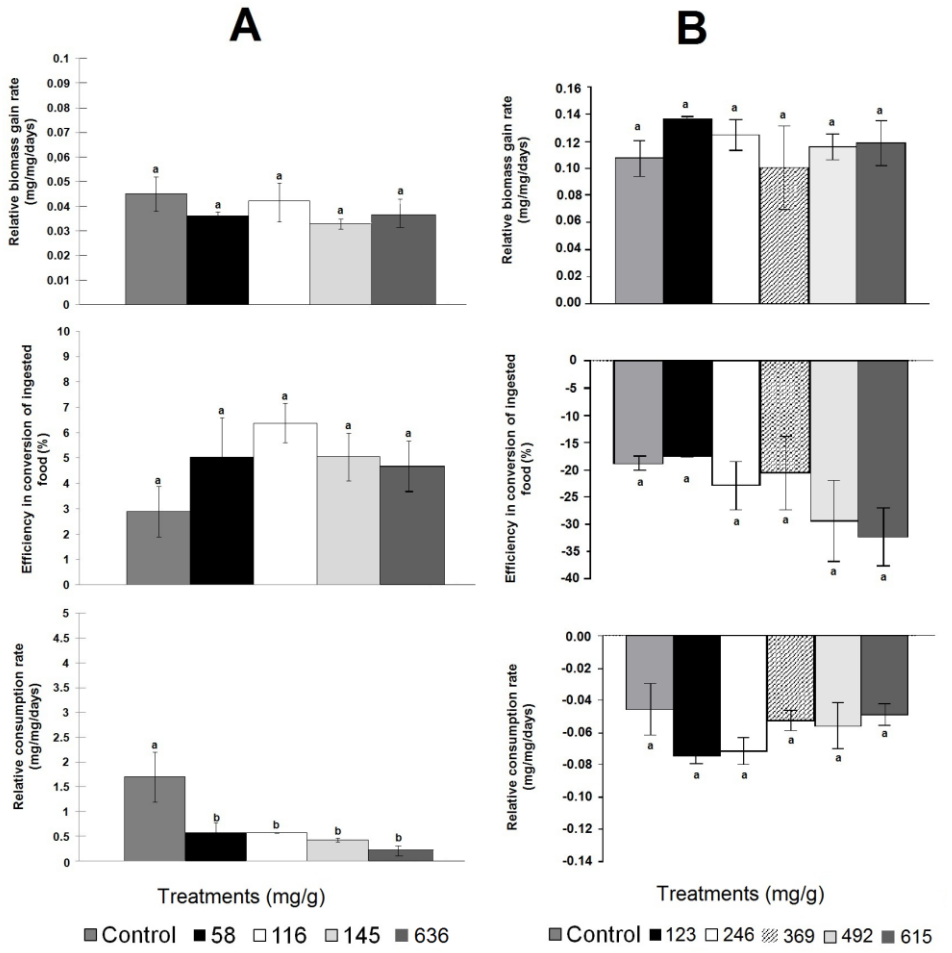


**Figure 1.** Nutritional parameters of *S. zeamais* adults reared on artificial diets containing (A) aqueous extract from *M. oleifera* whole seeds (58 to 636 mg of protein per g of wheat flour) or (B) aqueous extract from *M. oleifera* seeds cake (123 to 615 mg of protein per g of wheat flour). The relative biomass gain rate indicates the amount of biomass in mg gained every day per mg of initial body weight. The efficiency in conversion of ingested food (%) indicates the amount of ingested food incorporated by insects as biomass. The relative consumption rate indicates the amount of food consumed in mg per mg of insect body weight per day. Each bar corresponds to the mean ± SD of four replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

