

Structural characterization of coagulant *Moringa oleifera* Lectin and its effect on hemostatic parameters



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ABSTRACT

Lectins are carbohydrate recognition proteins. cMoL, a coagulant *Moringa oleifera* Lectin, was isolated from seeds of the plant. Structural studies revealed a heat-stable and pH resistant protein with 101 amino acids, 11.67 theoretical pI and 81% similarity with a *M. oleifera* flocculent protein. Secondary structure content was estimated as 46% α -helix, 12% β -sheets, 17% β -turns and 25% unordered structures belonging to the α/β tertiary structure class. cMoL significantly prolonged the time required for blood coagulation, activated partial thromboplastin (aPTT) and prothrombin times (PT), but was not so effective in prolonging aPTT in asialofetuin presence. cMoL acted as an anticoagulant protein on *in vitro* blood coagulation parameters and at least on aPTT, the lectin interacted through the carbohydrate recognition domain.

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1. Introduction

Lectins are a group of proteins which recognize and bind mono- and oligosaccharides [1,2]. They can bind to carbohydrate moieties on the surface of erythrocytes and agglutinate the cells, without altering the carbohydrate structure [3]. Lectins exist in viruses and all forms of life, however the best known are extracted from plants, especially seeds, organ of stock, which is a major source to obtain these molecules [4]. Hundreds of lectins have been purified and their sugar specificities identified, which has enabled their development into powerful tools for the purification, separation, and structural analysis of glycoproteins [5], as well as recognition

molecules inside cells, on cell surfaces, and in physiological fluids [6]. They have shown inhibitory activity against fungi and bacteria [7,8], insects [9], viruses [10] and cytotoxic effects against tumor cells lines [11].

Physical-chemistry characterization of lectins is important to explain the function in different biological processes [12]. Structural biology of macromolecules searches products potentially useful for solving biochemical problems and eventually develops new therapeutical agents [13]. To study structures of macromolecule, spectroscopic techniques such as circular dichroism (CD) is used, where optically active substances will absorb differently left and right circularly polarized light, and the difference in absorption of components is measured. The method has been extensively used to unravel secondary structure of proteins giving information on the effect of added ligands [14]. Proteins with high α -helical content shows CD spectrum in the far UV region as two negative CD bands around 208–210 nm and 222–228 nm as well as one positive band near 190–195 nm [15].

Moringa oleifera, known as horseradish or drumstick tree, is widely found throughout India, Asia, some parts of Africa and America, belonging to the Moringaceae family [16,17]. Its constituents such as leaf, flower, fruit and bark have been anecdotally used

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as herbal medicines in treatments for inflammation, paralysis and hypertension [18]. The seeds contain edible oils and water soluble substances (coagulant proteins) that can be used in drinking water clarification [19–22] or for treatment of selected dyeing effluents [23]. Lectins with coagulant properties were purified from *M. oleifera* seeds. Santos et al. [24] purified and partially characterized coagulant *M. oleifera* lectin, cMoL, the first lectin with coagulant properties for contaminants in water. cMoL is a basic protein, active at pH range 4.0–9.0 and complex sugar specificity which recognized mainly the glycoproteins azocasein and asialofetuin. WSMoL, Water-Soluble *M. oleifera* Lectin, was detected by Santos et al. [25] as an acid glycoprotein, with higher hemagglutination activity at pH 4.5, recognizing mainly fructose and porcine thyroglobulin. Ferreira et al. [26] referred that this lectin has coagulant activity and is a natural coagulant for contaminants in water, reducing turbidity and bacterial proliferation. WSMoL and cMoL showed insecticidal activity against *Aedes aegypti* [27] and *Anagasta kuehniella* [28], respectively. Katre et al. [29] reported the presence of a *M. oleifera* lectin, MoL, a homodimer with molecular mass of 14 kDa and subunits (7.1 kDa) linked by disulfide bond(s). MoL is also a glycoprotein with high stability and agglutinates human as well as rabbit erythrocytes.

cMoL is a protein with important biological activities [24,28], however its structural characterization was not completely elucidated. In this article, we report the primary structure, CD characterization and for the first time the anticoagulant properties of a coagulant lectin from *M. oleifera* on *in vitro* hemostatic parameters of human blood coagulation.

