UNIVERSIDADE FEDERAL DE PERNAMBUCO
DEPARTAMENTO DE BIOQUÍMICA
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MOLÉCULAS BIOATIVAS EXTRAÍDAS DE SEMENTES DE *Moringa oleifera*

RODRIGO DA SILVA FERREIRA

Orientadora: Profª. Drª. Patrícia Maria Guedes Paiva
Co-orientadora: Profª. Drª. Maria Luiza Vilela Oliva

Recife
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RODRIGO DA SILVA FERREIRA

MOLÉCULAS BIOATIVAS EXTRAÍDAS DE SEMENTES DE *Moringa oleifera*

Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do grau de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Aprovado com distinção por:

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Profa. Dra. Patrícia Maria Guedes Paiva (Presidente)

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Profa. Dra. Vera Lúcia de Menezes Lima, UFPE

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Profa. Dra. Maria do Socorro de M. Cavalcanti, UPE

Ata da defesa de dissertação do Mestrando **Rodrigo da Silva Ferreira,** realizada em 29 de fevereiro de 2008, como requisito final para obtenção do título de Mestre em Bioquímica e Fisiologia da UFPE.

Às 14:30 horas, do dia vinte e nove de fevereiro de 2008, foi aberto, no Auditório Prof. Marcionilo Lins – Depto. de Bioquímica, do Centro de Ciências Biológicas, da Universidade Federal de Pernambuco, o ato de defesa de dissertação do mestrando **Rodrigo da Silva Ferreira**, aluno do Curso de Mestrado em Bioquímica e Fisiologia/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. **Vera Lúcia de Menezes Lima** fez a apresentação do aluno, de sua orientadora Profa. Dra. Patrícia Maria Guedes Paiva, bem como da Banca Examinadora composta pelos professores doutores: Patrícia Maria Guedes Paiva, na qualidade de Presidente, Luana Cassandra Breitenbrach Barroso Coelho, Vera Lúcia de Menezes Lima, ambas do Depto. de Bioquímica/UFPE e Profa. Dra. Maria do Socorro de Mendonça Cavalcanti, do ICB/UPE. Após as apresentações, a Profa. Dra. Patrícia Maria Guedes Paiva convidou o aluno para a apresentação de sua dissertação intitulada: "**Moléculas Bioativas Extraídas de Sementes *de Moringa oleifera*",** e informou que de acordo com o Regimento Interno do Curso, o candidato dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas será de 30 (trinta) minutos. O aluno procedeu à explanação e comentários acerca do tema em **30 (trinta)** minutos. Após a apresentação do mestrando, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra a primeira examinadora, Profa. Dra. Maria do Socorro Mendonça Cavalcanti que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente passou a palavra para a Profa. Dra. Vera Lúcia de Menezes Lima, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, a referida professora deu-se por satisfeita. Logo após, a Sra. Presidente passou a palavra para a Profa. Dra. Luana Cassandra Breitenbrach Barroso Coelho, que agradeceu ao convite, fez alguns comentários e sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente, na qualidade de orientadora, usou da palavra para tecer alguns comentários a respeito do trabalho do aluno, agradecer à Banca Examinadora e parabenizar o candidato. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovado com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 29 de fevereiro de 2008.

Vera Lucia M. Lima.

“Não te glories do dia de amanhã”,

porque não sabes o que trará à luz.

Pv 27:1

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LISTA DE ABREVIATURAS

CD	Dicroísmo circular, do ingles “circular dichroism”.
Con A	lectina purificada de sementes de *Canavalia ensiformis*, do inglês concanavalin agglutinin”.
MoW	extrato aquoso de sementes de *Moringa oleifera*, do inglês “*M. oleifera* water”.
NAF	preparação não adsorvida da coluna de quitina, do inglês “Non-adsorbed fraction”
WSMoL	Lectina purificada de sementes de *Morinaga oleifera*, do inglês “Water soluble *M. oleifera* lectin”

Artigo

λ_{max}	fluorescence emission maximum.
ATCC	American Type Culture Collection.
CD	circular dichroism.
HA	Hemagglutinating activity.
IPA	Instituto de Pesquisas Agropecuárias de Pernambuco.
MoW	*M. oleifera* water.
NAF	non-adsorbed fraction from Chitin chromatography.
NTU	Nephelometric Turbidity Units.
WSMoL	Water soluble *M. oleifera* lectin.

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RESUMO

Moléculas bioativas têm sido isoladas de sementes de *Moringa oleifera.* As sementes têm sido usadas para tratamento da água para consumo humano devido às suas propriedades coagulantes. Adicionalmente a ausência de contaminação bacteriana em água tratada com sementes de *Moringa* tem sido descrita. Sementes de plantas são fontes de lectinas, proteínas que interagem com carboidratos e promovem aglutinação de eritrócitos. A capacidade de interação de lectinas com carboidratos resulta nas atividades antimicrobiana e inseticida detectadas nessas proteínas. Atividade hemaglutinante (AH) foi identificada no extrato de sementes de *M. oleifera* e a lectina (WSMoL) foi isolada por cromatografia em coluna de quitina. Os objetivos deste trabalho foram determinar parâmetros físico-químicos em águas tratadas com extrato aquoso de sementes (MoW), isolar WSMoL através de protocolo previamente estabelecido, caracterizar WSMoL, avaliar as atividades coagulante, antimicrobiana sobre *Staphylococcus aureus* e *Escherichia coli* e inseticida sobre *Callosobruchus maculatus de preparações de sementes de Moringa*. Para isolamento de WSMoL, o extrato de sementes (10%) foi fracionado com sulfato de amônio e a fração (F) de maior AH específica (F0-60%) foi cromatografada em coluna de quitina. WSMoL foi eluída com ácido acético 1 M. Para caracterização estrutural de WSMoL foram realizados ensaios de AH em diferentes condições experimentais, fluorescência e dicroísmo circular (DC). Parâmetros físico-químicos das águas foram alterados após tratamento com MoW. WSMoL com atividade coagulante foi isolada de outros compostos coagulantes por cromatografia em coluna de quitina. O espectro de fluorescência de WSMoL não foi alterado na presença dos ions Mg^{2+} and Zn^{2+} ions. DC de WSMoL was típico de uma proteína α-hélice. As atividades antibacteriana e inseticida foram detectadas nas preparações de *M. oleifera*. As atividades biológicas detectadas indicam o potencial biotecnológico de WSMoL.

Palavras Chave: Lectina; *Moringa oleifera*; Caracterização; Atividade antibacteriana.

ABSTRACT

Bioactive molecules have been isolated from seeds of *Moringa oleifera*. The seeds have been used to treat water for human consumption due to its coagulant properties. Additionally the absence of bacterial contamination in water treated with *Moringa* seeds has been described. Seeds of plants are a source of lectins, proteins that interact with carbohydrates and promote agglutination of erythrocytes. The ability to interaction of lectins with carbohydrates results in antimicrobial and insecticide activities found in these proteins. Activity hemaglutinanting (HA) has been identified in seed extract of *M. Oleifera* and lectin (WSMoL) was isolated by chromatography on chitin column. The objectives of this study were to determine physical-chemical parameters in water treated with aqueous extract of seeds (MoW), isolate WSMoL through previously established protocol, characterize WSMoL, evaluate the activities coagulant, antimicrobial on *Staphylococcus aureus* and *Escherichia coli* and insecticide on Callosobruchus maculates of *Moringa* seed preparations. For isolation of WSMoL, the seed extract (10%) was fractionated with ammonium sulfate and the fraction (F) with highest specific AH (F0-60%) was chromatographed on column of chitin. WSMoL was eluted with acid acétio 1 M. For structural characterization of WSMoL trials were conducted of AH in different experimental conditions, fluorescence and circular dichroism (CD). Physico-chemical parameters of water were altered after treatment with MoW. WSMoL was isolated of others coagulant compounds by chromatography on chitin column. The fluorescence spectrum of WSMoL was not altered in presence of Mg^{2+} and Zn^{2+} ions. CD spectrum of WSMoL was typical of a α-helice protein. Antibacterial and insecticide activities were found in preparations of *M. Oleifera*. The biological activities detected indicate the biotechnology potential of WSMoL.

Keywords: Lectin; *Moringa oleifera*; characterization; antibacterial activity.

1. INTRODUÇÃO

1.1 LECTINAS

Stilmarck em 1888, estudando a toxidade de extratos de *Ricinus communis* (mamona), observou que uma proteína presentes nos extratos da planta, denominada ricina, apresentava a capacidade hemaglutinante (Gaofu *et al*., 2007). No ano seguinte, 1889, Helin observou o mesmo resultado de hemaglutinação a partir do extrato tóxico de *Abrus precatorius* (jequiriti), denominando a proteína de abrina (Sharon e Lis, 1988). As proteínas que estavam presentes em plantas e eram capazes de hemaglutinar eritrócitos foram inicialmente denominadas de fitohemaglutininas, hemaglutininas, fitoaglutininas, ou aglutininas de plantas por Sharon e Lis (1988). Em 1969, Inbar e Sachs, descobriram que a lectina de *Canavalia ensiformis*, concanavalina A (Con A) aglutinava preferencialmente células malignas, foi quando realmente as lectinas tiveram um impulso em sua aplicação.

