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Removal of tetracycline from contaminated water by *Moringa oleifera* seed preparations

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ABSTRACT
The aim of this study was to evaluate tetracycline antibiotic (TA) removal from contaminated water by *Moringa oleifera* seed preparations. The composition of synthetic water approximate river natural contaminated water and TA simulated its presence as an emerging pollutant. Interactions between TA and protein preparations (extract; fraction and lectin) were also evaluated. TA was determined by solid-phase extraction followed by high-performance liquid chromatography-mass spectrometry. *Moringa* extract and flour removed TA from water. The extract removed TA in all concentrations, and better removal (40%) was obtained with 40 mg L^{-1}; seed flour (particles < 5 mm), 1.25 and 2.50 g L^{-1} removed 28% and 29% of tetracycline, respectively; particles > 5 mm (0.50 g L^{-1}) removed 55% of antibiotic. Interactions between TA and seed preparations were assayed by haemagglutinating activity (HA). Specific HA (SHA) of extract (pH 7) was abolished with tetracycline (5 mg L^{-1}); fraction (75%) and lectin HA (97%) were inhibited with TA. Extract SHA decreased by 75% at pH 8. Zeta potential (ZP) of extract 700 mg L^{-1} and tetracycline 50 mg L^{-1}, pH range 5–8, showed different results. Extract ZP was more negative (−10.73 to −16.00 mV) than tetracycline ZP (−0.27 to −20.15 mV); ZP difference was greater in pH 8. The focus of this study was achieved since *Moringa* preparations removed TA from water and compounds interacting with tetracycline involved at least lectin-binding sites. This is a natural process, which do not promote environmental damage.

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Tetracycline; water treatment; *Moringa oleifera*; seed flour; lectin

1. Introduction

Emerging pollutants are a group of new products or chemicals without defined regulatory status whose effects on environment and human health are unknown.[1] Municipal wastewater contains a large number of emerging contaminants, among them active pharmaceutical ingredients and personal-care products used in large quantities all over the world.[2] Antibiotics such as tetracycline are currently detected in wastewater.[3] Miège et al. [4] showed that the minimum and maximum concentrations of tetracycline in contaminated water were 0.240 and 0.790 µg L^{-1}. This class of drug is bacteriostatic with broad spectrum of microbes and being the most commonly used in livestock farming.[5] The extensive use of antibiotics has potential adverse effects to public health, mainly due to the increase in resistant bacteria present in ecosystems and humans. There are few studies to evaluate the effectiveness of conventional treatments of contaminated water for pharmaceuticals and endocrine disruptors' removal. Complex processes such as ozonation [6] and electrocoagulation [7] have been employed for removal of tetracycline of contaminated water. Choi et al. [5] studied the removal of this antibiotic by coagulation using poly-aluminium chloride (5–60 mg L^{-1}) and granular activated carbon.

A different strategy for contaminant removal from contaminated waters is the use of natural coagulants, which are user-friendly and without detrimental side effects. *Moringa oleifera* (Lam) is a plant belonging to the *Moringaceae* family with seeds that showed coagulant properties [8–10] and have been used in water and wastewater treatments.[11–13] Bhuptawat et al. [12] used a water extract of *M. oleifera* seed in pre-design studies towards a full-scale application. The results showed that 50 and 100 mg L^{-1} of *Moringa* extract dose removed 50% of influent chemical oxygen demand. Seed proteins are among the molecules responsible for water clarification.[8,9] Santos et al. [8] and Coelho et al. [14] purified two distinct haemagglutinating proteins from *M. oleifera* seeds with coagulant

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properties, namely cMoL (coagulant *M. oleifera* lectin) and water-soluble *M. oleifera* lectin.

Lectin is a group of haemagglutinating protein that interacts with carbohydrates and are mainly obtained from the seeds, from other tissues of leguminous plants [9,15] and from animals. The presence of lectin in a sample detected by haemagglutination assay is performed by a serial dilution of the sample and incubation with erythrocytes [16]; if the lectin is present in the sample, a network will be formed among the lectin-binding sites and carbohydrates present on erythrocyte surface.

