Moringa oleifera Lam.: Protease activity against blood coagulation cascade

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ABSTRACT

Background: The present study evaluated the protease activity of aqueous extracts of *Moringa oleifera* (Moringaceae) leaf (MOL) and root (MOR). **Materials and Methods**: Protease activity was assayed using casein, human plasma clot and human fibrinogen as substrates. **Results**: Caseinolytic activity of MOL was significantly higher ($P \leq 0.05$) than that of MOR. Similar observations were found in case of human plasma clot hydrolyzing activity, wherein MOL caused significantly higher ($P \leq 0.05$) plasma clot hydrolysis than MOR. Zymographic techniques were used to detect proteolytic enzymes following electrophoretic separation in gels. Further, both the extracts exhibited significant procoagulant activity as reflected by a significant decrease ($P \leq 0.05$) in recalcification time, accompanied by fibrinogenolytic and fibrinolytic activities; clotting time was decreased from 180 ± 10 sec to 119 ± 8 sec and 143 ± 10 sec by MOL and MOR, respectively, at a concentration of 2.5 mg/mL. Fibrinogenolytic (human fibrinogen) and fibrinolytic activity (human plasma clot) was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), plate method and colorimetric method. Zymographic profile indicated that both the extracts exerted their procoagulant activity by selectively hydrolyzing Aα and Bβ subunits of fibrinogen to form fibrin clot, thereby exhibiting fibrinogenolytic activity. However, prolonged incubation resulted in degradation of the formed fibrin clot, suggesting fibrinolytic like activity. **Conclusions**: These findings support the traditional usage of *M. oleifera* extracts for wound healing.

Key words: Caseinolytic, fibrinogenolytic, fibrinolytic, *Moringa oleifera*, procoagulant

INTRODUCTION

It is well known that proteases hydrolyze peptide bonds of proteins and they differ in their substrate specificity and ability to hydrolyze various peptide bonds. Proteases are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated reactions such as blood cascade, complement system, and apoptosis pathways.[1] Several plant latex proteases are known to interfere in hemostasis as procoagulants, suggesting their unique substrate preference over other proteases.[2,3] Cysteine proteases from the Asclepiadaceae plants latex were reported to exhibit thrombin and plasmin like activities.[4] Proteases interfering in blood coagulation and fibrin hydrolysis have been isolated and characterized from several plant latexes. Ficin, a cysteine protease from *Ficus carica*, and a serine protease from *Synadenium grantii*

were shown to involve in blood coagulation and fibrin hydrolysis.[3,5]

Coagulation is a complex process by which blood forms clots. It is an important part of hemostasis (the cessation of blood loss from a damaged vessel), wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and begin repair of the damaged vessel. Disorders of coagulation can lead to an increased risk of bleeding (hemorrhage) or clotting (thrombosis). In patients with advanced liver disease, bleeding and thrombosis are dangerous complications, particularly in those who are challenged by infection or who require surgery.[6]

Moringa oleifera is a tree of the Moringaceae family, widely cultivated throughout the tropics and subtropics, and its leaves, seed pods, seeds, seed oil, roots, bark, flowers, and sap are commonly consumed as food and used in traditional medicines. Its use as an inexpensive component in foods, nutritional supplements, or medicines by patients with HIV/AIDS has been advocated by several African

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governments.[7] It possesses anti-inflammatory, antioxidant, antimicrobial, antifertility, anticancer, antihepatotoxic and antiulcer activities.[8]

Reports have shown that the products of traditionally used plants such as *Bryophyllum pinnatum* and *M. oleifera* promoted healing in experimental animals.[9,10] The *M. oleifera* roots and seeds are prescribed for the treatment of snake bites and scorpion stings.[11] *M. oleifera* leaves are also reported to have hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity in rats.[12] These plants contain a mixture of several hydrolytic enzymes, in which proteases are the key enzymes responsible for the observed pharmacological actions. Hence, the aim of the present study was to investigate the protease activity of aqueous extracts of *M. oleifera* leaf and root against the blood coagulation cascade.

MATERIALS AND METHODS

Materials
Human fibrinogen and thrombin were purchased from Sigma Aldrich, Bangalore, India. Casein was purchased from Sisco Research Laboratory, Mumbai, India. Fresh human blood samples were collected from healthy volunteers. All other chemicals and reagents used in the study were of analytical grade.

Collection of the sample and preparation of the extracts
The root and the leaves of *M. oleifera* were collected from Mysore district during November 2009, identified by Dr. Shivprasad Hudeda, and a voucher specimen was retained in the laboratory for future reference. The samples were thoroughly cleaned, dried in hot air oven at 37°C overnight, powdered, passed through 60 mesh sieve and stored at 4°C until further use. The root was cleaned, cut into small pieces, dried in the oven at 37°C overnight, powdered, passed through 60 mesh sieve and stored at 4°C until further use. Aqueous extracts were prepared by extracting the powdered samples (50 g) with distilled water (1:8 w/v) in a mechanical shaker for 24 h at room temperature. The extracts were then filtered and freeze dried.