2. Materials and methods

2.1. Isolation of coagulant *M. oleifera* lectin (cMoL)

M. oleifera seeds were collected on the campus of Universidade Federal de Pernambuco, in Recife city, Northeast of Brazil. The extract, fraction and lectin were prepared according to Santos et al. [24], with modifications. Seeds were ground to flour, which was extracted with 0.15 M NaCl at room temperature (25 °C) for 6 h, and a saline extract was obtained. Proteins were precipitated with ammonium sulphate (60%) at room temperature for 4 h; the fraction obtained after centrifugation (12,000 × g for 20 min at 4 °C) was dialyzed against water and 0.15 M NaCl. The fraction was applied (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.1 M NaCl. cMoL was eluted with a saline gradient of 0.15, 0.3, 0.5 and 1 M NaCl. UV absorbance was used to monitor samples. cMoL active fractions eluted with 0.3 M NaCl were pooled, analyzed by HPLC and used in the experiments. cMoL protein concentration was evaluated according to Lowry et al. [30] using bovine serum albumin as standard at a range of 0–500 µg/mL and absorbance at 720 nm.

2.2. Reversed-phase HPLC

cMoL was subjected to reverse-phase column C4 on HPLC system (Shimadzu) for purity analysis. The column was equilibrated with solvent A [0.1% (v/v) trifluoroacetic acid (TFA) in H₂O] and eluted using solvent B (90% acetonitrile in 0.1% TFA) in a linear gradient, where B = 5% when $t = 0$ min, B = 5% at $t = 5$ min, B = 100% at $t = 60$ min, B = 0% when $t = 65$ min. The elution profile was monitored at 215 and 280 nm.

2.3. Hemagglutination activity (HA)

Glutaraldehyde-treated rabbit erythrocytes were obtained as described by Bing, Weyand, and Stavinsky [31]. The lectin (50 µL) was serially two-fold diluted in microtiter V-plates containing

0.15 M NaCl before addition of 50 µL 2.5% (v/v) suspension of treated rabbit erythrocytes. The results were read after about 45 min when the control, containing only erythrocytes fully precipitated, appeared as a dot at the bottom of the well. HA (inverse of the titer) was defined as the highest sample dilution showing full hemagglutination [4,32].

2.4. Primary sequence determination

Edman degradation [33] was performed with an automatic gas-phase sequencer (492cLC; Applied Biosystems) using conditions recommended by the manufacturer. Samples (0.8 mg/mL) for sequencing were reduced in 200 µL of 0.25 M Tris–HCl buffer, pH 8.5 containing 6 M guanidine–HCl, 1 mM EDTA and 5 mg of DTT, and alkylated with iodacetamide [34]. Then, the protein was separated by reversed phase chromatography HPLC. The similarity of sequences was searched using the BLAST protein sequence database [35] and the sequences were aligned with the MULTALIN program [36]. Theoretical pI was calculated by ExPASy ProtParam tool through the primary sequence of the protein.

2.5. Spectroscopic measurements

CD data were recorded on a Jasco J-810 spectropolarimeter. Samples were placed in a 0.5 mm path length circular quartz cuvette. Lectin concentration was 0.2 mg/mL in phosphate–borate–acetate (PBA) buffer for measurements in the far UV region (250–190 nm), as an average of 8 scans. Data were expressed in terms of mean residue ellipticity [θ]. The secondary structure estimative was calculated by deconvoluting the CD spectrum using the CDPro software package, which contains three CD analysis programs, CONTINLL, SELCON3 and CDSSTR [37]. The three programs were used with a reference protein set consisting of 56 proteins, thus increasing the reliability of deconvolution. Results were expressed as a mean of the three programs. CDPro package also contains the Cluster program that was used to determine the tertiary structure class of cMoL [38]. To study the pH effect on cMoL, the protein (0.2 mg/mL) was incubated in phosphate–borate–acetate buffer (PBA), 10 mM, for 10 min, at pH values of 2.0, 4.0, 6.0, 7.0, 8.0, 10 and 12. The temperature effect on cMoL secondary structure was also analyzed. Protein samples were heated at 40, 60, 80 and 100 °C for 30 min and at 100 °C for 1 h. CD measurements were recorded as described above.

