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ORIGINAL ARTICLE

Coagulant and antibacterial activities of the water-soluble seed lectin from *Moringa oleifera*

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Abstract

Aims: The aim of this work was to analyse the coagulant and antibacterial activities of lectin isolated from *Moringa oleifera* seeds that are used for water treatment.
Methods and Results: The water-soluble *M. oleifera* lectin (WSMoL) was separated from nonhemagglutinating components (NHC) by chitin chromatography. WSMoL fluorescence spectrum was not altered in the presence of ions that are often present in high concentrations in polluted waters. Seed extract, NHC and WSMoL showed coagulant activity on a turbid water model. Both NHC and WSMoL reduced the growth of *Staphylococcus aureus*, but only WSMoL caused a reduction in *Escherichia coli*. WSMoL was also more effective in reducing the growth of ambient lake water bacteria.
Conclusions: Data obtained from this study indicate that WSMoL is a potential natural biocoagulant for water, reducing turbidity, suspended solids and bacteria.
Significance and Impact of the Study: *Moringa oleifera* seeds are a material effective in the treatment of water.

Introduction

The chlorination method in use in developing nations to water disinfection may originate several by-products with long-term harmful effects. In this sense, alternative disinfection procedures using natural products have become the object of investigation (Aquino and Teves 1994; Suarez *et al.* 2003; Gopal *et al.* 2004; Ghebremichael *et al.* 2005; Moura *et al.* 2011). Seeds of *Moringa oleifera* Lam. (often called horseradish tree) are widely used as an alternative water treatment method in developing countries. A suspension of triturated seed reduces turbidity and improves water quality, making it more suitable for human consumption. A protein extract from *M. oleifera* seeds can remove humic acids from water reducing total and organic matter as well as aromatic content and colour; the authors suggested that coagulant mechanism involves adsorption and neutralization of charges (Santos *et al.* 2011).

Plant seeds are sources of hemagglutinins – lectins or carbohydrate-binding proteins – which showed antibacterial activity (Oliveira *et al.* 2008; Sá *et al.* 2009; Costa *et al.* 2010). Lectins specifically bind teichoic and teichuronic acids, peptidoglycans and lipopolysaccharides in bacterial cell walls (Ratanapo *et al.* 2001).

Santos *et al.* (2009) reported that *M. oleifera* seeds are sources of a lectin that showed coagulant activity (cMoL). Santos *et al.* (2005) reported that the fructose-inhibited hemagglutinating activity (HA) identified in seed aqueous extracts is linked to the so-called water-soluble *M. oleifera* lectin (WSMoL). Coelho *et al.* (2009) purified the lectin by chitin column chromatography and reported that

WSMoL could kill *Aedes aegypti* larvae, which showed a single polypeptide band on SDS-PAGE and similarity with M02.1 and M02.2 (identification number gi|127215) proteins from *M. oleifera* seeds. Genotoxicity assessment of an extract of *M. oleifera* seed powder and WSMoL revealed that seed extract at concentration (0·2 μg μl^{-1}) recommended to treat water was not genotoxic by Ames, Kado and cell-free plasmid DNA assays but mutagenic effect at doses higher than 0·4 μg μl^{-1} was detected; WSMoL was nonmutagenic by used assays (Rolim *et al.* 2011).

The objectives of this study were to separate nonhemagglutinating components (NHC) from purified WSMoL using chitin chromatography, evaluate the effect of ions often present in high concentrations in polluted waters on HA and the fluorescence spectrum of WSMoL and examine the coagulant and antibacterial activities of WSMoL.

Materials and methods

Protein evaluation

The protein concentration was estimated with the method described by Lowry *et al.* (1951); bovine serum albumin (31–500 μg ml^{-1}) was used as a standard. Absorbance at 280 nm was also measured.

Hemagglutinating activity (HA)

HA was assessed in microtitre plates (Kartell S.P.A., Noviglio, Italy), according to the method described by Santos *et al.* (2005) using suspension (2·5% v/v) of rabbit erythrocytes treated with glutaraldehyde (Bing *et al.* 1967). HA (titre), the reciprocal of the highest dilution of the sample promoting full agglutination of erythrocytes, was defined as one hemagglutination unit (Chumkhunthod *et al.* 2006). Specific HA was defined as the ratio between the titre and protein concentration (unit mg^{-1}). The assay was also performed with 200 mmol l^{-1} fructose and 5, 10, 20 or 30 mmol l^{-1} $MgCl_2$ or $ZnCl_2$.