Numerous studies showed coagulation ability of *Moringa* seeds. Sengupta et al. [11] revealed that *M. oleifera* seed extract reduced water turbidity. Bhuptawat et al. [12] and Bhatia et al. [17] showed that this property is due to the presence of active polyelectrolytes, which neutralize the negative charge of colloids present in wastewater. Seeds of *M. oleifera* possess the capacity of decontaminating water with arsenic,[18] cadmium,[19] chromium,[20] and can be used as a domestic technology being environmentally safe for water treatment. Beltrán-Heredia et al. [21] observed dyes removed from wastewater by these seeds. Santos et al. [16] showed that *M. oleifera* seed preparations interact with humic acid and could be used in water treatment to remove these organic compounds.

The aim of this work was to characterize the affinity of protein preparations obtained from *M. oleifera* seeds (extract, fraction and cMoL) to bind tetracycline and to study tetracycline removal by a natural, innovative and environmentally friendly technology using *M. oleifera* seed extract and flour.

2. Materials and methods

2.1. *M. oleifera* seed preparations

M. oleifera seeds were collected from a tree in Recife City (Northeast Brazil), and a sample was kept as voucher specimen number 63184, IPA, at the herbarium 'Dárdano de Andrade Lima' (Empresa Pernambucana de Pesquisa Agropecuaria, Recife, Brazil). The seeds were washed with distilled water, dried at room temperature and stored at 22°C until further use. They were ground to a flour, which was used in both sifted (particles <5 and >5 mm) and without sifted forms.

A protein extract, fraction and lectin were prepared as described by Santos et al. [9]; the seed flour extracted with 0.15 M NaCl for 6 h at room temperature (r.t., 25° C) and a protein saline extract were obtained. Elemental composition of extract was determined with an elemental analyzer (Leco CHNS model 932). The proteins present in the extract were precipitated with ammonium sulphate (390 mg mL^{-1} at r.t.) for 4 h and a protein fraction was obtained. The fraction was isolated (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL h^{-1} flow rate) with 0.15 M NaCl. The lectin, named cMoL, was eluted with 1.0 M NaCl. The extract, fraction and cMoL were used in the experiments described below.

2.2. *Haemagglutinating activity (HA) and HA inhibition assay*

The HA assay was performed in microtiter plates in accordance with Correia and Coelho [22]. The *M. oleifera* seed extract, fraction and cMoL (50 µL) were twofold serial diluted in 0.15 M NaCl before addition of 50 µL suspension of rabbit or human erythrocytes previously treated with glutaraldehyde $\varphi_r = 2.5\%$. After an incubation period of 45 min, the titer was expressed as the highest dilution resulting in haemagglutination. Assays were performed with extract at different concentrations (0.1, 0.7, 0.8 and 1.0 mg mL^{-1}) and different pH values (5, 6, 7 and 8); fraction and cMoL were used at 1 mg mL^{-1}.

The HA inhibition assay followed the same protocol as the HA, with an additional incubation step with the inhibiting substance (tetracycline 5.0 mg L^{-1}) for 15 min, before addition of the erythrocytes' suspension. The effect of pH in an interval from 5.0 to 8.0, and that of manganese, calcium and potassium (10 mM of $MnCl_2$, $CaCl_2$ or KCl solutions prepared in 10 mM sodium phosphate buffer) on the extract HA were assessed in the presence of tetracycline (5.0 mg L^{-1}).

The results of HA and HA inhibition are presented as specific haemagglutinating activity (SHA), which was calculated by dividing HA by protein concentration (mg mL^{-1}).

2.3. *Diffusion assay*

A diffusion assay was carried out in agarose gel (10 mg mL^{-1}) prepared in 0.15 M NaCl in a Petri dish. A tetracycline solution (30 µL) of a concentration of 5 mg L^{-1} was placed in a central well, while peripheral wells were filled with 15 µL (1000 mg mL^{-1}) of *M. oleifera* seed extract, fraction and cMoL. Diffusion experiments were performed in a humid chamber at 4°C for 48 h. Subsequently, the gels were thoroughly washed with 0.15 M NaCl and stained for 2 h with 1 mg mL^{-1} Coomassie Brilliant Blue, prepared in a mixture of ethanol $\varphi r = 45$ vol. % and acetic acid $\varphi r = 10$ vol. %.

2.4. *Preparation of synthetic contaminated water*

Synthetic contaminated water was prepared as described by Rodrigues et al. [23] with few modifications.