Lectinas é um grupo de proteínas que liga-se à açúcares e reconhece especificamente estruturas de carboidratos aglutinando células através de ligação à superfície celular de gliconjugados (Watanabe *et al*., 2007).

Lectinas de plantas são proteínas que possuem pelo menos um domínio não-catalítico, que liga reversivelmente e especificamente um mono-ou oligossacarídeo (Peumans e Van Damme, 1995) e têm sido amplamente utilizadas em processos médicos e biológicos (Ghosh *et al*., 1999).

As lectinas vegetais foram as primeiras a serem descobertas e seu fácil isolamento, comparado ao de outras fontes, faz com que, ainda hoje, sejam muito estudadas e caracterizadas em grande número (Rüdiger e Gabius, 2001).

Lectinas ligadoras de quitina têm sido isoladas de diversas fontes, incluindo bactérias, insetos, plantas e mamíferos e muitas delas apresentam atividade antifúngica, uma vez que a quitina é o componente-chave da parede celular de fungos (Trindade *et al.,* 2006).

1.1.2 Detecção

A presença de lectina pode ser detectada pela capacidade que apresentam de interagir com carboidratos. A atividade hemaglutinante é decorrente da interação da lectina via seus sítios de ligação com os carboidratos da membrana dos eritrócitos. Os eritrócitos utilizados para detecção podem ser de sangue humano ou de outras espécies, tratados enzimaticamente ou quimicamente (Coelho e Da Silva, 2000; Kabir, 1998; Nomura *et al.,* 1998) ou não tratados (Mo *et al.,* 1993; Sampaio *et al.,* 1998).

1.1.3 Classificação das lectinas

A interação com carboidratos, permite a classificação de lectinas em grupos de especificidade: galactose (Rameshwaram e Nadimpalli, 2007), glicose/manose (Wong e Ng, 2005), manose (Holmberg *et al.,* 2007; Gibson *et al.,* 2007, Marzi *et al*., 2007, Van de Geijn *et al*., 2007), glicose, galactose e galactosamina acetiladas

(Chumkhunthod *et al*., 2006), ácido siálico (Bhowal *et al*., 2005; Gerlach *et al*., 2002; Ratanapo *et al*., 1998), Xilose (Liu *et al*., 2006), lactose (Han *et al*., 2005), arabinose (Wang e Ng, 2005) e raminose (Nitta *et al*., 2007).

Com relação, a estrutura global, as lectinas de plantas podem ser classificadas em merolectinas, hololectinas, quimerolectinas (Peumans e Van Damme, 1998) e superlectinas (Peumans *et al*., 2001). Merolectinas são proteínas monovalentes que possuem apenas um sítio de ligação para carboidratos, desta forma não podem precipitar glicoconjugados nem aglutinam células. Hololectinas possuem 2 ou mais sítios de ligação a carboidratos, sendo idênticos ou homólogos, precipitam glicoconjugados e ou aglutinam células. Grande parte das lectinas de plantas pertencem ao grupo das hololectinas. Quimerolectinas são proteínas que apresentam um ou mais sítios para carboidratos e outro sítio independente que não liga-se a carboidrato. Superlectinas, são proteínas que apresentam pelo menos 2 sítios de ligação para carboidratos diferentes.

1.1.4 Ocorrência

Lectinas são amplamente distribuídas na natureza, podendo ser encontradas em plantas (Wong e Ng, 2005), animais invertebrados (Sun *et al*., 2007), vertebrados (Hogenkamp *et al*., 2007; Ourth *et al*., 2007) e fungos (Thakur *et al*., 2007).

1.1.5 Aplicação das lectinas

A versatilidade demonstrada pelas lectinas estimula a exploração do seu potencial aplicativo em várias áreas de pesquisas médica e bioquímica. Existem diversos estudos em que a propriedade aglutinante das lectinas é utilizada para a obtenção de dados da composição sacarídica da superfície de outros tipos celulares, como por exemplo de células tumorais, sendo utilizadas em histoquímica com marcadores de tecidos neoplásicos (Melo-Júnior *et al*., 2006; Beltrão *et al*., 2003; Brooks, 2000).

Uma ferramenta importante para identificação de oligossacarídeos específicos armazenados no interior da célula é a análise histoquímica com lectinas. Em casos de doença de armazenamento, a análise auxilia no diagnóstico ao identificar os açúcares específicos acumulados no citoplasma celular (Kader, 1997; Pinedo *et al*., 1993; Jach *et al*., 1995).

As propriedades biológicas das lectinas incluem aglutinação de células bacterianas (Zheng *et al*., 2007), agregação plaquetária (Radis-Baptista *et al*., 2006), atividade gastrointestinal (Coutiño-Rodríguez *et al*., 2001), atividade mitogênica (Zheng *et al*., 2007), antimitogênica (Liu *et al*., 2006), inseticida (Kaur *et al*., 2006; Coelho *et al.,* 2007), ação antifúngica (Yan *et al*., 2005) e antibacteriana (Tunkijjanukij e Olafsen,1998).

A seletividade das interações entre lectinas e carboidratos teve notável importância histórica pela contribuição na descoberta dos grupos sangüíneos humanos (Sharon e Lis, 2005). Como algumas lectinas são específicas para

determinado eritrócitos humanos, elas aglutinam apenas um dos tipos sangüíneos e portanto podem ser utilizadas na tipagem sangüínea (Mo *et al*., 2000).

1.2 PURIFICAÇÃO

Os métodos de separação de proteínas utilizam as propriedades que variam de uma proteína para a outra. O primeiro passo em qualquer procedimento de purificação é o rompimento das células que contêm as proteínas, liberando-as em uma solução denominada extrato bruto (Lehninger *et al*., 2006).

A partir do extrato bruto, as proteínas podem ser fracionadas por métodos tais como precipitação seletiva de proteínas com sais (Paiva e Coelho, 1992) ou elevadas temperaturas (Bezerra *et al*; 2001). Geralmente, o extrato é submetido a tratamentos que separam as proteínas em frações diferentes, baseados em alguma propriedade como carga ou tamanho, processo denominado fracionamento. As etapas iniciais do fracionamento empregam diferenças na solubilidade das proteínas, que dependem de diversos fatores como o pH, a temperatura e a concentração salina, entre outros (Lehninger *et al*., 2006). A adição de sal é um dos procedimentos mais usados em função das proteínas possuírem muitos grupos carregados, sua solubilidade depende da concentração dos sais dissolvidos logo aumentando a proporção que os sais são adicionados (*salting in*) e volta a decrescer a medida que mais sal é adicionado (*salting out*).

A cromatografia de afinidade é uma técnica amplamente utilizada. Neste processo cromatográfico a amostra é aplicada na coluna e após retirado o material

que não interagiu com o ligante pela lavagem da coluna com a solução de equilíbrio, segue-se a etapa de eluição para onde a proteína adsorvida é retirada da coluna através da alteração do pH, adição de um agente que compete com a proteína ou aumento da força iônica.

Uma das classes de ligantes que vem sendo explorada na cromatografia de afinidade para isolamento de glicoconjugados são as lectinas (Monzo *et al*., 2007). Elas são imobilizadas em suportes inertes e usadas para isolamento de compostos que contêm carboidratos, tais como glicolipídeos (Lima *et al*., 1997).

A cromatografia de afinidade para isolamento de lectina tem como base de separação a propriedade da proteína se ligar especificamente a matrizes polissacarídicas através de ligações não covalentes. Matrizes são construídas pela incorporação, a um suporte insolúvel, de um ligante, pelo qual a proteína de interesse tem afinidade. A proteína de interesse é obtida com alto grau de pureza (Sun *et al*., 2007).

1.3 DICROÍSMO CIRCULAR

Dicroísmo circular (CD) é uma técnica que permite determinar estruturas e monitorar mudanças estruturais de biomoléculas (Venyaminov and Yang, 1996). Proteínas, carboidratos e ácidos nucléicos são macromoléculas biológicas de muitas unidades opticamente ativas que exibem sinal de CD. Essas moléculas opticamente ativas interagem com a luz polarizada e provocam alteração na polarização da luz incidente. O CD detecta essa alteração através da diferença da

absorção da luz circularmente polarizada à direita e à esquerda após esta passar através de uma amostra.

A forma do espectro de CD de proteína, figura 1, depende do seu conteúdo de estrutura secundária. Permitindo que sejam determinadas as proporções de α-hélices, estruturas β, β + α, α / β e estrutura aleatória (Brahms e Brahms, 1980), Figura 2.