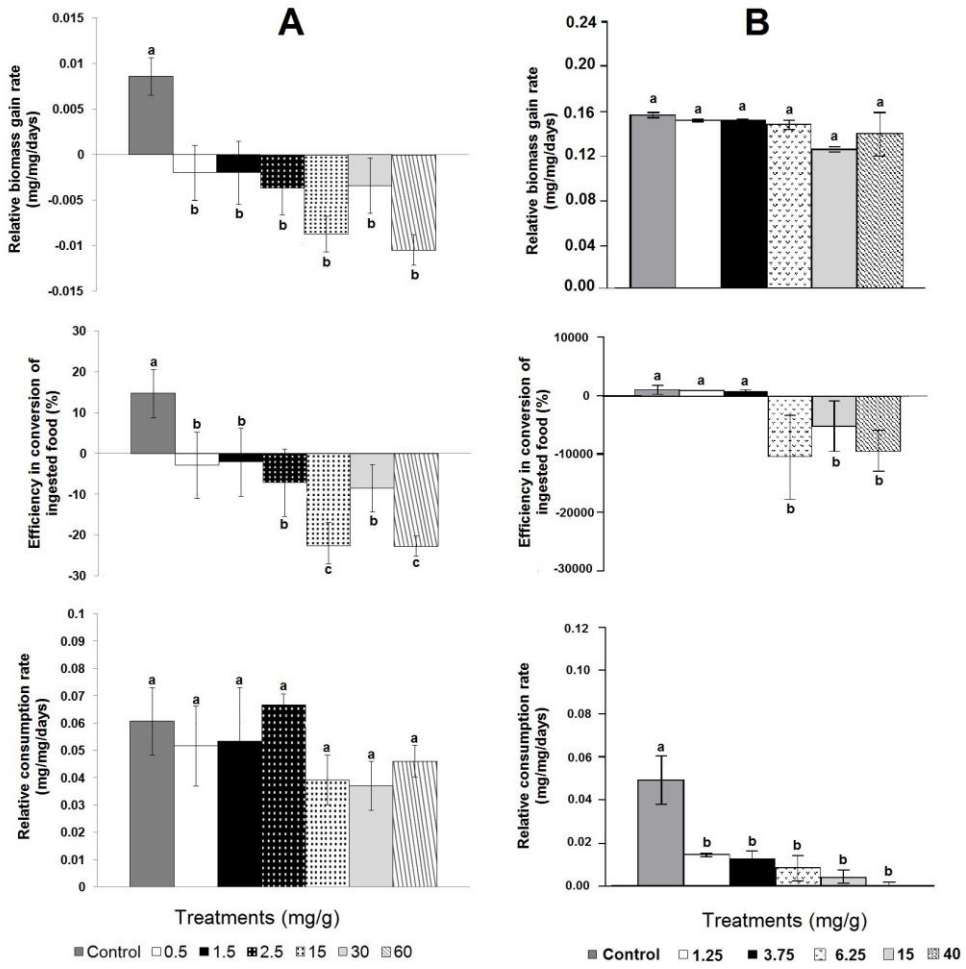


**Fig. 2.** Nutritional parameters of *S. zeamais* adults reared on artificial diets containing (A) WSMoL (0.5 to 60 mg per g of wheat flour) or (B) $WSMoL_C$ (1.25 to 40 mg per g of wheat flour). The relative biomass gain rate indicates the amount of biomass in mg gained every day per mg of initial body weight. The efficiency in conversion of ingested food (%) indicates the amount of ingested food incorporated by insects as biomass. The relative consumption rate indicates the amount of food consumed in mg per mg of insect body weight per day. Each bar corresponds to the mean ± SD of four replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