It was composed of 0.06 mg L^1 KH_2PO_4, 0.76 mg L^{-1} NH_4Cl, 0.67 mg L^{-1} KNO_3, 27.6 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 19.54 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 1.88 mg L^{-1} $(NH_4)_2$ $[Fe(SO_4)_2$. $]{\cdot}6H_2O$, 3.87 µg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.153 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 3.18 g L^{-1} $CuSO_4{\cdot}5H_2O$, 0.48 ng L^{-1} $CoCl_2{\cdot}6H_2O$, 2.4 mg L^{-1} $NaB_4O_7.10H_2O$, 2.0 mg L^{-1} $NaMoO_4.2H_2O$, 1.05 µg L^{-1} $NiCl_2{\cdot}6H_2O$, 0.009375 g L^{-1} HNa_2O_4P and 0.0225 g L^{-1} H_2KO_4P. Tetracycline (5 mg L^{-1}), purchased from Sigma-Aldrich (St. Louis, MO, USA), was added to water. To adjust the pH of the synthetic water, 1 M solutions of NaOH and HCl were used.

2.5. *Jar test*

Jar tests were performed with synthetic water containing tetracycline 5 mg L^{-1} and seed flour in both sifted (particles >5 and <5 mm) and without sifted forms (0.50, 0.75, 1.00, 1.25 and 2.50 g L^{-1}) as well as with the extract in different protein concentrations (1, 20, 30, 40 and 50 mg L^{-1}). The effects of different seed amounts, extract concentrations and pH in a range of 5–8 were assessed. Assays were conducted at room temperature (±22°C). The test consisted of instantaneous addition of seed flour or extract to 400 mL of synthetic water, which is followed immediately by a rapid mix at 120 rpm for 1 min, slow mix at 30 rpm for 15 min and sedimentation for 30 min. A negative control with only synthetic water was included. Distilled water was used in the solution preparations.

2.6. *Analysis of tetracycline*

Tetracycline was determined by solid phase extraction (SPE) followed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) as described in Matos et al. [24]. In SPE treatment, the samples were filtered through a 0.45 µm pore size filter (Advantec, Dublin, CA, USA). Then, the pH of the sample was adjusted to <3 with 40% H_2SO_4; also, 50 µL of 5% Na_2EDTA as well as 50 µL of 50 µg mL^{-1} chlortetracycline solution (internal standard) were added to the sample. The SPE procedure consisted in the precondition of an Oasis HLB cartridge (30 mg mL^{-1}, Waters, Milford, MA, USA) with 1 mL of methanol, 1 mL of 0.5 N HCl and 1 mL of deionized water, sequentially. Then, 5 mL of sample was extracted with the HLB cartridge at a flow rate of approximately 5 mL min^{-1} and the cartridge was washed with 1 mL of 5% methanol aqueous solution. Finally, the antibiotic was eluted with 1 mL of methanol. The extract was dried under a gentle stream of N2 and the residue was dissolved with 1 mL of mobile phase B (0.1% acid formic in acetonitrile). Tetracycline was determined by HPLC-MS. The HPLC system consists of a HPLC Pump (Finnigan Surveyor Plus), an autosampler (Finnigan) and a PDA detector (Finnigan). Tetracycline and the internal standard were separated using a Kinetex 2.6 mm C18 column (100 mm × 4.6 mm). A multistep binary elution gradient composed of 0.1% acid formic in water (mobile phase A) and in acetonitrile (mobile phase B) was pumped through the column with a flow rate of 0.3 mL min^{-1}. The separation of tetracycline and internal standard was achieved with the following mobile phase gradient programme: at 0 min A/B = 90/10; 15 min A/B = 20/80; 19 min A/B = 90/10; and 22 min A/B = 90/10. The MS system consisted of a linear ion trap mass spectrometer (Thermo Lxq) equipped with an electrospray ionization (ESI) source operated in the positive ionization mode. The optimal conditions for the ESI source, obtained with a standard solution of tetracycline, were 275°C capillary temperature and 13 V capillary voltage. Selected ion monitoring mode was chosen to quantify the antibiotic.

The removal efficiency for tetracycline was calculated from Equation (1), where Ci and Cf are the initial and final concentrations, respectively, expressed as follows:

$$\% \text{ removal efficiency} = 100 - (\text{Cf} \ . \ 100/\text{Ci}). \quad (1)$$

2.7. *Protein evaluation*

The protein was estimated according to Lowry et al. [25] using the Sigma protein assay kit (Sigma Diagnostics, St Louis, MO, USA).