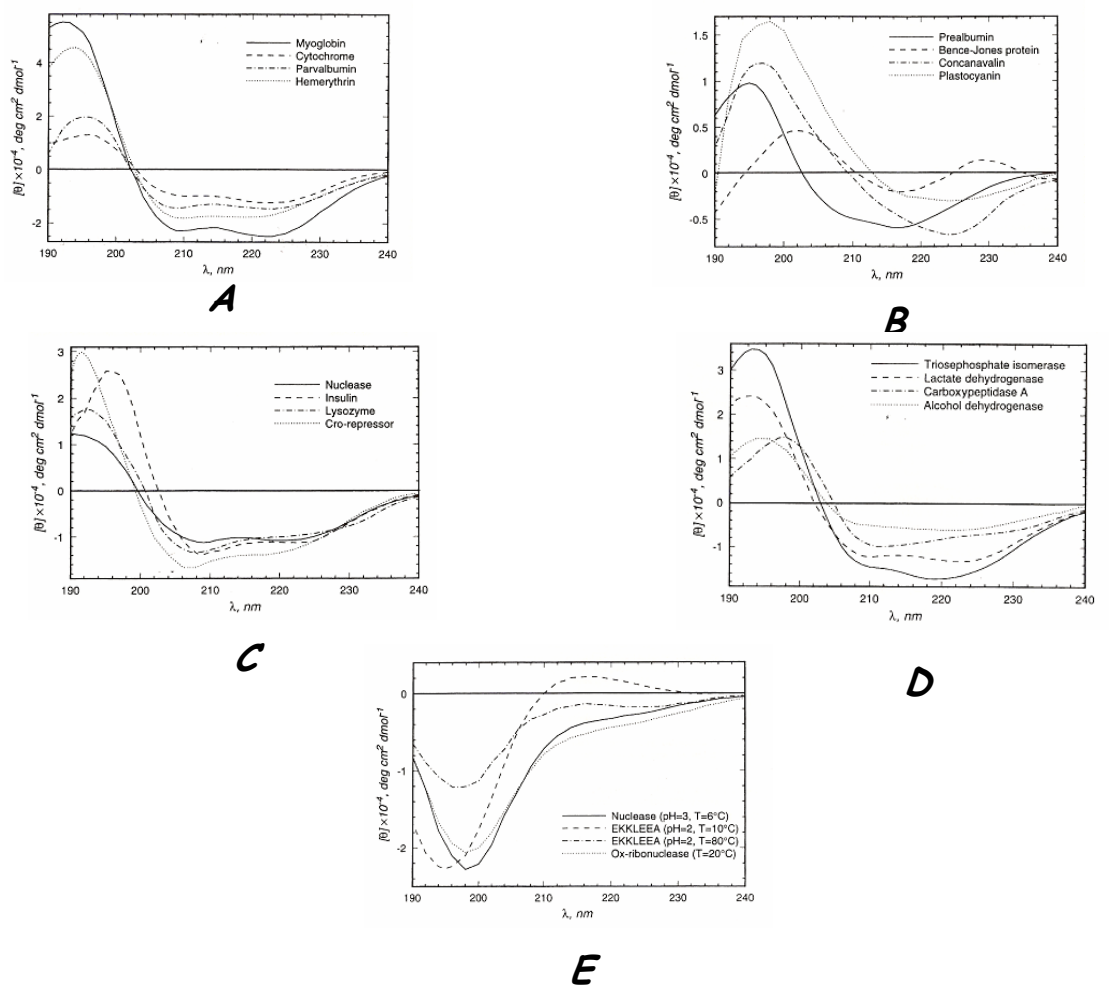


Figura 1 – Espectro de CD de proteínas toda em α-hélice **(A)**. Toda em β-folha **(B)**. Estrutura secundária em α + β **(C)**. Estruturas em α / β **(D)**. Proteínas com estrutura desordenada **(E)**. Fonte - Livro: Venyaminov e Yang, Determination of proteins secondary structures, 1996.

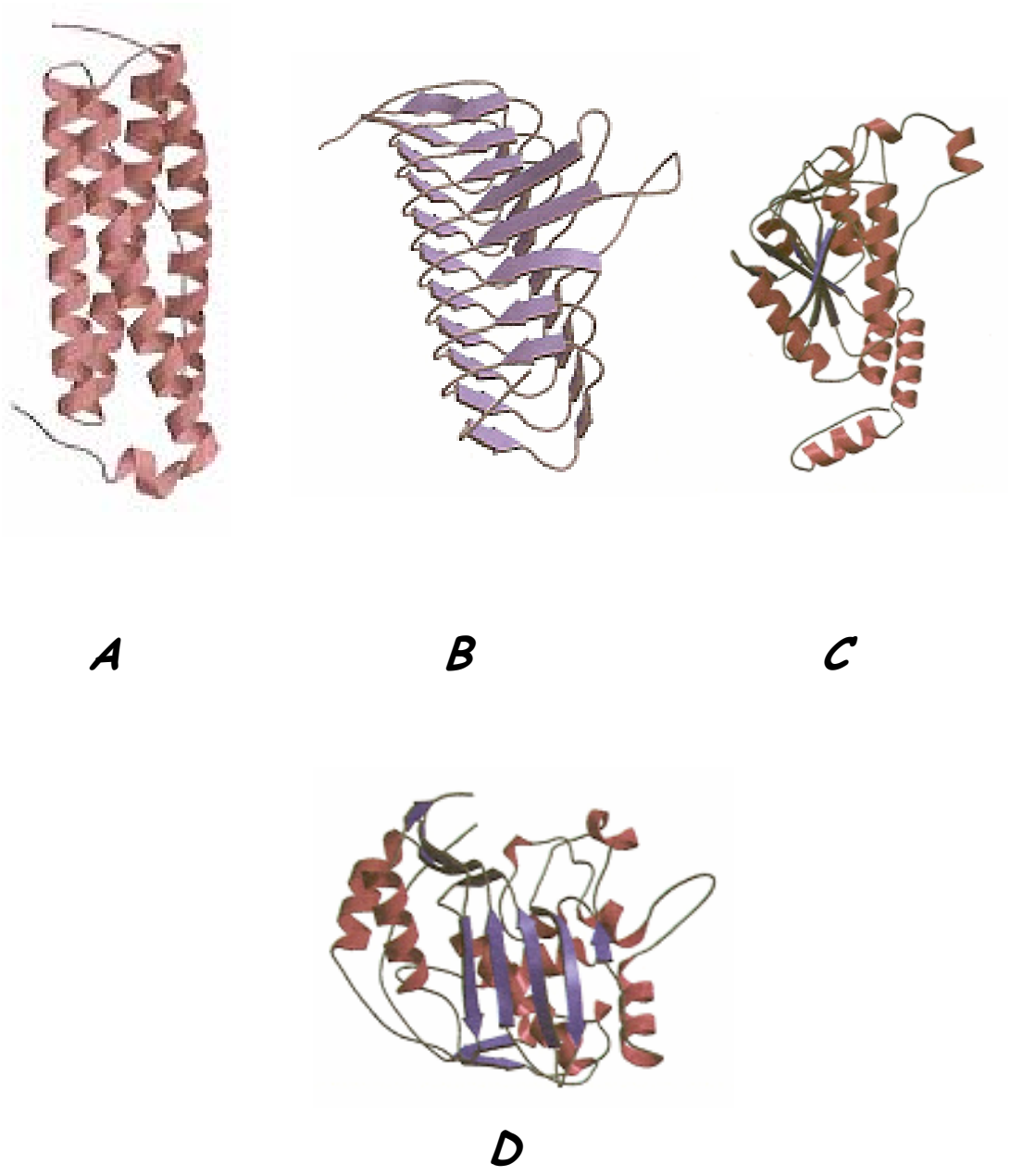


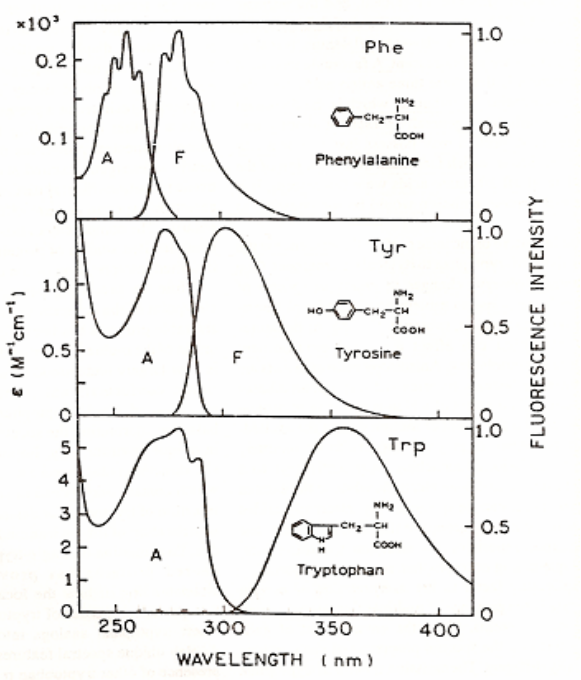
Figura 2 – Estruturas secundárias de proteínas. Proteína com estrutura toda em α-hélice **(A)**. Toda em β-folha **(B)**. Estrutura secundária em α + β **(C)**. Estruturas em α / β **(D)**.

Fonte – Livro: Lehninger, A. L., Princípios de Bioquímica, 2006.