Chromatography on the chitin column: separation of NHC from WSMoL

Mature seeds from cultured *M. oleifera* plants were collected in the city of Recife, State of Pernambuco in north-east Brazil. Taxonomy was confirmed at the *Instituto Agronômico de Pernambuco* (Recife, Brazil), and a voucher specimen was archived under number 73 345. Seeds were dried at room temperature (28°C), milled to a fine powder (10 g) and then homogenized with distilled water (100 ml) in a magnetic stirrer (200 rev min^{-1}, 16 h at 4°C). Following homogenization, the mixture was filtered through cotton gauze and centrifuged at 3000 ***g*** for 15 min. The supernatant was treated with a 60% saturated ammonium sulfate solution (Green and Hughes 1955). The precipitated protein (0–60 fraction) was collected by centrifugation (3000 ***g***, 15 min, 4°C), dissolved in 0·15 mol l^{-1} NaCl and submitted for dialysis (3·5-kDa cut-off membrane) against 0·15 mol l^{-1} NaCl (6 h at 4°C). The dialysed 0–60 fraction (50 mg of proteins) was then applied to a chitin column (18 × 1·5 cm) equilibrated with 0·15 mol l^{-1} NaCl (0·3 ml min^{-1} flow rate). The column was washed with the equilibrium solution, and fractions that showed >0·200 absorbance at 280 nm were pooled (NHC). The adsorbed HA (WSMoL) was eluted with 1·0 mol l^{-1} acetic acid and dialysed (3·5-kDa cut-off membrane) against distilled water by 6 h at 4°C (Coelho *et al.* 2009). The HA of WSMoL was inhibited by fructose.

WSMoL fluorescence spectroscopy

Fluorescence was measured on a Hitachi F2500 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan) using quartz cuvettes with a path length of 1 cm. The excitation wavelength was 295 nm which selectively excites tryptophan (Lakowicz 1999), and the emission spectra were recorded in the range of 310–450 nm as an average of four scans. WSMoL (0·05 mg) was analysed in 10 mmol l^{-1} sodium phosphate buffer (pH 7·0) containing 0·5 mol l^{-1} magnesium (Mg^{2+}) and zinc (Zn^{2+}).

Seed extract

Macerated *M. oleifera* seeds without coats (0·2 g) were added to 1 l of distilled water and manually agitated for 5 min. The resulting suspension was filtered through cotton gauze, and the filtrate (seed extract) was used for analysis. Additional dilutions of 0·1 and 0·05 g l^{-1} were prepared by adding appropriate amounts of distilled water to the original 0·2 g l^{-1} extract.

Coagulant activity of seed extract, NHC and WSMoL

Continuous recording of optical density at 500 nm was used to evaluate active coagulants and to observe settling characteristics of the flocs (Ghebremichael *et al.* 2005). A clay suspension was prepared by adding 10 g of kaolin clay to 1 l of distilled water, stirring (200 rev min^{-1}) the mixture for 30 min and allowing it to settle for 24 h to achieve complete hydration. The desired optical density was obtained by dilution with distilled water. Aliquots (0·3 ml) of seed extract (0·2, 0·1 or 0·05 g l^{-1}), NHC (1 mg ml^{-1} of protein), WSMoL (1 mg ml^{-1}), 5%

aluminium sulfate (positive control) or distilled water (negative control) were added to a 4-ml plastic cuvette (10 × 10 × 44 mm; Cral®, São Paulo, Brazil) containing 2·7 ml of the clay suspension. The solution was agitated for 3 min using a 1-ml pipette (HTL, Poland). Samples were allowed to settle for 1 h at 27°C, and an aliquot of 900 μl from the top of the solution was transferred to a 1·5-ml plastic cuvette (10 × 4 × 44 mm; Cral®). Absorbances were measured at 500 nm using a UV–visible spectrophotometer (Femto 700 S; Femto Indústria e Comércio de Instrumentos, São Paulo, Brazil) at time 0 (initial absorbance), every 5 min for 60 min and then every 10 min for 100 min. Reduction in absorbance relative to the negative control defined coagulation activity. Coagulant activity was defined as decline in absorbance in regard to absorbance in the negative control. The assays were conducted in triplicate.