2.6. Determination of activated partial thromboplastin time – aPTT and prothrombin time – PT

aPTT and PT were determined in a semi-automated coagulometer BFT II (Dade Behring). Total plasma was obtained by centrifugation of human blood samples at 1726 × g, for 15 min (25 °C). PT assay control was made with 50 µL of saline and 50 µL of plasma, incubation for 60 s, with subsequent addition of 100 µL of reagent (Thromborel S-Dade Behring). cMoL in different concentrations (3.0, 15, 30, 37.5, 45 and 60 µg/mL) in 50 µL was incubated (60 s) with 50 µL of plasma, followed by subsequent addition of 100 µL of reagent. aPTT assay control was made with 50 µL of saline, 50 µL of plasma and 50 µL of aPTT reagent (Dade actin activated cephaloplastin reagent-Dade Behring), incubation for 120 s and subsequent addition (50 µL) of 0.025 M calcium chloride. cMoL (3.0, 15, 30, 37.5, 45 and 60 µg/mL) was incubated for 120 s with 50 µL of plasma and 50 µL of the aPTT reagent, followed by addition of 50 µL of 0.025 M calcium chloride. The blood coagulation assays were also performed with cMoL (50 µL) inhibited by glycoprotein. cMoL was previously incubated with asialofetuin (0.5 mg/mL) for 15 min e then the assay proceeded as described above. Each assay was made in duplicate and results were expressed as average of

3 independent protocols (\pm s.e.m). All experiments were approved by the Ethics Committee of the Universidade Federal de São Paulo, number CEP 1793/11, according to Brazilian federal law.

2.7. Statistical analysis

Differences between means values were analyzed using one-way ANOVA followed by Tukey's multi-comparison test in the coagulation assays. A p value < 0.05 was considered significant.

3. Results and discussion

3.1. Structural analysis of cMoL

cMoL is a coagulant *M. oleifera* lectin, purified by Santos et al. [24] after saline extraction and guar gel column chromatography. The coagulant property of this lectin is related to reduction of contaminants in water; cMoL promoted turbidity reduction of approximately 92% (in relation to negative control) similar to the positive control aluminum sulphate on water with high and low turbidity of kaolin clay [24]. By hemagglutination assays, the protein is resistant to change in pH, thermostable, agglutinates erythrocytes from rabbit and human blood types [24] and have insecticidal activity [28]. By a new and simple protocol, Santos et al. [24] purified and partly characterized a lectin different from those already reported, WSMoL [25] and MoL [29]. The knowledge of the structure and physicochemical properties of cMoL is needed to understand the mechanisms of action involved in the biological activities developed by the lectin and thus integrate the study of structure-function of the protein. Reverse phase chromatography was made to assess the purity of the fractions obtained in guar gel column. Fig. 1 shows the cMoL profile obtained by HPLC. The peak represents cMoL fractions from guar gel column eluted with 0.3 M NaCl. The detection of a single peak shows homogeneity in the purification, so this step purified efficiently the lectin, biologically active.

Highly pure fractions of proteins are important for the performance of amino acid sequence and circular dichroism analysis. In this study, the complete sequence of cMoL was obtained and compared with other protein sequences of the NCBI-BLAST data bank; the search indicated similarity with the sequence of M.O_{2.1}, a flocculent active protein from *M. oleifera* [19]. These sequences were aligned using MULTALIN program (Fig. 2). The sequence revealed that cMoL is a protein with 101 amino acids, two chains, and has 81% of similarity with M.O_{2.1}, which presents 6.5 kDa and isoelectric point above pH 10 [19]. It is important to note that cMoL have eight cysteine residues, which may be involved in the disulfide bonds.

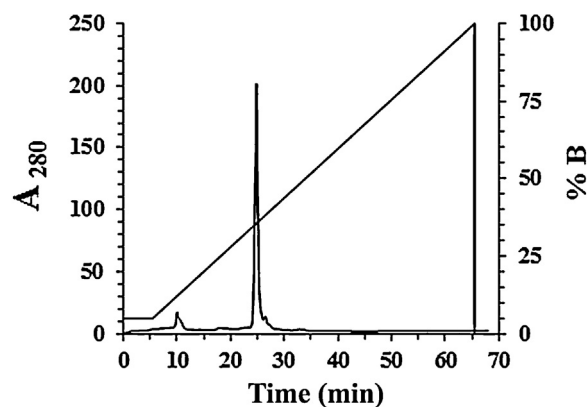


Fig. 1. cMoL profile by reverse phase chromatography using VYDAC C4 column in a HPLC system. Fractions eluted with 0.3M NaCl on an affinity guar gel column were assessed for homogeneity evaluation. Absorbance was performed at 280 nm. The protein fraction was eluted using a linear gradient: solvent B (90% acetonitrile in 0.1% TFA), where B = 5% when $t=0$ min, B = 5% at $t=5$ min, B = 100% at $t=60$ min, B = 0% when $t=65$ min.