1.4 FLUORESCÊNCIA

Em proteínas os aminoácidos responsáveis pela fluorescência são os aromáticos, tirosina, fenilalanina e triptofano. O triptofano é o aminoácido que mais contribui para o espectro de emissão nas proteínas. A emissão máxima do triptofano em água ocorre por volta de 350 nm e absorve em torno de 295 nm. A emissão máxima da fenilalanina e tirosina é na faixa de 282 e 303 nm respectivamente (Lakowicz, 1999), figura 3.

Quando esses aminoácidos não estão presentes na proteína de interesse ou eles não estão expostos nas condições estudas utiliza-se a fluorescência extrínseca. Nesse caso faz-se necessário a utilização de sondas extrínsecas que irá se ligar a proteína de interesse.



Figuras 3 – Espectros de absorção (A) e emissão (F) dos aminoácidos aromáticos em água, pH 7,0.

Fonte – Livro: Lakowicz, J. R. Principles of Fluorescence Spectroscopy, 1999.

1.5 *Callosobruchus maculatus* x *Vigna unguiculata*

O *Callosobruchus maculatus*, devido ao seu potencial depreciativo e ocorrência mundial, é considerado a principal praga do feijão *Vigna* armazenado, reduzindo o peso e a qualidade dos grãos, bem como o poder germinativo e qualidade das sementes (Dongre *et al.,* 1996). Esses fatos conduzem à necessidade de se estabelecer medidas de controle de pragas, por meio de métodos alternativos, sem desencadear os problemas causados pelos inseticidas sintéticos químicos (Faroni *et al.,* 1995).

Vigna unguiculata tem sido consumida em países em desenvolvimento da África, da Ásia e da América Latina, onde é especialmente valioso como fonte de proteínas, vitaminas e minerais (Singh *et al*. 2003).

Várias classes de proteínas têm sido sugerido como mecanismo de resistência contra patógenos de plantas (Shewry e Lucas 1997). Essas proteínas incluem lectinas (Peumans e Van Dame 1995), quitinases (Chang *et al*. 1995), inibidores de proteinases (Geoffroy *et al*. 1990), proteinases (Pinedo *et al*. 1993) e proteínas inativadoras de ribossomos (Jach *et al*. 1995).

1.6 *Moringa oleifera*

Moringa oleifera (Lam.) é uma planta tropical pertencente à família Moringaceae. Tecidos da planta como folhas e vagens têm elevado valor nutricional e têm sido consumidos pela população (Chumark *et al*., 2007).

As sementes de *M. oleiferaI*, Figura 4, apresentam propriedade coagulante e têm sido utilizadas como método alternativo para tratamento de água, Figura 5.

A facilidade de cultivo em regiões de baixa precipitação pluviométrica, o não requerimento de manejo agrícola para cultivo e a eficiência na redução da turbidez da água barrenta têm levado ao uso das sementes. O método tem sido estimulado por organizações não governamentais que distribuem as sementes e orientam o seu uso.

A Moringa oleifera tem sido amplamente estudada (Katayon *et al*., 2006; Bhuptawat *et al*., 2007) devido as suas propriedas floculantes (Ghebremichael *et al*., 2005; Gassenschmidt *et al.*, 1995), antioxidantes (Santos *et al*., 2005) e diminuição da formação de placas ateroscleróticas em coelhos (Chumark *et al*., 2007).

Figura 4 – Sementes de *Moringa oleifera*

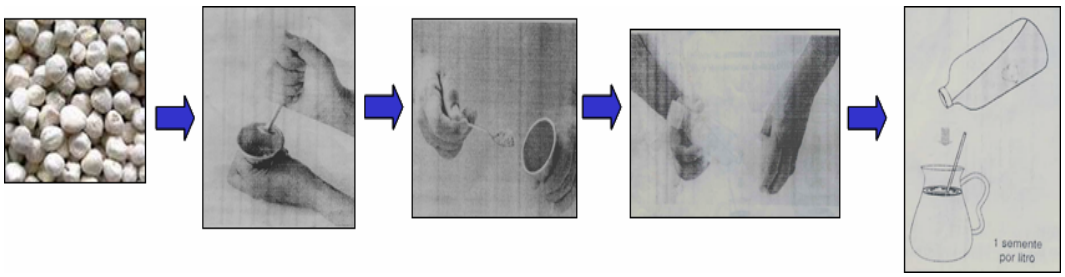


Figura 5 – Preparação da água de Moringa e tratamento de água não potável.

2 OBJETIVOS

2.1 Objetivo Geral

Extração e caracterização da lectina (WSMoL) e avaliações de atividades biológicas de moléculas bioativas extraídas de sementes de *moringa oleifera*.

2.2 Objetivos Específicos

1. Determinar parâmetros físico-químicos de amostras de águas tratadas com extrato aquoso (MoW) de sementes de *M. oleifera*.

2. Isolar WSMoL através de cromatografia em coluna de quitina.

3. Avaliar as atividades coagulantes e antimicrobiana de MoW e de preparação não adsorvida (NAF) e adsorvida (WSMoL) a coluna de quitina.

4. Caracterizar WSMoL por determinação da fluorescência, dicroísmo circular.

5. Atividade inseticida de WSMoL.

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4. ARTIGO

ARTIGO A SER SUBMETIDO À REVISTA INTERNACIONAL "WATER RESEARCH"

Bioactive molecules extracted from *Moringa oleifera* seeds

Bioactive molecules extracted from *Moringa oleifera* seeds

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Abstract

Seeds of *Moringa oleifera* contain lectin (WSMoL) and have been used to treat water for human consumption due to its coagulant property. This work describe the physical-chemical parameters of distilled or lake waters treated with *M. oleifera* seed extract (MoW), the separation of coagulant compounds by chromatography, the fluorescence and circular dichroism (CD) of WSMoL as well as antibacterial and insecticide activities from seeds. Physico-chemical parameters such as turbidity, conductivity, hardness, chloride and sulphate concentration as well pH were altered in waters treated with MoW. Chitin chromatography isolated WSMoL with coagulant activity of other coagulants present of non-adsorbed fraction (NAF). CD spectrum revealed WSMoL as a α-helice protein. Mg^{2+} increased WSMoL hemagglutinating activity but fluorescence and CD spectrum of WSMoL was not altered at presence of Mg^{2+} and Zn^{2+} ions. WSMoL showed antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* while MoW and NAF were only active on *S. aureus.* WSMoL was insecticide on *Callosobruchus maculatus*.

Keywords: Lectin; *Moringa oleifera*; characterization; antibacterial activity.

1. Introduction

Seeds of *M. oleifera* are widely used as an alternative method of water treatment and coagulant molecules such as organic polyelectrolyte and protein were already isolated from seeds. Proposed coagulation mechanisms to involve ionic interactions between coagulant molecule and its counter ions on the particles in suspension leading to formation of insoluble matter (Gassenschmidt et al., 1995; Ndabigengesere et al., 1995; Okuda et al., 2001a; Ghebremichael et al., 2005). The coagulant activity from *M. oleifera* seeds was detected using synthetic or natural turbid waters (Gassenschmidt et al., 1995; Okuda et al., 2001b).

Seeds of plants are source of lectins, proteins that interact with carbohydrates and promote the agglutination of erythrocytes used for its detection. The ability to bind carbohydrates makes them active proteins in biological processes involving cell-cell interaction. Antimicrobial activity has been speculated be due to interaction of lectin with teicoic and teicuronic acids, peptidoglycans and lipopolysaccharides present in cellular bacteria walls (Ratanapo et al., 2001). Chitin binding lectins have shown insecticide activity due its interaction with chitin present on perithrophic membranes of insects. Toxics effect has been described on Coleoptera, Hemiptera, Homoptera and Lepidoptera (Macedo et al., 2004; Habibi et al., 2000; Powell et al., 1998).

Water soluble *M. oleifera* lectin, WSMoL, was detected in aqueous extract of seeds (Santos et al., 2005). The objectives of this study are to determine physical-chemical parameters in distilled or lake waters treated with aqueous extract of seeds (MoW), separate coagulant molecules present in the seeds, characterize WSMoL by fluorescence and

circular dichroism (CD) assays and to evaluate coagulant, antibacterial and insecticide activities from different preparations from seeds.

2. Materials and Methods

2.1 Protein evaluation

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

2.2 Hemagglutinating activity (HA)

Hemagglutinating activity (HA) of was evaluated according to Santos et al. (2005) using glutaraldehyde-treated rabbit erythrocytes. The HA was obtained by mixing a twofold serial dilution of samples (50 μl) in 0.15 M NaCl followed by the addition of a 2.5% (v/v) suspension of erythrocytes (50 μl), in microtiter plates (Kartell S. P. A., Italy) and incubation (45 min). HA was also performed by mixing a twofold serial dilution of samples (50 μl) in 0.15 M NaCl containing 5, 10, 20 or 30 mM $MgCl_2$ or $ZnCl_2$. Titer was defined as the lowest sample concentration, which showed hemagglutination. Specific HA (SHA) was calculated from the ratio of titer to protein concentration (mg ml^{-1}).