Antibacterial activity of seed extract, NHC and WSMoL

Antibacterial activity of *M. oleifera* preparations was evaluated on Gram-positive *Staphylococcus aureus* (WDCM 00034) and Gram-negative *Escherichia coli* (WDCM 00013), obtained from the *Instituto de Ciências Biológicas*, *Universidade de Pernambuco* (Recife, Brazil). Stationary cultures were maintained on nutrient agar (NA) and stored at 4°C. Bacteria were cultured in nutrient broth (NB) and incubated at 37°C for 3 h. Culture concentrations were adjusted turbidimetrically at a wavelength of 600 nm to 10^5–10^6 colony-forming units (CFU) per ml. Two hundred microlitres of seed extract (10 mg ml^{-1} of protein), NHC (1 mg ml^{-1} of protein), WSMoL (1 mg ml^{-1}) or Milli-Q® (Millipore Company, Billerica, MA, USA) water (negative control) was added to 200 μl of each incubation medium. The mixtures were shaken and incubated at 37°C for 24 h. NA medium (20 ml) was distributed to sterile Petri plates (90 × 15 mm) and allowed to solidify. From each of the incubation mixtures, 50 μl was withdrawn from either near the surface (top) or the sediment and smeared on NA plates. The plates were incubated at 37°C for 12 h, after which bacterial growth was observed. The assays were conducted in triplicate.

The antibacterial activity of seed preparations was also evaluated using *Cavouco* lake water collected at the *Universidade Federal de Pernambuco*. Aliquots (0·5 ml) of seed extract (10 mg ml^{-1} of protein), NHC (1 mg ml^{-1} of protein), WSMoL (1 mg ml^{-1}) or Milli-Q® water (negative control) were added to lake water, diluted 1 : 4 with Milli-Q® water and incubated at 37°C. After 14 h of incubation, 50 μl of each mixture was smeared on NA plates containing NA and incubated at 37°C for 24 h. The assay was performed in quintuplicate.

The minimal inhibitory concentration (MIC) was determined for *E. coli* and *Staph. aureus*. A 1/1000 dilution in NB of a 10^5–10^6 CFU overnight culture was made. Samples of seed extract (10 mg ml^{-1} of protein), NHC (1 mg ml^{-1} of protein) and WSMoL (0·5 mg ml^{-1}) were diluted 1 : 2 in NB and submitted to a series of ten double dilutions, to a final ratio of 1 : 2048. A 180-μl aliquot of each dilution was dispensed into a microtitre plate well. All wells were inoculated with 20 μl of the 1/1000 bacterial inoculum and incubated at 37°C for 24 h. Assays for each concentration were made in triplicate. After incubation, the optical density at 605 nm (OD_{605}) was measured using a microplate reader. MIC was determined as the lowest concentration at which there was ≥50% reduction in optical density relative to the control well OD_{605} (Amsterdam 1996).

To determine the minimum bactericide concentration (MBC), inoculations from the wells of the treatments that were found to inhibit bacterial growth were transferred to a NA plate and incubated at 37°C for 24 h. The lowest concentration showing no bacterial growth was recorded as the MBC. Amoxicillin (1 mg ml^{-1}) was used as the positive control. The assay was performed in triplicate.

Statistical analysis

GraphPad Prism, version 4.02 (GraphPad Software, La Jolla, CA, USA), was used for statistical analysis. Data were expressed as a mean ± standard deviation (SD). Data from the coagulant assay were analysed with a Student's *t*-test ($P \leq 0{\cdot}05$) to determine significant differences between treatments, using Origin 6.0 (OriginLab, Northampton, MA, USA).