cMoL is a basic protein with a theoretical pI of 11.67 indicating a strong positive charge on the surface and confirming 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. Several protein families with flocculent/coagulant activity are present in *M. oleifera* seeds [19,39]. cMoL must develop its coagulant activity in water due to interactions among charges, as proposed to MO_{2.1} [19,20] and not through binding to carbohydrate recognition site. Further studies on the genome and proteome of *M. oleifera* seeds could contribute to unravel the function of products from a unique gene(s) in the plant [24].

The native cMoL molecular weight obtained by Santos et al. [24] through Sephacryl S-300 size exclusion chromatography revealed a single peak corresponding to an apparent molecular mass of 30,000 Da; however, the molecular weight estimated to cMoL through the primary sequence revealed that the protein showed 11,928 Da. These results suggested that cMoL is a trimer consisting of three subunits of 11,928 Da. cMoL is not a glycosylated protein, which was confirmed through Schiff's reagent staining by Santos et al. [24].

Analyses of CD spectrum shows a typical shape and features of a mainly α -helical secondary structure, i.e., two negative bands at 222 and 209 nm, and a positive band at 192 nm (Fig. 3). The secondary structure content of cMoL was estimated at 46% α -helix, 12% β -sheets, 17% β -turns and 25% unordered structures with root mean

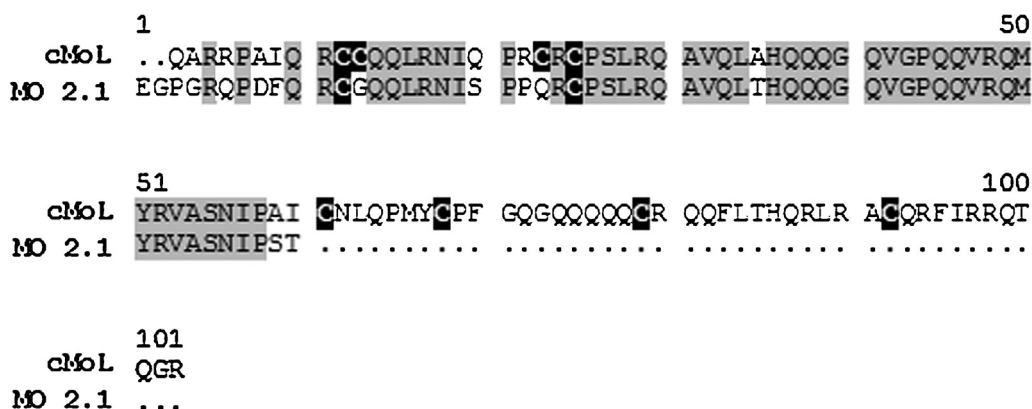


Fig. 2. Analysis of cMoL sequence and multiple sequence alignment of cMoL with flocculent *M. oleifera* protein MO_{2.1} (2111235A). Identical residues among them are displayed in gray background. The cysteine residues of both proteins are indicated in black background.

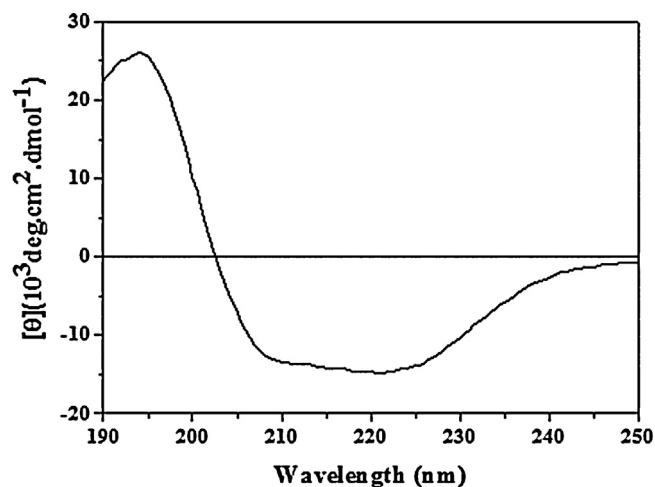


Fig. 3. CD spectrum of cMoL in 10 mM PBA, pH 7.0. Measurements were recorded as an average of 8 scans for protein solutions of 0.2 mg/mL, at 25 °C. CD spectrum deconvolution indicated 46% α -helix, 12% β -sheet, 17% β -turn, 25% unordered structure.