2.3 M. oleifera water (MoW)

Mature seeds from *M. oleifera* were collected in Recife city, State of Pernambuco, Brazil Northeast. Taxonomic identification was performed and voucher specimens were

deposited under number 73,345 (IPA – Instituto de Pesquisas Agropecuárias de Pernambuco).

M. oleifera water was obtained according to protocol for treatment of turbid water used by Brazilian people. Macerated shelled seeds (0.2 g) were added to distilled water (1000 ml) and manual agitation (5 min) was made. Following, this *M. oleifera* suspension was through on gauze. The filtered suspension (MoW) was then immediately used. Additional extracts were prepared by MoW 0.2 g l^{-1} dilution in distilled water for 0.1 and 0.05 g l^{-1}.

2.4 Physical-chemical analysis of waters treated with MoW

Water was taken of lake in the Federal University of Pernambuco, Recife city, Brazil Northeast. MoW (0.2 g l^{-1}, 1000 ml) was added to lake water (1000 ml) or distilled water (1000 ml) and manual agitation (5 min) was made. Following, the mixtures were kept at rest by 3 h for decanting of organic matter in suspension and the supernatants as well as distilled or water lake without MoW were used to physical-chemical measurement as described in the Standard Methods, 1998.

2.5 Chromatography on Chitin column: Non-adsorbed fraction (NAF) and WSMoL separation

M. oleifera seeds were dried at room temperature (26° C). Once dried, the seeds were milled to a fine powder (10 g) that was then homogenised in a magnetic stirrer (16 h at 4°C) with distilled water (100 ml). Following the mixture was filtered through gauze and centrifuged at 3,000 x *g* (15 min). The supernatant, extract, was treated with ammonium

sulphate at saturation of 60% (Green and Hughes, 1955) and the precipitated protein (0-60 fraction) was collected by centrifugation, dissolved in 0.15 M NaCl and furthermore submitted to dialysis (5 kDa cut-off membrane) against 0.15 M NaCl (6 h at 4°C). The dialysed 0-60 fraction (50 mg of proteins) was then applied onto a chitin column (18 x 1.5 cm) equilibrated with 0.15 M NaCl (0.3 ml min^{-1} flow rate). After extensive washing with the equilibrium solution until absorbance at 280 nm lesser than 0.025 (non adsorbed fraction, NAF), adsorbed WSMoL was eluted with 1.0 M acetic acid and dialysed (5 kDa cut-off membrane) against distilled water (6 h at 4°C). Spectrophotometry at 280 nm was used to follow protein elution.

2.6 Coagulant activity of MoW, NAF and WSMoL

Coagulation assay was performed according to Ghebremichael *et al*. (2005). Initially, synthetic turbid water was prepared. Distilled water (1 l) was treated with kaolin clay (10 g), stirred for 30 min and allowed to settle for 24 h to achieve complete hydration. Desired turbidity was obtained by dilution. Aliquot (0.3 ml) of MoW (0.2 g l^{-1}), MoW (0.1 g l^{-1}), MoW (0.05 g l^{-1}), NAF or WSMoL containing 1 mg ml^{-1} of protein as well as 5% aluminium sulphate (positive control) was added to clay suspension (2.7 ml, 250-300 NTU, Nephelometric Turbidity Units). Samples were allowed to settle for 1 h at 27 °C. In order to reduce background effect, a sample volume of 900 µl from the top was transferred to the cuvette and absorbance measured at 500 nm using a UV-Visible spectrophotometer FEMTO 700 S corresponded to initial absorbance (time 0). Then, absorbance was determined every 5 min up to 60 min and to each 10 min up to 140 min. Reduction in

absorbance relative to control defines coagulation activity. The assays were performed three times.

2.7 Antibacterial activity of MoW, NAF and WSMoL

Antibacterial activity of *M. oleifera* preparations was evaluated on Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *E. coli* (ATCC 25922). Stationary cultures were maintained into Nutrient Agar (NA) and stored at 4 ºC. Bacteria were cultured in Nutrient broth and incubated at 37 ºC by 3 h. Following 200 µL of MoW, NAF (1 mg ml-^{1}) or WSMoL (1 mg ml-^{1}) were added to 200 µL of each incubation medium or milli-Q water (negative control) and the mixtures were shaking and incubated under at 37 ºC by 12 h. Muller Hinton medium (20 ml) were distributed in sterile Petri plates (90 x 15 mm) and allowed to solidify. After 50 µL of top or sediment of each mixture above described were distributed in the Petri plates and incubation at 37º C by 12 h was performed. The effect of *M. oleifera* preparations on bacterial growth was than observed compared to control.

2.8 Fluorescence spectroscopy of WSMoL

Fluorescence measurements were realized on a Hitachi F2500 spectrofluorimeter. Quartz cuvettes of 1 cm path length were used for the measurements. The excitation wavelength was 295 nm and the emission spectra were recorded in the range 310-450 nm as an average of four scans. WSMoL concentration 0.05 at 280 nm in sodium phosphate buffer 10 mM, pH 7.0 and in the presence of 0.5 M Mg^{+2} and Zn^{+2} ions were analyzed.

2.9 Circular dichroism of WSMoL

Circular dichroism (CD) data were performed using a Jasco J-810 spectropolarimeter (Jasco Corporation, Japan) in the wavelength range of 190–250 nm as an average of 8 scans. The samples for CD experiments were the same used in the fluorescence experiments. All measurements were made at a lectin concentration of 0.05 at 280 nm.

2.10 Insecticide activity of WSMoL

Insecticide activity was evaluated on *Callosobruchus maculatus*. Artificial seeds were made mixing WSMoL (0.008 g) and *Vigna unguiculata* seeds flour (0.392 g) using a hand press. Control artificial seed (0.4 g) contained no WSMoL. Each treatment had one artificial seeds and was replicated three times. The seeds were offered to fertilized females and after allowing 24 h for oviposition, the number of eggs per seed was reduced to three. Following incubation for 18 days at 28˚C, the seeds were opened and the mass and number of larvae were determined.

3. Results and discussion

Seeds of *M. oleifera* contain active compounds involved in its known coagulant property that is widely used for drinking water treatment (Gassenschmidt *et al*., 1995; Ndabigengesere *et al.*, 1995; Okuda *et al*., 2001a; Ghebremichael *et al.*, 2005). Additional advantage for use of seeds is the decreasing of bacterial contamination detected in the water treated with the seeds (Ghebremichael *et al*., 2005).

Physical-chemical parameters were determined in waters before and after treatment with MoW (Table 1). Turbidity of lake water was decreased after treatment probably due to coagulant property of MoW. When distilled water was treated its turbidity was increased reflecting the presence of organic components extracted from seeds. This variation in the concentration of organic material into these two waters after treatment was also detected by measuring the conductivity that decreased in the lake water and increased in the distilled water. The measurement of hardness of the water was evaluated and was detected that after treatment it was reduced in the water lake and increased in the distilled water. The concentration of ions was changed, reducing chloride and increasing sulphate. The pH was also changed; the determined values were lower in distilled and lake waters treated with MoW than that without treatment. The decreasing of water lake turbidity occurred at pH 7.53 (table 1) and thus at pH value lower than that given to organic polyelectrolyte isolated from *M. oleifera* seeds that was able to remove water lake turbidity at pH 9.0 (Okuda et al. 2001b).

MoW (0.2 g l^{-1}), the seed preparation obtained according protocol used by people for water treatment, had SHA of 6.0 revealing the presence of lectin. Santos et al. (2005) reported the presence of WSMoL in extracts obtained by water soaking of *M. oleifera* intact seeds by 5, 15 and 37 h by determination of SHA of 0.9, 0.1 and 0.15, respectively. Thus the protocol indicated for water treatment and used here to prepare MoW yielded more active WSMoL.

Aiming to separate from *M. oleifera* seeds active components soluble in water protocol was established using seed flour, protein extraction with water, protein fractionation by treatment of extract with ammonium sulphate and chromatography of

hemagglutinating 0-60% fraction on chitin column. The chromatographic matrix was able to isolate WSMoL (eluted fraction) of non-hemagglutinant compounds collected in the NAF (Figure 1). The interaction between WSMoL and chitin, polysaccharide matrix, probably involved carbohydrate-binding sites present in WSMoL, similarly occurred in the purification of lectin by affinity chromatography on polysaccharide support (Trindade et al., 2006).

The chitin chromatography yielded WSMoL with higher SHA (4,096) than that detected in the extract (3.5) or ammonium sulphate fraction (56). The SHA of WSMoL (4,096) was increased at presence of 20 and 30 mM Mg^{+2} for 8,192 and 16,384, respectively. Zinc ion interfered on HA assay promoting erythrocytes dispersion and thus the SHA determination was not possible.