Results

D(+)-Fructose totally inhibited the HA of seed extract. Chromatography of the 0–60% fraction (specific HA of 56) on chitin column resulted in the separation of the coagulant NHC from WSMoL (eluted coagulant fraction, Fig. 1a). The specific HA of WSMoL (4096) increased in the presence of 20 mmol l^{-1} (8192) and 30 mmol $^{-1}$ (16 384) of Mg^{2+}. Conversely, Zn^{2+} was found to promote erythrocyte dispersion and thus interfere with WSMoL HA. WSMoL exhibited a maximum fluorescence emission (λ_{max}) of about 346 nm upon excitation at 295 nm (Fig. 1b). The lectin fluorescence data were not altered in the presence of Mg^{2+} or Zn^{2+} (λ_{max} 346 nm), indicating that these ions did not modify protein structure in a tryptophan environment (Fig. 1b).

Lectin was detected only in the 0·2 g l^{-1} extract (specific HA of 6·0), and there was a significant ($P < 0{\cdot}05$) reduction in water turbidity in both the 0·1 and 0·2 g l^{-1}

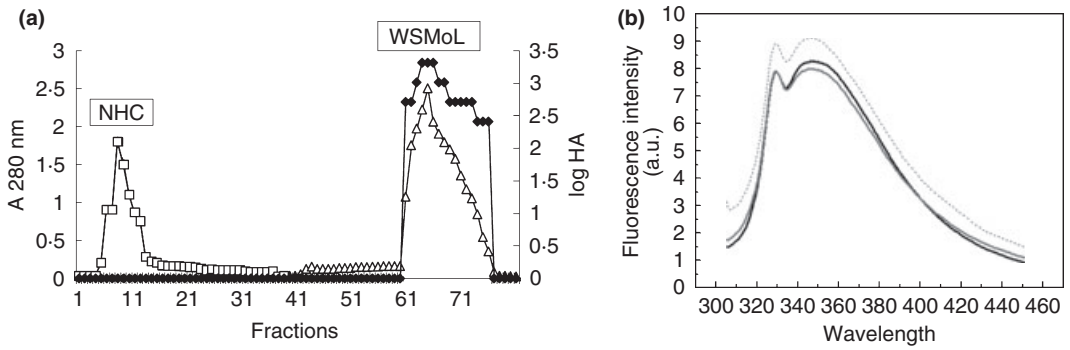

Figure 1 (a) Chromatography on the chitin column: nonhemagglutinating components (NHC) and water-soluble *Moringa oleifera* lectin (WSMoL) separation. Sample of dialysed 0–60 fraction (50 mg of proteins) was applied to the column (18 × 1·5 cm) and equilibrated with 0·15 mol l^{-1} NaCl (0·3 ml min^{-1} flow rate). Arrows indicate when eluents were added. Fractions (2·0 ml) were collected. NHC corresponded to the pool of fractions from washing step that showed >0·200 absorbance at 280 nm. WSMoL corresponded to the adsorbed hemagglutinating activity (HA) eluted with 1·0 mol l^{-1} acetic acid. Absorbance at 280 nm (□, Δ) and HA (♦) are represented. (b) Fluorescence spectra of WSMoL in sodium phosphate buffer at 25°C excited at 295 nm. Maximum emission was around 345·5 nm. (·····) WSMoL; (——) WSMoL with Mg^{2+} and (——) WSMoL with Zn^{2+}.

extracts as well as in NHC (Fig. 2). These coagulant activities were significantly ($P < 0·05$) lower than in the aluminium sulfate treatment (positive control). The coagulant activity was also detected in WSMoL, which was not significantly different ($P > 0·05$) from positive control (Fig. 2).

Table 1 summarizes the effect of *M. oleifera* preparations on bacterial growth. The suspension of *Staph. aureus* treated with seed extract, NHC or WSMoL showed lower bacterial growth in comparison with the Milli-Q® water (negative control). Growth of *E. coli* decreased only in the surface sample from the WSMoL treatment. The number of CFU in sediments from seed extract, NHC and WSMoL treatments was similar to that detected in negative control. In lake water, seed extract and NHC were not effective in reducing the growth of bacteria; in contrast, WSMoL was effective.