2.8. *Zeta potential*

The zeta potentials (ZP) of extract (0.7 mg L^{-1} of protein) and tetracycline (50 mg L^{-1}) were determined using a Malvern Zetasizer instrument equipped with the ZP cell DTS1060 at 20°C. ZP values were derived from the electrophoretic mobility using the Smoluchowski approximation.[26]

3 Results and discussion

3.1. *M. oleifera seed extract caracterization*

The elemental composition of the *M. oleifera* seed extract determined in the present study was 30.87% of C, 4.98% of H and 3.70% of N. Coagulant properties of carbohydrate-binding proteins, lectins purified from the extract, have already been described.[8,9] Ndabigengesere et al. [27] analysed shelled and non-shelled seeds and showed that the elemental composition of shelled seeds consisted of 55% C, 8.5% H and 6% N. The remaining 31% consisted of O and trace elements. The non-shelled seeds trail closely the shelled seeds in all the

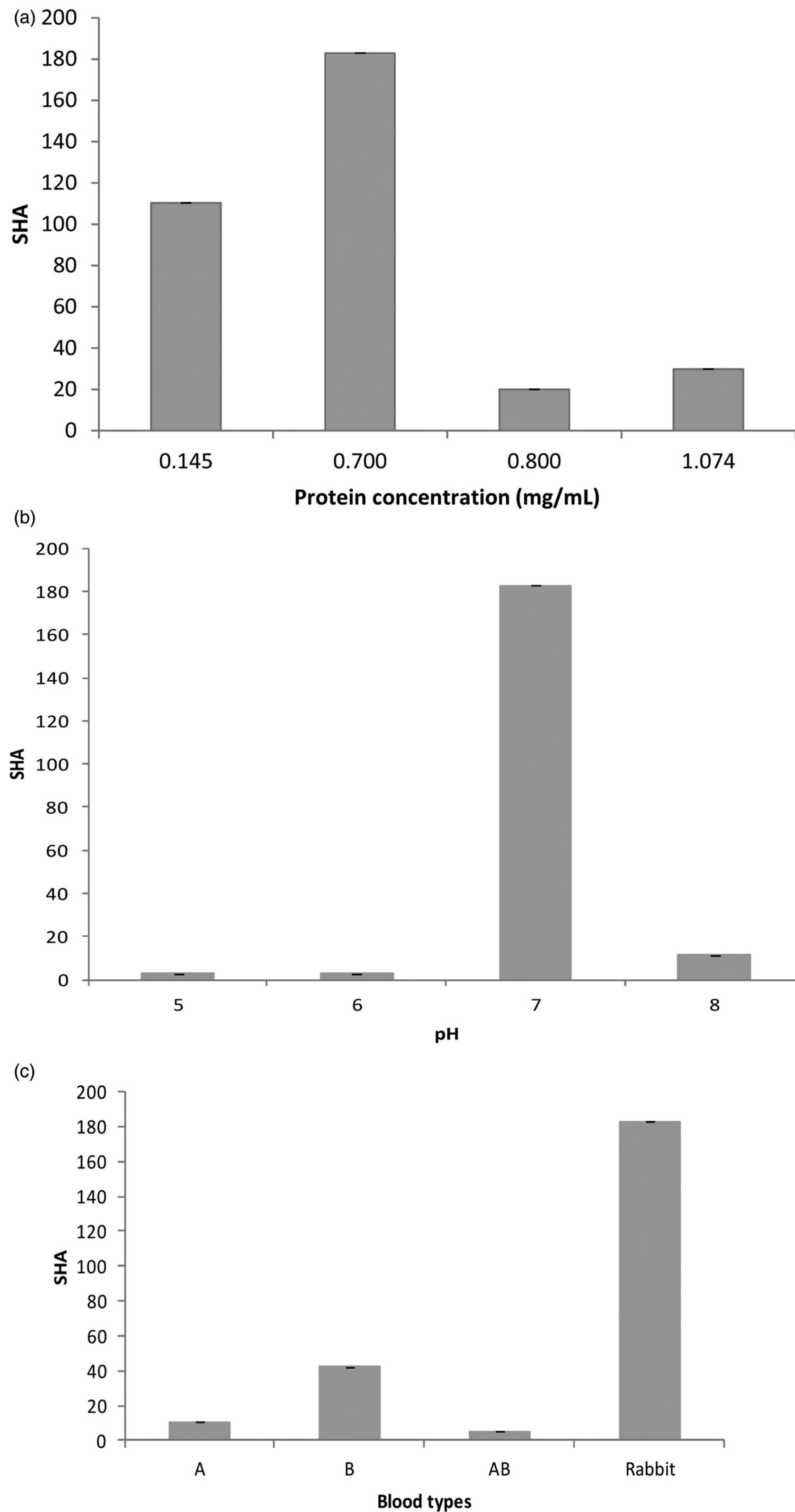

Figure 1. SHA of the *M. oleifera* seed extract in different protein concentrations (a), distinct pH values with 0.7 mg mL^{-1} extract (b) and different human and rabbit blood cells (c). Erythrocytes previously treated with glutaraldehyde $\varphi r = 2.5\%$ were used. Values represent the mean of three assays (± standard deviation).

elements analysed with inferior percentage. In addition, shelled and non-shelled seeds contained about 37% and 27% of proteins as well as 35% and 21% of lipids, respectively; carbohydrates (as oligosaccharides) represented about 5% of both kinds of seeds.

3.2. *HA and HA inhibition assays*

Among the extract concentrations tested, the best HA (SHA: 182.8) was obtained with 0.7 mg mL^{-1} and pH 7 (Figure 1(a)–(c)). Coelho et al. [12] showed that