The coagulant activity of MoW at concentrations (g l^{-1}) 0.2, 0.1 and 0.05, NAF and WSMoL were evaluated using synthetic turbid water. Kaolin water absorbance at absence of *M. oleifera* preparations (negative control) was maintained at all investigated period. It was detected clarification of water by all tested samples except MoW 0.05 g l^{-1} (Figure 2). This result indicates that the protocol used to obtain MoW extracted coagulant compounds that probably promoted a reduction in turbidity of the lake water (Table 1). MoW preparation of highest protein concentration (0.2 g l^{-1}) was more efficient in reduce the water turbidity than the others more diluted MoW (Figure 2). Coagulant activity was also detected in NAF and WSMoL. The results revealed that chitin chromatography was able to separate WSMoL with coagulant property of others coagulant molecules already described (Okuda *et al*., 2001a; Gassenschimdt *et al*., 1995; Nadabgengesere *et al.*, 1995;

Ghebremichael *et al.*, 2005). WSMoL showed activity higher than NAF and similar to that detected for aluminium sulphate (positive control).

The figures 3 and 4 show that preparations from *M. oleifera* seeds were effective in reducing the concentration of *E. coli* and *S. aureus*. Muller Hinton medium containing the suspensions treated with MoW (Fig. 3 1B, 2B; Fig. 4 1B, 2B), WSMoL (Fig. 3 1C, 2C; Fig. 4 1C, 2C) or NAF (Fig. 3 1D, 2D; Fig. 4 1D, 2D) had a smaller number of colonies compared suspension treated with water (Fig. 3 1A, 2A; Fig. 4 1A, 2A).

Differences were found in relation to the effect of the samples from the top or sediment in the growth of bacteria Gram positive and Gram negative. Decrease in the concentration of *E. Coli* was detected for WSMoL from top (Fig. 3 1C) while WSMoL from sediment (Fig. 3 2C) as well as MoW and NAF from top (Fig. 3 1B, 1D) and sediment (Fig. 3 2B, 2D) showed pattern of growth similar to the control. When *S. Aureus* was assessed was observed that MoW (Fig. 4 1B, 2B), WSMoL (Fig. 4 1C, 2C) and NAF (Fig. 4 1D, 2D) from top and sediment were effective in reducing the concentration of bacteria detected by the smaller number of colonies in relation to the control (Fig. 4 1A, 2A). The result shows that *M. oleifera* seeds contain different antimicrobial agents that were separated from each other by chromatography on chitin column. WSMoL was able to inhibit Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacteria while NAF was only active on *S. Aureus*. Additionally the antimicrobial assay revealed that the protocol used to prepare MoW extracted only the component active on *S. Aureus*. It has been reported that the protein coagulant of molecular mass 6.5 kDa isolated from *M. oleifera* seeds has the ability to reduce microbial populations as a flocculant (Ghebremichael *et al.*, 2005). Therefore it is possible that the antimicrobial activity detected in NAF is due to the

presence of this compound. Surface water normally contains high turbidity and microorganisms that cause illness. *E. coli* and *S. aureus* are bacteria that cause diarrhoea in humans. The effect of WSMoL on both strains may contribute to the decreasing of bacterial contamination of water treated with *M. oleifera* seeds already reported (Ghebremichael *et al*., 2005). Antibacterial activity against *S. aureus* was already described for lectin isolated from *Eugenia uniflora* seeds and has been reported that the binding of legume lectins to muramic acid and *N*-acetylmuramic acid present in the bacterial cell wall result in antimicrobial activity (Ayouba et al., 1991).

WSMoL exhibited a fluorescence emission maximum (λ_{max}) about 346 nm upon excitation at 295 nm typical of highly exposed tryptophan residues (Lakowicz, 1999). As shown in Figure 5 the fluorescence data of the lectin conformation did not alter in presence of ions tested (λ_{max} 346 nm). The presence the ions did not bring any appreciable change in tryptophan environment because tryptophan fluorescence can be selectively excited at 295-305 nm (Lakowicz, 1999). Thus, the protein may not have specific binding sites for Mg^{+2} and Zn^{+2} ions since the absence of any shift in the fluorescence emission maximum indicates that its structure is not sensitive to the tested ions.

Coagulant protein of 13 kDa isolated from *M. Oleifera* seeds showed no change or change of fluorescence when the experimental conditions of the medium were altered. The coagulant exhibited no significant change in the protein fluorescence intensity in ionic solutions with different concentrations (Kwaambwa and Maikokera, 2007) while showed changes of fluorescence intensity in the presence of SDS on excitation at 295 nm. The later results indicate that the tryptophan environment in the coagulant protein changes due to strong interaction with SDS (Maikokera and Kwaambwa, 2007).

The emission proteins is dominated by tryptophan, which absorbs at the longest wavelength, because of its long wavelength, energy absorbed by phenylalanine and tyrosine residues is often transferred to the tryptophan residues in the same protein (Lakowicz, 1999). The aromatic amino acid fluorescence of proteins is a sensitive probe for studying conformational transitions (Feis et al., 2004). The data of fluorescence intensity and fluorescence emission maximum of tryptophan residues to protein are susceptive to local environment of tryptophan (Sultan and Swamy, 2005).

CD spectra of WSMoL shows a strong double minimum at 220-225 nm and 208-210 nm and a stronger maximum at 190-195 nm (Fig. 6), which are characteristic of an α helix (Venyaminov, 1996). Lectins show distinct spectroscopic properties (Okuda *et al*., 2001). Indeed, the CD revealed a broad negative trough centered around 218 nm and a negative- to- positive crossover at 203 nm of the lectin BmoLL (from *Bauhinia monandra*), but the shape of Con A (Concanavalin A) spectrum confirmed its typical *β*-plated sheet-rich structure (Andrade et al., 2005). A lectin MoL isolated from *M. oleifera* seeds is an alpha-beta protein (Katre et al., 2008). The secondary structure of WSMoL was not affected by Mg^{+2} and Zn^{+2} ions, which are present in large quantities in polluted waters. Evaluation of WSMoL structure by fluorescence and CD studies to suggest that WSMoL may not have sites for Mg^{+2} and Zn^{+2} ions and thus the highest HA of WSMoL at Mg^{+2} presence was probably due to stabilization of the link between amino acids of the lectin site and carbohydrates of the erythrocyte surface promoted by ion (Delatorre et al., 2006).

Insecticide activity of WSMoL on *C. maculatus* was detected by absence of larval development at presence of *V. unguiculata* seed flour enriched with 2% WSMoL. The larvae in artificial seed control containing only *V. unguiculata* seed flour weighed 0.0240 g.

Based on studies with plant lectins that showed insecticide activity, the effect of WSMoL on *C. maculatus* probably involved the interaction between carbohydrate binding site of lectin and glycoconjugates from epithelial cell surfaces in the digestive tract of insects (Macedo et al., 2004; Gatehouse et al., 1998; Sauvion et al., 2004). It has been reported that the susceptibility of common bean (*Phaseolus vulgaris* L.) to *Zabrotes subfaciatus* infection was correlated with the lectin content of the bean but not with seed hardness, seed coat thickness, tannin, trypsin inhibitor or protein content (Guzman-Maldonado et al., 1996).

The protocol used by people for water treatment was effective for extraction of antibacterial, coagulant, hemagglutinating and insecticide properties from *M. oleifera* seeds. The detection of coagulant as well as antibacterial activity of NAF and WSMoL indicates the presence of different bioactive molecules in *M. oleifera* seeds that was can separate by chromatography on chitin column. The effect of WSMoL on lake water turbidity and on *E. coli* and *S. aureus* growth it is an evidence of lectin as molecule involved in the water treatment effect. Although the precise mode of insecticidal action of plant lectins is not fully understood it is possible that WSMoL abolished the larval development due its chitin binding property.

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Table 1. Physical-chemical parameters determined in waters before and after treatment with MoW.

Water	Turbidity (NTU)	Conductivity ($\mu s\ cm^{-1}$)	Hardness ($mg\ ml^{-1}$)	Chloride ($mg\ ml^{-1}$)	Sulphate ($mg\ ml^{-1}$)	pH
Lake	21.49	250.0	66.5	52.45	8.66	8.02
Lake + MoW	13.09	195.6	35.0	48.99	9.68	7.53
Distilled	0.11	37.0	2.5	11.35	2.59	7.40
Distilled + MoW	5.69	49.8	4.5	3.95	3.93	6.43

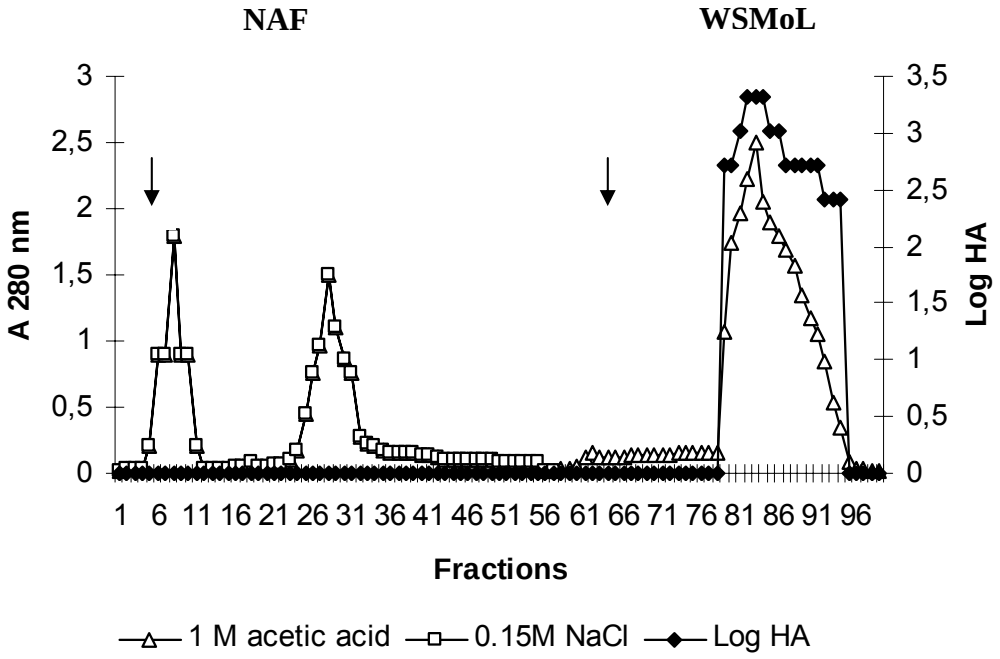

Fig 1. Chromatography on Chitin column: Non-adsorbed fraction (NAF) and WSMoL separation.