Staphylococcus aureus MICs were 7·8 μg ml^{-1} (WSMoL), 62·5 μg ml^{-1} (seed extract) and 625 μg ml^{-1} (NHC). WSMoL had the lowest MIC (250 μg ml^{-1}) for *E. coli*, while the NHC and seed extract values were much higher (500 and 5000 μg ml^{-1}, respectively). MBC assays revealed bactericidal activity only in WSMoL (*Staph. aureus*, MBC of 300 μg ml^{-1}).

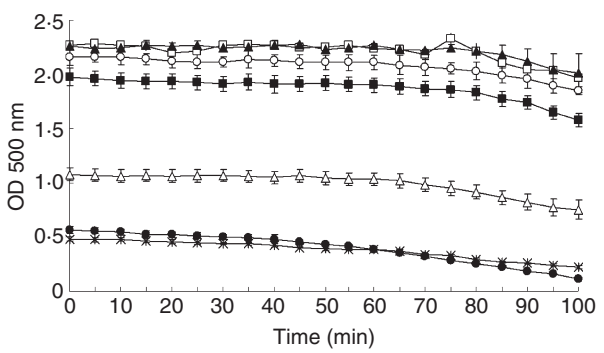

Figure 2 Coagulant activities of 0·2 (■), 0·1 (○) and 0·05 (▲) g l^{-1} seed extract, 1 mg ml^{-1} nonhemagglutinating components (△) and 1 mg ml^{-1} water-soluble *Moringa oleifera* lectin (•) using a clay suspension. Positive (*) and negative (□) controls were 5% aluminium sulfate and the clay suspension, respectively. The values represent the mean of three assays (±standard deviation). Significant differences between groups were determined at $P \leq 0·05$. See the abbreviations in Fig. 1.

Table 1 Growth of *Escherichia coli*, *Staphylococcus aureus* and ambient lake water bacteria after treatment with seed extract, nonhemagglutinating components (NHC) and water-soluble *Moringa oleifera* lectin (WSMoL)

	Colony-forming units (CFU) per ml		
Samples	*Staph. aureus*	*E. coli*	Lake water bacteria
Seed extract			
Surface	0	>10 000	>10 000
Sediment	1060	>10 000	>10 000
NHC			
Surface	400	>10 000	>10 000
Sediment	4860	>10 000	>10 000
WSMoL			
Surface	80	20	144
Sediment	40	>10 000	220
Control			
Surface	>10 000	>10 000	>10 000
Sediment	>10 000	>10 000	>10 000

Samples from the surface and sediment of each mixture were evaluated. Seed extract (10 mg ml^{-1}), NHC (1 mg ml^{-1}) and WSMoL (1 mg ml^{-1}). Negative control: Milli-Q® water.

Discussion

The quality of water treated with *M. oleifera* seed flour is improved through the proteins that promote coagulation (Gassenschmidt *et al.* 1995; Ndabigengesere *et al.* 1995; Ghebremichael *et al.* 2005; Santos *et al.* 2009). Coagulant proteins also demonstrated an ability to reduce the density of *E. coli*, *Bacillus thurigiensis* and *Pseudomonas aeruginosa* populations (Ghebremichael *et al.* 2005). Pritchard *et al.* (2009) reported that *M. oleifera* seed extract reduced the turbidity and number of faecal coliforms in water samples from shallow wells.

Extraction of *M. oleifera* seed proteins with water prevents the solubilization of the protein cMoL in high concentrations. The cMoL is a lectin soluble in saline solution (0·15 mol l^{-1} NaCl) with physicochemical properties and molecular mass distinct from WSMoL (Coelho *et al.* 2009; Santos *et al.* 2009; Rolim *et al.* 2011). The total inhibition of HA from seed extract by fructose indicates the presence of WSMoL and suggests that there is no contamination with cMoL, which is not inhibited by fructose.

Fluorescence intensity and maximum fluorescence emission of tryptophan residue data have often been used to study conformational transitions in protein structure (Sultan and Swamy 2005). Mg^{2+} increased the HA of WSMoL but fluorescence spectroscopic data indicate that this ion did not modify protein structure in a tryptophan environment. The apparent Mg^{2+}-mediated increase in WSMoL HA was as a result of stabilization of the interaction between the lectin carbohydrate binding site and erythrocyte surface. Mg^{2+} is often present in high concentrations in polluted waters (Akaninwor *et al.* 2007), and this fact can be a factor in the effectiveness of WSMoL in improving water quality.