square deviation (RMS) lower than 2% for all structures. The Cluster analysis showed that cMoL belongs to the α/β tertiary structure class, corroborated by results of secondary structure estimative. For α/β proteins, the 222 nm band generally has a higher intensity than 208–210 nm band, whereas for the $\alpha+\beta$ proteins the reverse is observed [38]. The content of α -helices estimated for cMoL was similar to that determined for MO, the coagulant protein purified by Ndabigengesere and Narasiah [40]. Kwaambwa and Maikokera [41] reported that this protein presents secondary structural components of 58% α -helix, 10% β -sheet and 33% unordered structures, with RMS lower than 4% for all structures. But different from MoL, an alpha–beta lectin, isolated by Katre et al. [29] which containing 28% α -helix, 23% β -sheet, 20% turn and 28% of unordered structure. The secondary structure content of cMoL and MoL revealed that these lectins have different content of secondary structures but consist of a mixture of alpha and beta structures.

Generally, the functional stability is accompanied by structural stability. Fig. 4a shows CD spectra of cMoL in pH values of 2.0, 7.0 and 12. The secondary structure of the protein did not change in acidic and alkaline conditions as shown in the CD spectra, corroborating with the data on the stability of biological activity in function of pH. The content of each secondary structure of cMoL at different pH range is shown in Table 1. No significant change due to pH was observed in the estimated secondary structures from CD spectra. Sample spectra from other pH values were omitted since

Table 1
cMoL secondary structure estimated from CD spectra at different pH values.

pH	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Unordered (%)
2.0	43	13	18	26
7.0	46	12	17	25
12.0	39	15	19	27

Quantitative predictions were performed using CDPro software.

Table 2
cMoL secondary structure estimated from CD spectra at different temperatures.

Temperatures	α -Helix (%)	β -Sheet (%)	β -Turn (%)	Unordered (%)
40 °C	51	9	16	25
60 °C	51	8	16	25
80 °C	42	10	21	27
100 °C (30 min)	18	25	23	33
100 °C (1 h)	7	26	27	40

Quantitative predictions were performed using CDPro software.

small changes were detected. cMoL and MoL have as a common feature activity in acidic and alkaline conditions [24,29]. Other lectin with similar pH stability is a chitin-binding lectin from rhizomes of *Setcreasea purpurea* (SPL). SPL is a homotetrameric protein with hemagglutinating activity stable in pH range of 2.0–9.0 [42].

Treating cMoL at 100 °C and subsequent evaluation by HA revealed the lectin as a thermostable protein [24], however by accurate CD analysis, the protein conformation is maintained only until 80 °C for 30 min (Fig. 4b). When cMoL was subjected to heating at 80 °C, change in α -helix content was verified, followed by a gentle increase of β -structures while unordered structures did not alter. The estimated unordered structure content increased when cMoL was heated at 100 °C for 30 min and 1 h (Table 2). High temperature is a powerful denaturing agent leading to protein unfolding through breaking of hydrogen bonds that maintain protein structure [43]. Thermostability is a common feature of proteins from *M. oleifera* seeds. MOCP, a coagulant protein isolated by Ghebremichael et al. [21] remained active after 5 h heat treatment at 95 °C. Katre et al. [29] also reported that MoL is a heat-stable protein, which retains the activity even after incubating at 85 °C up to 30 min at pH 7.2.

3.2. cMoL effect on hemostatic parameters

The influence of cMoL on blood coagulation was determined by activated partial thromboplastin time (aPTT) and prothrombin time (PT) (Fig. 5). The positive control used in the assays was plasma of healthy volunteer donors with normal values for clotting times. A variety of new anticoagulants are being developed and tested