A sample of dialysed 0-60 fraction (50 mg of proteins) was applied to the column (18 x 1.5 cm) equilibrated with 0.15 M NaCl (0.3 ml min^{-1} flow rate). Arrows indicate the addition of eluents. Fractions of 2.0 ml were collected. Absorbance at 280 nm (○), HA (●).

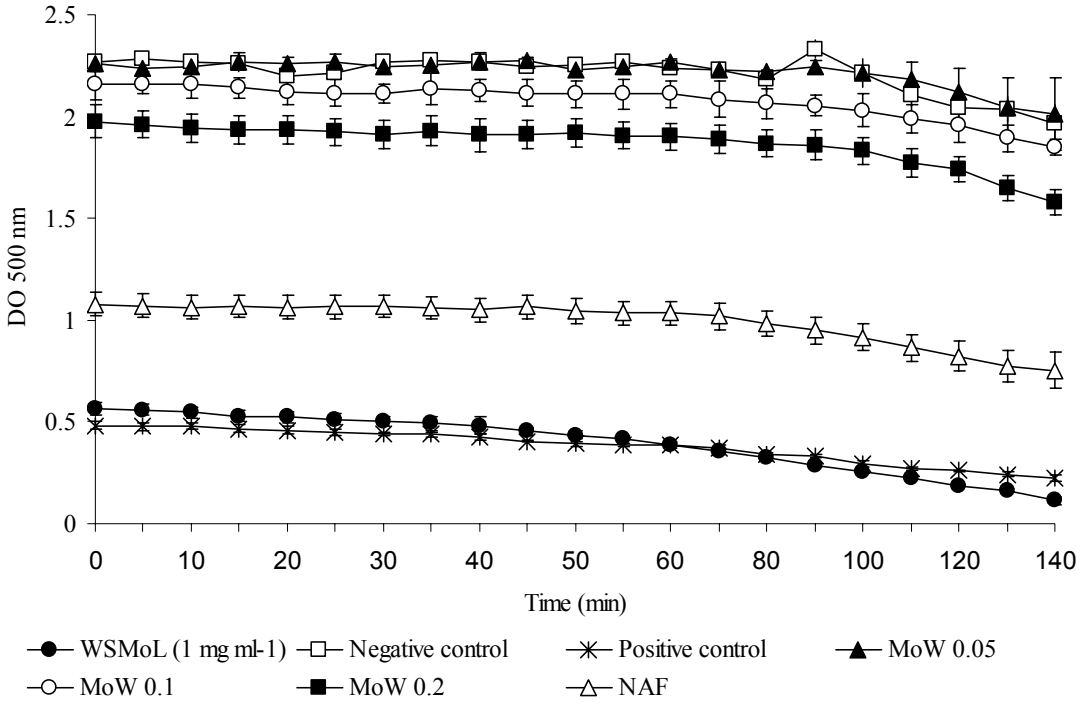

Fig 2. Coagulant activities of MoW (g l^{-1}), NAF (1 mg ml^{-1}) and WSMoL. (1 mg ml^{-1}) using synthetic turbid water. Positive and negative controls were 5% aluminium sulphate and clay suspension, respectively. The values represent the mean of three assays (± standard deviation): significant differences between groups were determined at $\rho < 0.05$.

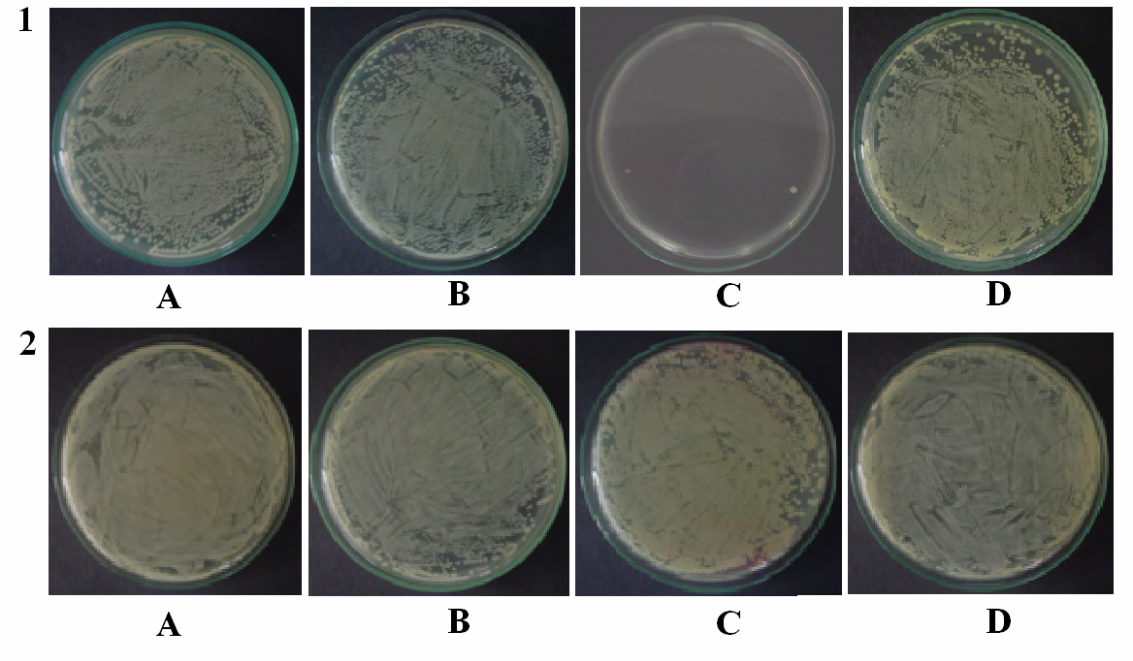

Figure 3. *E. coli* growth after treatment of bacterial suspension with water (A), MoW (B), WSMoL (C) and NAF (D). Samples of top (1) and sediment (2).

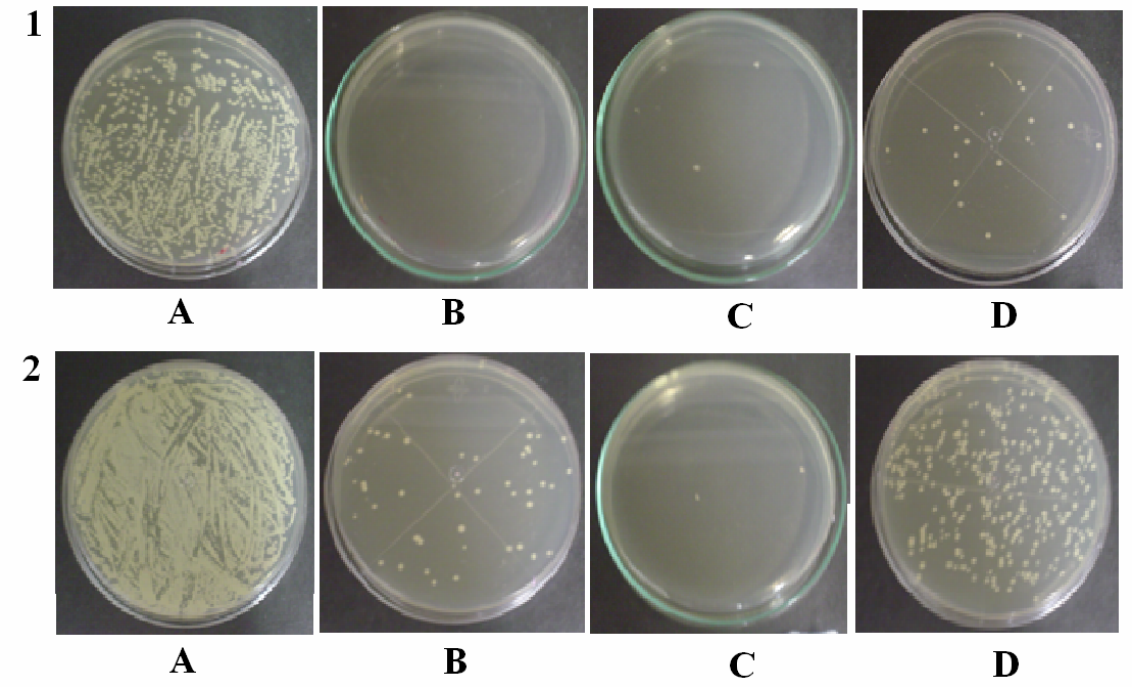

Figure 4. *S. aureus* growth after treatment of bacterial suspension with water (A), MoW (B), WSMoL (C) and NAF (D). Samples of top (1) and sediment (2).