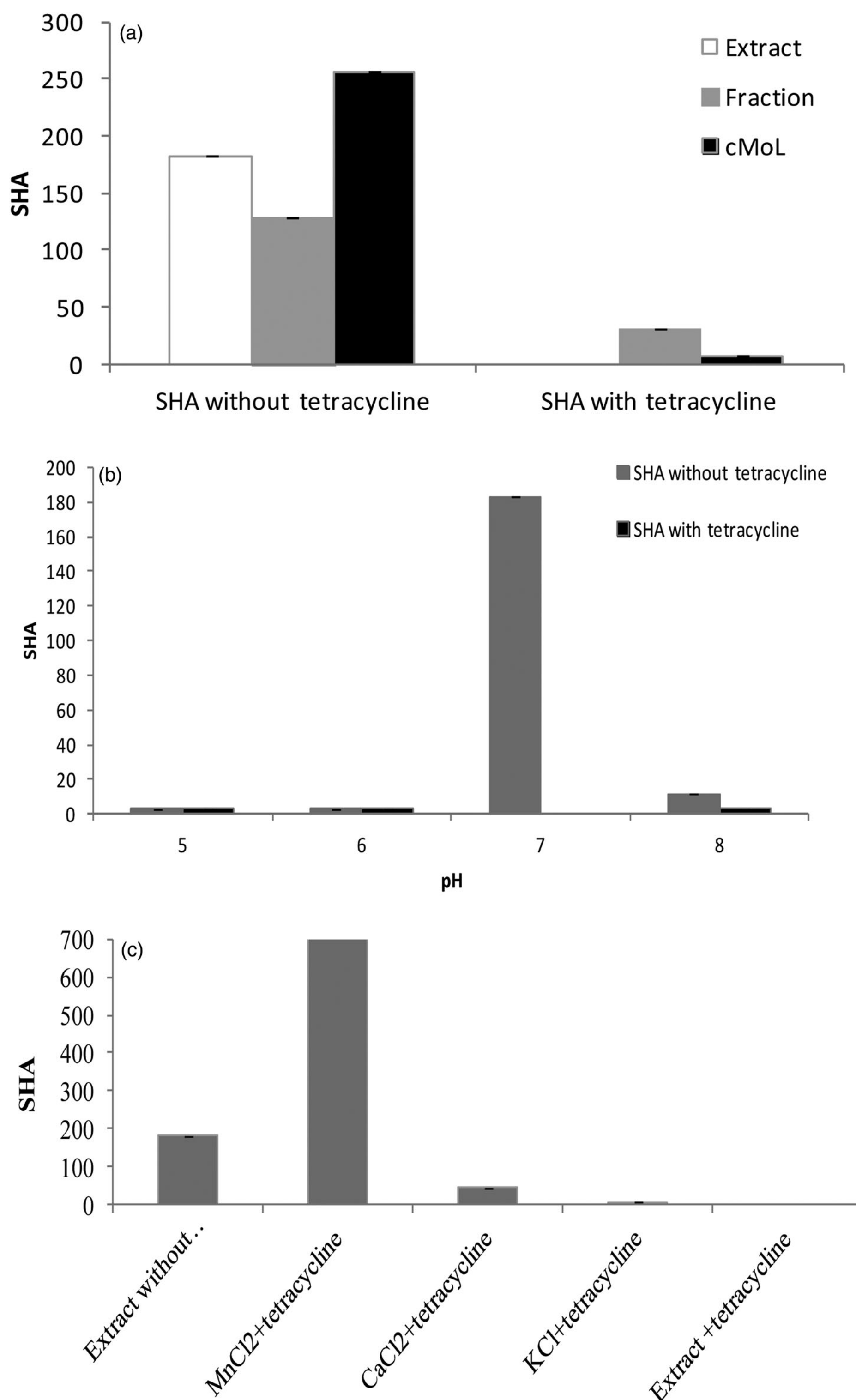

Figure 2. SHA of *M. oleifera* seed extract (0.7 mg mL^{-1}), fraction and cMoL (1 mg mL^{-1}) with and without tetracycline (a), SHA of extract (0.7 mg mL^{-1}) in different pH values (b) and in the presence of different ions (c). Rabbit erythrocytes previously treated with glutaraldehyde φr = 2.5% were used. Values represent the mean of three assays (± standard deviation).

aqueous extract from *M. oleifera* seeds (5.0 mg mL^{-1}) presented SHA of 128. Santos et al. [8] studied that saline seed extract from *Moringa* (4.9 mg L^{-1}) showed SHA:208, and this extract activity was inhibited by 0.2 M carbohydrates: D(+)-Raffinose, D(+)-Glucose, D (+)-Mannose and glycoproteins (0.5 mg mL^{-1}): fetuin, obalbumin, casein and azocasein; rabbit erythrocytes were used in the assays.

In the presence of 5 mg L^{-1} tetracycline at pH 7, SHA of *M. oleifera* seed extract (0.7 mg mL^{-1}) was abolished; activity of fraction and cMoL (1 mg mL^{-1}) were inhibited (75% and 97%, respectively) (Figure 2(a)). In addition, SHA of extract was inhibited (75%) at pH 8 (Figure 2 (b)); at pH 5 and 6, tetracycline did not inhibit extract SHA. The effect of mono- and divalent cations from extract SHA with tetracycline was depicted in Figure 2 (c). Then Mn^{2+}, Ca^{2+} and K^{+} (10 mM of $MnCl_2$, $CaCl_2$ and KCl) increased the extract SHA; tested ions were not able to increase the possible interactions between the lectins present in *M. oleifera* seed extract and tetracycline. Santos et al. [16] showed that the interaction between *M. oleifera* lectin and humic acid was increased by ions.

The single radial diffusion gel did not show precipitation bands, indicating that the extract, fraction and cMoL did not bind tetracycline through this assay.

3.3. *Tetracycline removal*

Seed flour (particles <5 mm) in the concentrations of 1.25 and 2.50 g L^{-1} removed 28% and 29% of tetracycline, respectively; with particles >5 mm and 0.50 g L^{-1}, removal of 55% of antibiotic was detected; without sifted seed flour did not remove tetracycline from contaminated water. *M. oleifera* seed extract removed tetracycline in all concentrations analysed and the best removal (39%) was observed with 40 mg L^{-1} (Table 1). Cosmetic ingredients and pharmaceuticals have been removed by coagulation–flocculation and flotation.[28] Choi et al. [5] studied the removal of tetracycline by coagulation using poly-aluminium chloride and granular activated carbon filtration, and both treatments were effective in removing this drug.

Table 1. Tetracycline removal by *M. oleifera* seed extract in different protein concentrations.

Extract concentration (mg L^{-1})	Tetracycline removal (%)
1	16
20	17
30	31
40	39
50	28

Notes: Assays were performed at pH 7. Values represent the mean of three assays (± standard deviation).