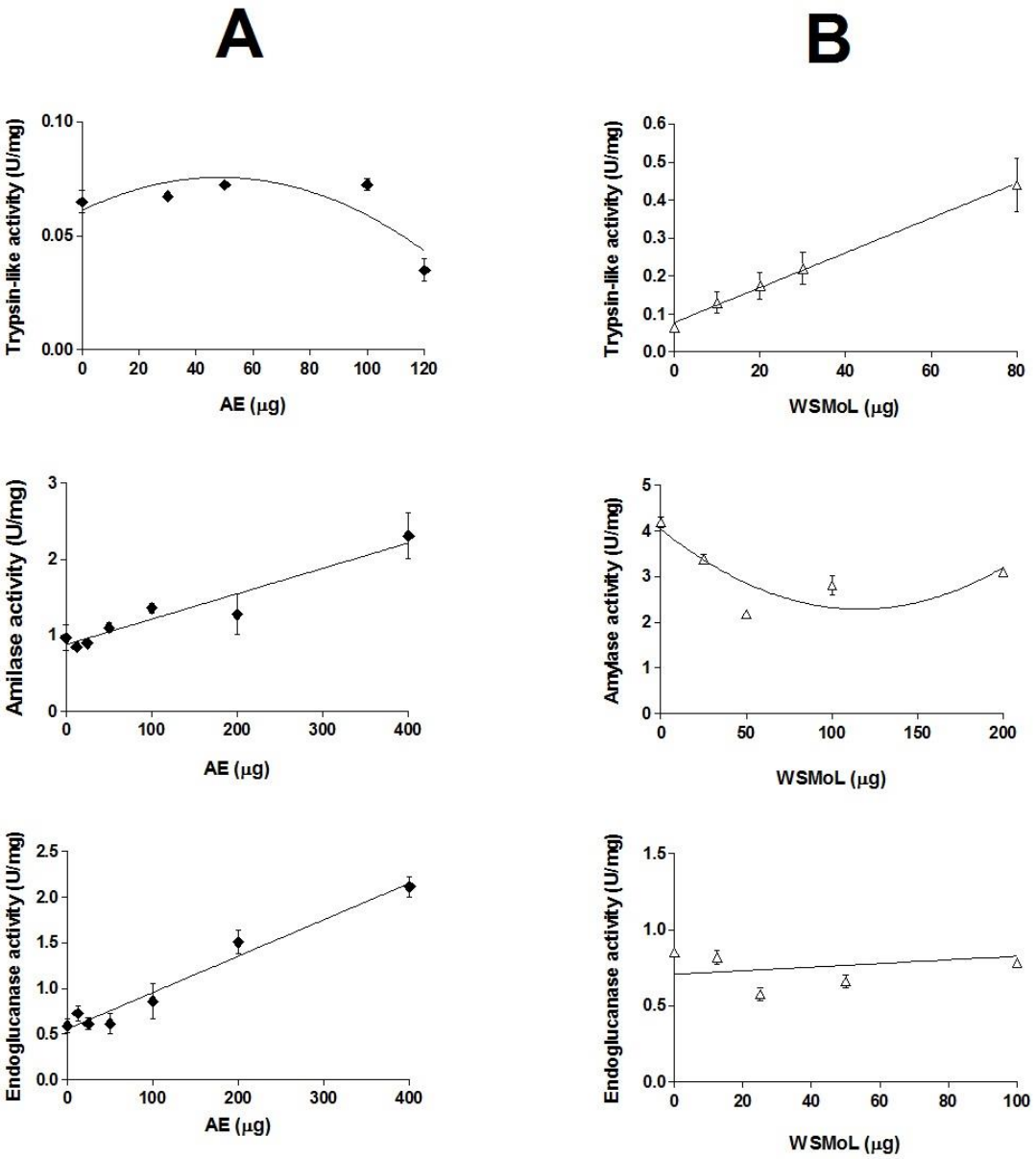


**Fig. 3.** Effect of (A) aqueous extract from *M. oleifera* whole seeds (AE), and (B) WSMoL on trypsin-like, α-amylase and endoglucanase activity from *S. zeamais* gut extracts.