Gel electrophoresis
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.[13] Polyacrylamide gel electrophoresis (PAGE 7.5%) under neutral conditions was carried out for aqueous extracts of leaf (MOL) and root (MOR).

Caseinolytic activity
Caseinolytic activity was assayed using denatured casein as substrate.[14] Briefly, 0.4 mL casein (2%) in 0.2 M Tris-HCl buffer, pH 8.5, was incubated separately with different concentrations of MOL and MOR in a final volume of 1 mL for 2 h at 37°C. The reaction was stopped by adding 1.5 mL of 0.44 M trichloroacetic acid (TCA) and the mixture was allowed to stand for 30 min. The reaction mixture was centrifuged at 1500 g for 15 min. An aliquot (1 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin reagent (1:2, v/v). The color developed was read at 660 nm. Activity was expressed as units/h. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h.

Zymography
Casein-zymography was performed according to the method of Kim *et al*,[15] with SDS-PAGE 7.5% containing 0.1% (w/v) casein. MOL and MOR (1–3 mg) were incubated with SDS-PAGE sample buffer without 2-mercaptoethanol for 30 min at 37°C and the samples were subjected to SDS-PAGE. After electrophoresis, the gels were washed in 2.5% (v/v) Triton X-100 in Tris buffer, pH 7.5, for 15 min twice and then incubated in the incubation buffer at 37°C for 12–24 h under shaking, and stained with Coomassie Brilliant Blue R and then destained. The area of digestion appears as clear bands against a darkly stained background where the substrate has been degraded by the enzyme.

Coagulant activity
Coagulant activity was determined according to the procedure described by Condrea *et al*,[16] Fresh human blood was mixed with 0.11 M trisodium citrate (1:9, v/v). The mixture was centrifuged for 15 min at 1500 g and the supernatant obtained was platelet poor plasma (PPP). To 300 µL of PPP, 30 µL of 0.01 M Tris-HCl buffer (pH 7.4) was added and pre-warmed to 37°C, following which 1 mg of sample was added and incubated for 1 min. The clot formation was initiated by adding 30 µL of 0.25 M $CaCl_2$. The time taken for the formation of visible clot from the time of addition of $CaCl_2$ was recorded. Control experiment was carried without the addition of sample.

Fibrinogenolytic assay
Fibrinogen hydrolyzing activity was estimated according to the method of Ouyang and Teng.[17] Human fibrinogen (50 µg) in 10 mM Tris-HCl buffer, pH 7.4, was incubated with 1 mg MOL and MOR in a reaction volume of 40 µL for 30 min at 37°C. The reaction was terminated by adding 20 µL of sample buffer containing 4% -mercaptoethanol, 1 M urea and 4% SDS, and subjected to SDS-PAGE (10%). The fibrinogen-hydrolyzing pattern was visualized by staining with Coomassie Brilliant Blue R-250.

Fibrinogen polymerization assay

Fibrinogen polymerization assay was carried out by slightly modifying the method of Joo *et al*,[18] Three milliliters of 1.2% agarose warmed to 50°C was mixed with 3 mL of 0.4% (w/v) fibrinogen dissolved in 0.1 M Tris-HCl (pH 7.4). Fibrinogen–agarose mixture was poured into a Petri plate and allowed to solidify for 1 h at room temperature. Wells of about 3 mm diameter were made on the plate, and 20 μL of buffer containing 1 mg of MOL and MOR was introduced into the well and incubated at 37°C for 2 h. The zone of fibrin rings formed due to the polymerization of fibrinogen was observed. 0.2 unit of thrombin was used as positive control and the buffer alone served as control.

Fibrinolytic activity

SDS-PAGE analysis of plasma clot hydrolysis

The assay was carried out as described by Rajesh *et al*,[19] Briefly, the washed plasma clot (cross-linked fibrin) was incubated with different amounts of MOL and MOR extracts (1–3 mg) separately in a reaction volume of 40 μL containing 10 mM Tris-HCl buffer, pH 7.4, for 12 h. The reaction was terminated by adding 20 μL of sample buffer containing 4% β-mercaptoethanol and 1 M urea, boiled for 3 min and centrifuged to settle the debris of plasma clot. An aliquot (20 μL) of the supernatant was subjected to 7.5% SDS-PAGE to analyze fibrin hydrolyzing pattern.

Plasma clot hydrolyzing activity

Plasma hydrolyzing activity was assayed according to the method by Rajesh *et al*,[19] Briefly, 100 μL of citrated human plasma was mixed with 30 μL of 0.25 M CaCl$_2$ and incubated for 2 h at 37°C. The clot obtained was washed thoroughly for 5–6 times with phosphate buffered saline (PBS) and suspended in 400 μL of 0.2 M Tris-HCl buffer, pH 8.5. The reaction was initiated by adding varying amounts of MOL and MOR (1–5 mg) in 100 μL of 10 mM Tris-HCl buffer, pH 7.4, and incubating for 2 h 30 min at 37°C. The undigested clot was precipitated by adding 750 μL of 0.44 M TCA and allowed to stand for 30 min and centrifuged for 15 min at 1500 *g*. The aliquots of 0.5 mL supernatant were transferred to clean glass tubes, followed by the addition of 1.25 mL of 0.4 M sodium carbonate and 0.25 mL of 1:3 diluted Folin and Ciocalteu's phenol (FC) reagent. The color developed was read at 660 nm after allowing the tubes to stand for 30 min. One unit of activity is defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h (37°C).