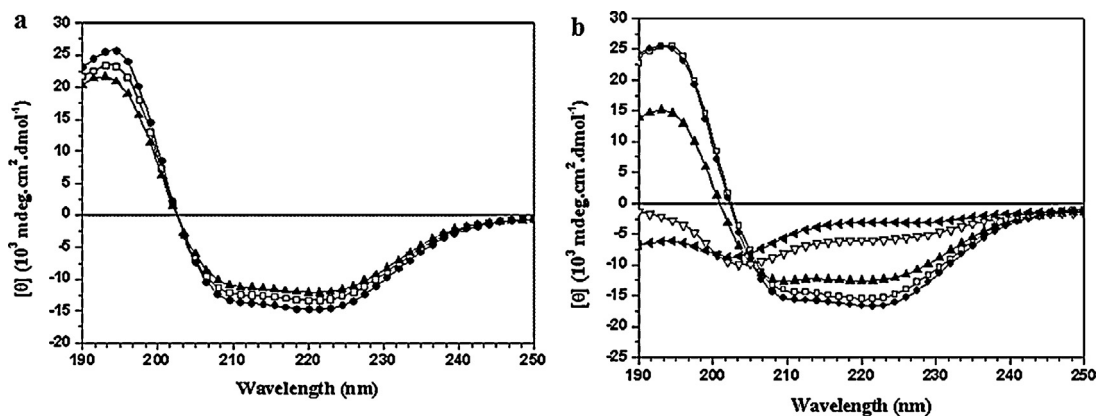


Fig. 4. (a) CD spectra of cMoL (0.2 mg/mL), in 10 mM PBA buffer, at pH 2.0 (□), 7.0 (●) and 12 (▲) and (b) cMoL spectra after heating at 40 (□), 60 (●), 80 (▲) and 100 °C for 30 min (▽). The spectra after heating at 100 °C for 1 h is also represented (◄).

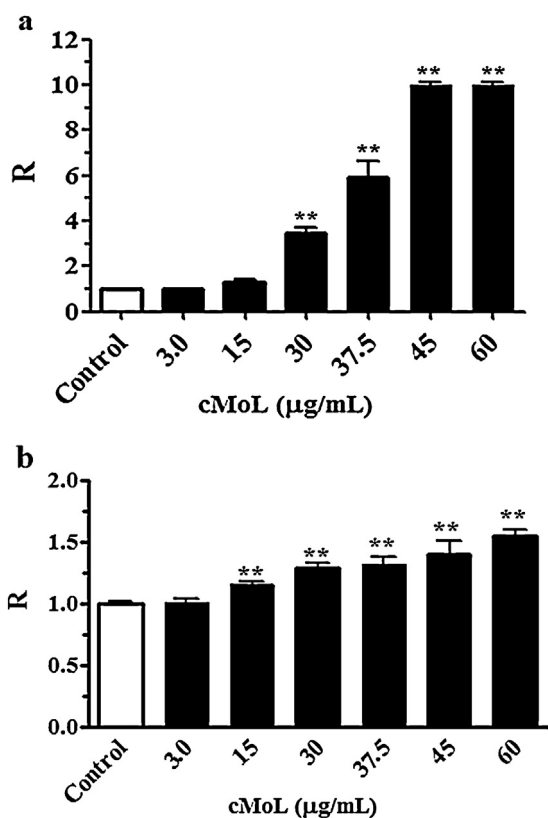


Fig. 5. *In vitro* effect of cMoL in hemostatic parameters. Activated partial thromboplastin time (aPTT, a) and prothrombin time (PT, b) were determined. The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. A p -value < 0.05 was considered to indicate significance. p -Value < 0.05 (*) and p -Value < 0.001 (**). R: ratio of sample coagulation time with control coagulation time. Data represent means \pm s.e.m., $n = 3$.

to inhibit the various steps in the coagulation cascade [44]. The determination of aPTT is particularly useful in monitoring the effect of heparin and determining deficiencies of factors VIII, IX, XI and XII. TP reflects the activity of factor II (prothrombin), V, VII and X, whose deficiency is accompanied by a prolongation of time required for clot formation [45].

cMoL significantly prolonged aPTT (to more than 300 s) and PT (Fig. 5b) coagulation time, at the assayed concentrations (3.0, 15, 30, 37.5, 45 and 60 μ g/mL) but the most affected parameter was aPTT (Fig. 5a). Prolongation of aPTT suggests inhibition of the intrinsic pathway and/or common blood coagulation, while prolongation of PT suggests inhibition of the extrinsic pathway [46]. The intrinsic and extrinsic pathways converge in the formation of factor Xa [47]. Other lectins also significantly prolongs both coagulation times. *Cratylia mollis* seed lectin (Cramoll 1,4), a mannose/glucose binding lectin, promotes almost two-fold increase of coagulation times [48]. *Bauhinia forficata* lectin, BfL, increased only aPTT coagulation time but this effect was not related to human plasma kallikrein or human factor Xa inhibition [49]. Thus cMoL showed an anticoagulant activity, since in determining the R, the ratio between sample coagulation time and control coagulation time [50] was higher than 1.0.