3.4. *Zeta potential*

To study the interaction between tetracycline and *M. oleifera* seed extract, the ZP was determined at a pH range 5–8, as depicted in Table 2. Different negative ZP values were observed with each sample. The ZP of tetracycline (50 mg L^{-1}) was different from the extract (700 mg L^{-1}) in each pH tested. With the interaction between extract and tetracycline, changes at the surface chemistry of extract particles were observed in all pH tested. These ZP variations are in accordance with the ionization of the carboxylic and phenolic acidic groups, and this parameter gives an indication of the potential stability of the system. The ZP of extract, tetracycline and extract-tetracycline suspensions ranged between +20 and −20 mV, and were characteristic of unstable suspensions that flocculated rapidly.[29] In the present work, the negative ZP differences of each sample analysed showed that the coagulation process occurred with *M. oleifera* seed extract and tetracycline might have induced attraction between different negatives surface charges. This difference was greater in pH 8 (−027 mV to the tetracycline and −10.43 mV to the extract); the results suggest that pH 8 may be the most appropriate to the interaction between lectins present in the extract and tetracycline.

3.5. *Proposal of coagulation mechanism of cMoL and tetracycline*

Santos et al. [8] described cMoL as a lectin with a molecular weight of 26.5 kDa, thermo stable and pH resistant. Luz et al. [30] showed that this lectin is composed of 101 amino acids and 2 polypeptide chains. In addition, cMoL have eight cysteine residues, which may be involved in the disulphide bonds. cMoL is a basic protein with a theoretical pI of 11.67, indicating a strong positive charge on the surface and confirming

Table 2. ZP in different pH values for extract (0.7 mg L^{-1} protein), tetracycline (50 mg L^{-1}) and extract–tetracycline solution.

Samples	pH	ZP (mV)
Extract	5	−10.73 ± 0.14
	6	−15.47 ± 0.24
	7	−16.00 ± 0.38
	8	−10.43 ± 0.29
Tetracycline	5	−15.75 ± 0.81
	6	−13.10 ± 0.07
	7	−20.15 ± 0.60
	8	−0.27 ± 0.00
Extract[a]–tetracycline solution after water treatment	5	−9.12 ± 0.14
	6	−10.90 ± 0.56
	7	−15.30 ± 0.35
	8	−8.19 ± 1.00

Note: Values represent the mean of three assays (± standard deviation).
[a]Extract protein concentration before treatment was 40 mg L^{-1}.

its cationic nature. High contents of glutamine (26.7%), alanine (6.9%), proline (6.9%) and 17 positively charged amino acids (16 arginines and 2 histidines) are also present in the lectin structure. Gassenchmidt et al. [31] and Ndabigengesere et al. [27] proposed that a flocculent protein from *M. oleifera* seeds namely $MO_{2.1}$ must develop its coagulant activity in water due to interactions among charges. Luz et al. [30] suggested that cMoL may act in a similar way and not through binding to a carbohydrate recognition site.

Santos et al. [32] proposed a mechanism involved in cMoL coagulation process and showed that cMoL, a basic positively charged protein,[8] can interact with colloidal particles such as kaolin, whose ZP is negative. Bridge formation can be the model used to explain the coagulant activity of high-molecular cationic polyacrylamide derivatives. Negatively charged coagulation particles result from binding of positively charged particles by Coulomb forces and neutralization of part from the surface charge. Flocks are formed when the electrostatic repulsion is reduced through interaction between negatively charged particles [31]; Tetracycline presented negative ZP in all pH tested (Table 2). Therefore, the mechanism to explain cMoL and tetracycline coagulation could be through interactions among different charges. Okuda et al. [33] proposed a model where coagulation by a purifed coagulant solution (MOC-SC-pc) from *M. oleifera* seeds occurs due to interaction of MOC-SC-pc with bivalent cations, forming *net-like* structures. Luz et al. [30] suggested that this model cannot be applied to cMoL since bivalent ions (Ca^{+2} and Mg^{+2}) did not improve the efficiency of coagulation, according to Santos et al. [8]. The ions Mn^{2+}, Ca^{2+} and K^{+} did not increase interactions between the lectins present in *M. oleifera* seed extract and tetracycline (Figure 2(c)). Then, the model suggested by Okuda et al. [33] also cannot be applied in this study.

4. Conclusions

M. oleifera seed preparations interacted with tetracycline, and cMoL can be the lectin responsible for this interaction. Removal of tetracycline by extract in all assayed concentrations, and by seed flour (particles <5 and >5 mm) indicates that *Moringa* preparations could be used to remove this antibiotic from contaminated water through a simple, natural and environmentally friendly technology.

Disclosure statement

No potential conflict of interest was reported by the authors.

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