Coagulant activity was detected in seed extracts, NHC and WSMoL; lectin coagulation was similar to that described for cMoL (Santos *et al.* 2009). The coagulant properties of *M. oleifera* seeds are likely to be significantly augmented by WSMoL activity.

Antibacterial assays indicated that *M. oleifera* seeds contain different antibacterial agents that had been successfully separated by chromatography on the chitin column. WSMoL was active on both Gram-negative (*E. coli*) and Gram-positive (*Staph. aureus*) bacteria, while NHC was active only on *Staph. aureus*. The growth of *E. coli* in sediment from the bacterial suspension treated with WSMoL suggests that lectin may promote bacterial coagulation, although it could not inactivate bacteria. WSMoL was highly effective against *Staph. aureus*, because the colony numbers from sediment decreased more than 250 times in comparison with negative control. The substantial reduction in *Staph. aureus* colony numbers in the surface and sediment from the incubation mixtures of all treatments indicates coagulation and inactivation of *Staph. aureus* cells. Mild inactivation conditions may lead to immediate reduction in cell numbers, while acute inactivation generally results in death of all bacterial cells (Tamplin 2005).

Although *M. oleifera* seeds are broadly used to treat water, the full composition of seed extract is not known, and the presence of water-soluble mutagenic agent from seeds has been reported (Suarez *et al.* 2003; Rolim *et al.* 2011). In this way, the identification of the active components responsible for coagulant and antibacterial effects is important. WSMoL has been reported to be nonmutagenic (Rolim *et al.* 2011), a characteristic that may appoint WSMoL as a sustainable, safe and environmentally friendly alternative and a competitive natural product, when expressed in recombinant forms for use in large scale. The use of *M. oleifera* seed polypeptides to replace commonly used coagulation and disinfecting agents was suggested by Suarez *et al.* (2003), who showed that recombinant and synthetic forms of *M. oleifera* seed cationic polypeptides promoted the sedimentation of suspended particles and bacteria in heavily contaminated water and that these polypeptides present bactericidal activity.

The results from MIC assay correlate with reduced *Staph. aureus* growth in surface and sediment samples from the cultures treated with WSMoL. Antibacterial activity against *Staph. aureus* has been described for *Eugenia uniflora* seed lectin with MIC of 1·5 μg ml^{-1} and MBC of 16·5 μg ml^{-1} (Oliveira *et al.* 2008) and *Myracrodruon urundeuva* heartwood lectin with MIC of 0·58 μg ml^{-1} and MBC of 8·1 μg ml^{-1} (Sá *et al.* 2009). The MIC value of WSMoL for *Staph. aureus* (7·8 μg ml^{-1}) is between the range of MICs determined for plant compounds (e.g. terpenes, phenols, flavonoids and alkaloids) considered potential antistaphylococcal agents with clinical relevance (Gibbons 2004). However, WSMoL is not suitable for practical application as a good antibacterial agent on *E. coli*.

WSMoL was highly active on Gram-positive *Staph. aureus*, probably due to the high level of peptidoglycan found in *Staph. aureus* cell wall. This peptidoglycan contains *N*-acetylglucosamine, which makes it a potential target for chitin-binding lectins such as WSMoL. The weak antibacterial activity of WSMoL on Gram-negative *E. coli* may be ascribed to the fact that it makes it more difficult for lectin to cross the lipid layer of the outer cell wall, reach the periplasmic space and, consequently, to interact with peptidoglycan (Nunes *et al.* 2011).

WSMoL was effective against lake water bacteria, and these data indicate that the lectin can contribute to disinfection of water promoted by *M. oleifera* seeds. A protein

preparation from *M. oleifera* flowers containing trypsin inhibitor activity could remove bacterial contamination from natural water (Moura *et al.* 2011).

The WSMoL, as component of *M. oleifera* seeds, is implicated on antibacterial effect against *Staph. aureus* and *E. coli* cell growth. Seed extract, NHC and WSMoL could promote the coagulation and inactivation of *Staph. aureus*. These, and similar, data on the efficacy of lectins may help broaden their use in water treatment.

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