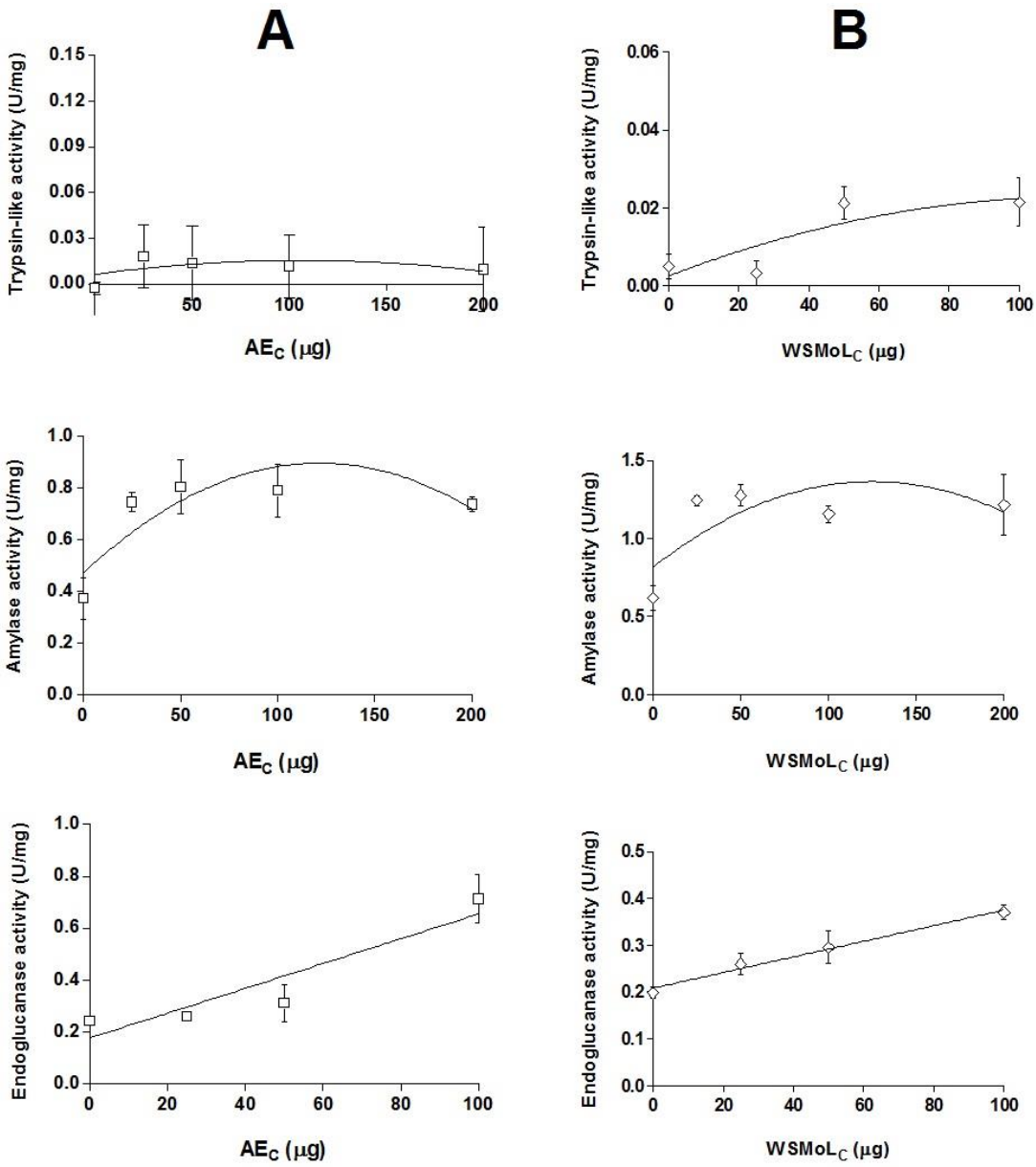


**Fig. 4.** Effect of (A) aqueous extract from *M. oleifera* seeds cake ($AE_C$) and (B) $WSMoL_C$ on trypsin-like, α-amylase and endoglucanase activity from *S. zeamais* gut extracts.

**Table 1.** Mortality rates of *S. zeamais* adults reared for 7 days on diets containing *M. oleifera* extracts and isolated lectins.

| Sample concentration (mg/g of wheat flour) | Mortality rate (%) after 7 days |
|---|---|
| AE | |
| 58 | 21.7 ± 7.0 a |
| 116 | 50.0 ± 7.0 b |
| 145 | 50.0 ± 0.0 b |
| 636 | 82.5 ± 6.4 c |
| Control | 5.0 ± 0.0 d |
| $AE_C$ | |
| 123 | 2.5 ± 2.3 a |
| 246 | 2.5 ± 2.3 a |
| 369 | 2.5 ± 2.3 a |
| 492 | 5 ± 4 a |
| 615 | 10 ± 8.6 a |
| Control | 2.5 ± 0.6 a |
| WSMoL | |
| 0.5 | 4.0 ± 1.5 a |
| 1.5 | 10.0 ± 6.1 a |
| 2.5 | 6.0 ± 2.0a |
| 15 | 5.0 ± 3.5 a |
| 30 | 5.0 ± 0.0 a |
| 60 | 12.0 ± 2.7 b |
| Control | 4.0 ± 1.5 a |
| $WSMoL_C$ | |
| 1.25 | 5 ± 4 a |
| 3.75 | 2.5 ± 2.3 a |
| 6.25 | 11.6 ± 10.1 a |
| 15 | 6.2 ± 2.3 b |
| 40 | 13.7 ± 4.9 b |
| Control | 1.25 ± 0.6 a |

AE: aqueous extract from *M. oleifera* whole seeds. $AE_C$: aqueous extract from *M. oleifera* seeds cake. Control treatments contained only wheat flour. Different letters indicate significant ($p<0.05$) differences between treatments.

# 7 CONCLUSÕES

- Lectina presente em sementes de *M. oleifera* resistiram ao processo de extração do óleo com n-hexano.
- A lectina $WSMoL_C$ foi purificada com elevada atividade hemaglutinante e apresentou perfis eletroforético e de reconhecimento de carboidratos similares à lectina WSMoL. Ainda, $WSMoL_C$ apresentou homologia em sua estrutura primária com outras proteínas de sementes de *M. oleifera.*
- A estrutura secundária de $WSMoL_C$ é predominantemente α-hélice.
- $WSMoL_C$ é uma lectina com ampla estabilidade estrutural frente a agentes desnaturantes químicos e físicos, dentre os quais ureia, temperatura e altas pressões.
- $WSMoL_C$ foi tóxica para larvas e ovos de *A. aegypti* e apresentou efeito estimulante sobre a oviposição por fêmeas desse mosquito.
- $WSMoL_C$ apresentou atividade termiticida contra soldados e operários de *N. corniger*.
- A atividade inseticida de $WSMoL_C$ contra larvas de *A. aegypti* e cupins pode estar relacionada com interferência na atividade de enzimas digestivas.
- O extrato aquoso da torta não causou a mortalidade de insetos adultos de *S. zeamais*.
- $WSMoL_C$ promoveu mortalidade e desregulou a fisiologia digestiva dos insetos, possivelmente por estimulação das atividades enzimáticas digestivas.
- A torta de moringa é uma importante fonte de lectina bioativa com notável valor biotecnológico, o que agrega valor à cadeia de produção do óleo.

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