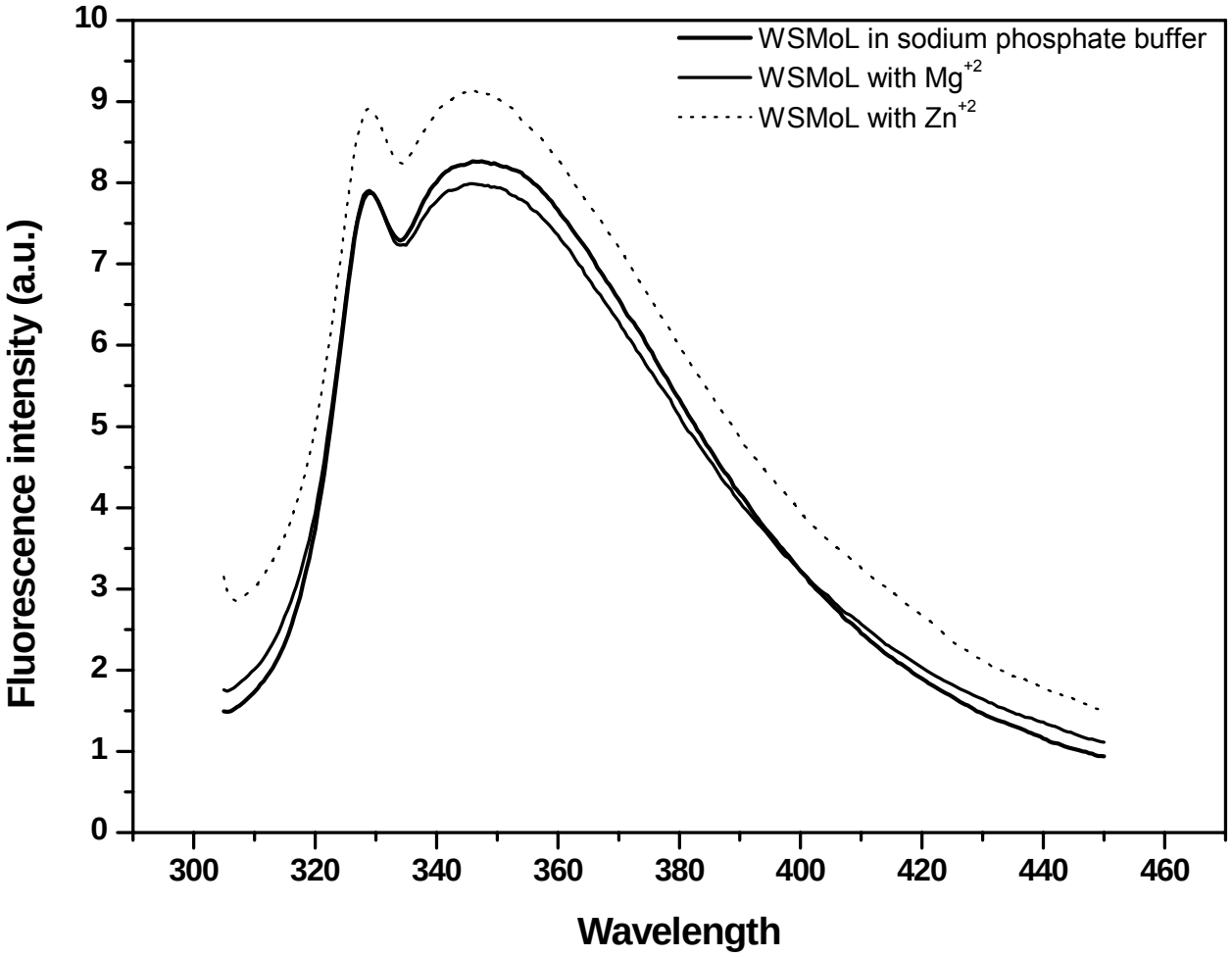

Figure 5. Fluorescence spectra of WSMoL at 25º C excited at 295 nm.

Emission maximum was around 345.5 nm.

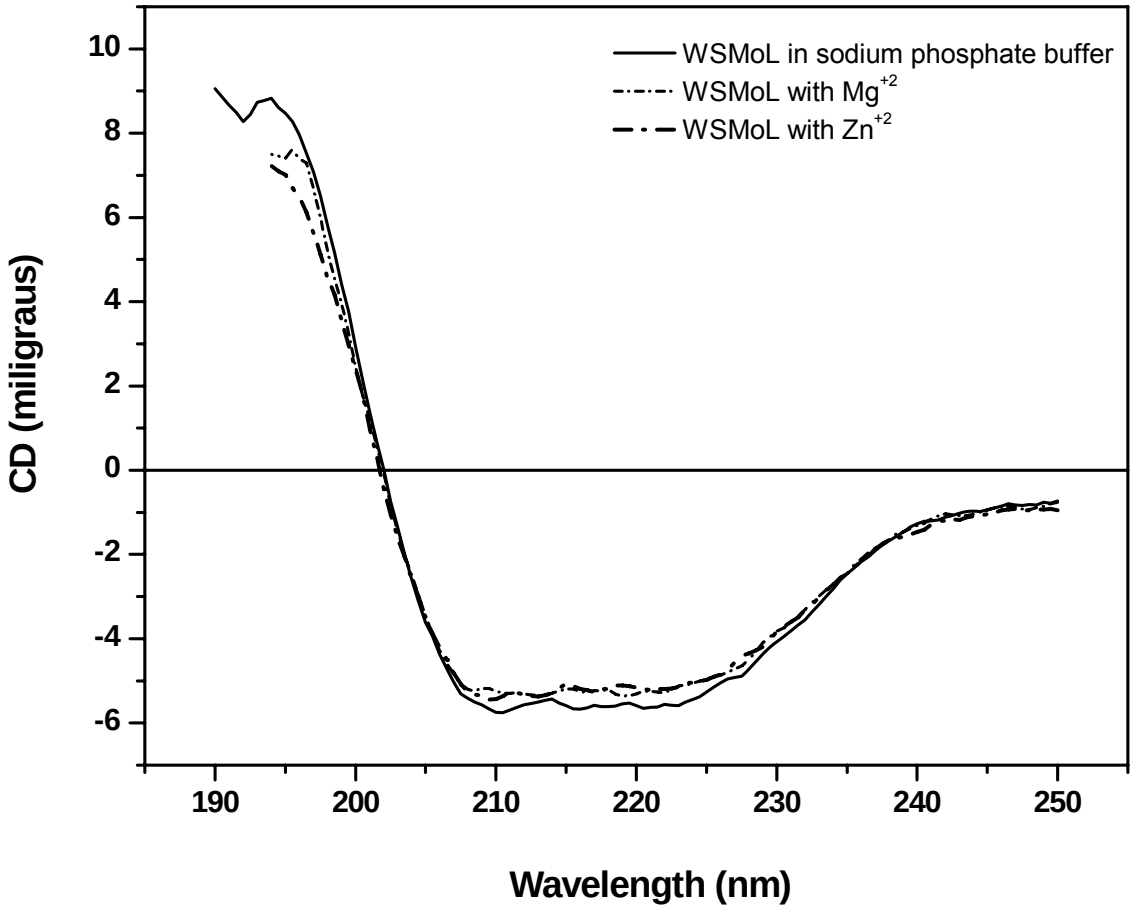

Figure 6. CD spectra of WSMoL in sodium phosphate buffer, pH 7.0 and at presence of Zn^{+2} an Mg^{+2}.

Measurements were recorded as an average of 8 scans for protein solutions of 0.05 mg/ml, at 25ºC.

5. CONCLUSÕES

MoW alterou valores físico químicos de amostras d' água.

O protocolo de purificação utilizado foi capaz de isolar lectina com elevada atividade hemaglutinante específica e biomoléculas sem AH.

WSMoL foi isolada por cromatografia de afinidade em coluna contendo quitina.

Mg^{+2} aumentou a atividade hemaglutinante de WSMoL.

MoW, WSMoL e NAF apresentaram atividade coagulante.

A coluna de quitina foi capaz de isolar biomoléculas com atividade antibacteriana para diferentes bactérias.

Os sítios hidrofóbicos de WSMoL onde estão localizados os triptofanos foram estáveis na presença de Mg^{+2} e Zn^{+2}.

A estrutura secundária predominante de WSMoL é α-hélice.

WSMoL pode ser utilizada como inseticida para *Callosobruchus maculatus*.

ANEXO

Normas para submissão de artigos para Water Research

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