Significant correlation between recognition and binding of lectins to carbohydrates or glycoconjugates on cell surface or free in biological fluids are involved in many lectin effects, such as activity against insects, viruses, bacteria and cytotoxicity [27,42,51,52]. Then, to investigate cMoL interaction with coagulation factors, blood coagulation assays were performed in the presence of asialofetuin. This glycoprotein blocks the site of carbohydrate recognition of the lectin. aPTT assays showed a reduction in the

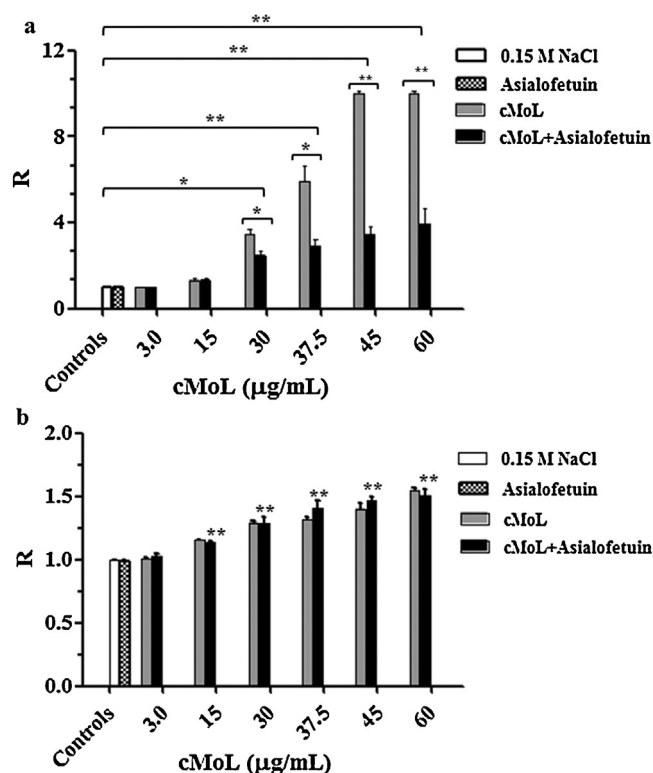


Fig. 6. Blood coagulation assays in the presence of cMoL inhibited by asialofetuin (0.5 mg/mL). Lectin was previously incubated (15 min) with asialofetuin. (a) Activated partial thromboplastin time (aPTT); (b) prothrombin time (PT). The statistical significance was evaluated using one-way ANOVA, followed by Tukey's test. A p -value < 0.05 was considered to indicate significance. $p < 0.05$ (*) and $p < 0.001$ (**) represent comparative analysis between cMoL inhibited by asialofetuin and controls. Statistical significance of cMoL in the presence and absence of glycoprotein was also showed. R: ratio of sample coagulation time with control coagulation time. Data represent means \pm s.e.m., $n = 3$.

time required for blood coagulation in the presence of the inhibited lectin by glycoprotein in the same assayed concentrations (Fig. 6a), while PT was not affected (Fig. 6b). These results suggest that cMoL interacts with the carbohydrate portion of serine proteases of the coagulation cascade from intrinsic pathway. No decrease in PT in the presence of inhibited lectin suggests that the lectin should act on the factors of the extrinsic pathway and/or under the factor Xa by a different domain from the carbohydrate recognition.

4. Conclusions

In this article structural characteristics of cMoL were described; also, for the first time, the anticoagulant activity was detected on human blood coagulation process by a coagulant *M. oleifera* seed lectin. Structural analysis revealed cMoL common features with another coagulant protein from *M. oleifera*. cMoL interactions in blood coagulation must occur at least partially, by the carbohydrate recognition domain. cMoL is the only currently described protein from *M. oleifera* seeds that exhibits anticoagulant activity.

Author contributions

Conceived and designed the experiments: LAL, MCCS, RSF, MLVO, LCBBC. Performed the experiments: LAL, MCCS, RSF, LAS, RM. Contributed to reagents and analysis tools: RM, MLVO, PMGP, LCBBC. Analyzed and interpreted the data: LAL, MCCS, RSF, RASL, MLVO, PMGP, LCBBC. Wrote the paper: LAL, MCCS, RASL, PMGP and LCBBC. All authors read and approved the final manuscript.

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