Plasma clot hydrolyzing activity (fibrinolytic activity)

Plate method

The method described by Gene *et al,* was followed with slight modification.[20] A mixture consisting of 2 mL of healthy human plasma and 3 mL of 1.2% molten agarose (45°C) in 10 mM Tris-HCl buffer containing 0.02% sodium azide, 70 mM (NH4)$_2$SO$_4$, 90 mM NaCl, 0.70 mM MgCl$_2$ and 200 μL of 0.2 M CaCl$_2$ was poured into 10 × 90 mm Petri dish and allowed to stand for 2 h at 27°C. Different concentrations of MOL and MOR (1–2 mg) were placed separately on the surface of the gel matrix and incubated for 12 h at 27°C. The diameters of the clear zones (plaque) were noted. 10 μL of 10 mM Tris-HCl buffer, pH 7.4, served as control.

Statistical analysis

Measurements made were mean of triplicate determinations, and wherever applicable, the data were analyzed by analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for significant difference using SPSS 16.0 software.

RESULTS AND DISCUSSION

The present study showed the protease activity of aqueous extracts of *M. oleifera* leaf and root in blood coagulation cascade. Presence of proteolytic activity is one of the important characteristic features of the plant. These crude extracts were subjected to electrophoresis under SDS-PAGE (7.5%) in a non-reducing condition. In both the extracts, clearly distinguishable protein bands were resolved in gel [Figure 1].

The extracts were assessed for proteolytic activity using denatured casein as substrate. Both the extracts showed proteolytic activity in a dose-dependent manner. The leaf extract was found to exhibit significantly higher ($P \leq 0.05$) activity compared to the root extract [Figure 2]. For further detecting the protease activity, we used the casein-zymographic technique [Figure 3]. This action is due to the presence of proteolytic
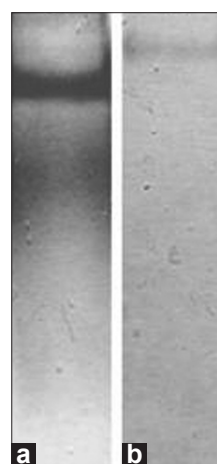

Figure 1: SDS-PAGE of MOL and MOR. 1 mg concentration of each of the samples was loaded onto the 7.5% SDS-PAGE and electrophoresis was performed in a constant current under non-reducing condition using Tris-glycine buffer. (a) MOL and (b) MOR
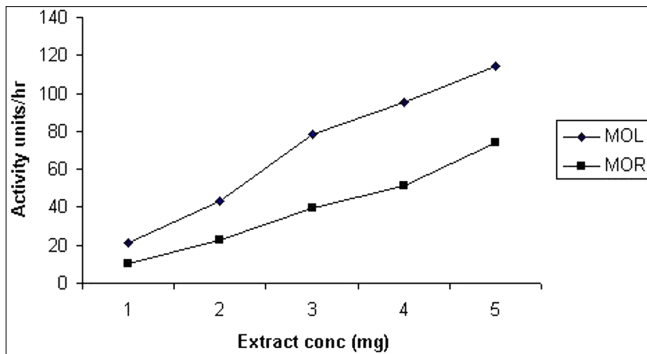
Figure 2: Caseinolytic activity of MOL and MOR extracts. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h. Caseinolytic activity of MOL (- -) and MOR (- -) extracts. One milliliter of reaction mixture contained 0.4 mL of casein 2% in 0.2 M Tris-HCl buffer, pH 8.5, incubated with different concentrations of extracts ranging from 1 to 5 mg for 2 h at 37°C
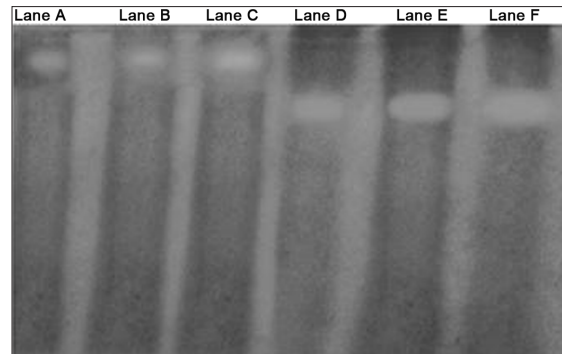
Figure 3: Protease activity of MOL and MOR by zymography method. Includes casein as a substrate copolymerized with the polyacrylamide gel, for the detection of enzyme activity. Substrate degradation products were resolved in 7.5% SDS-PAGE. Lane A, MOR (1 mg); Lane B, MOR (2 mg); Lane C, MOR (3 mg); Lane D, MOL (1 mg); Lane E, MOL (2 mg); Lane F, MOL (3 mg)
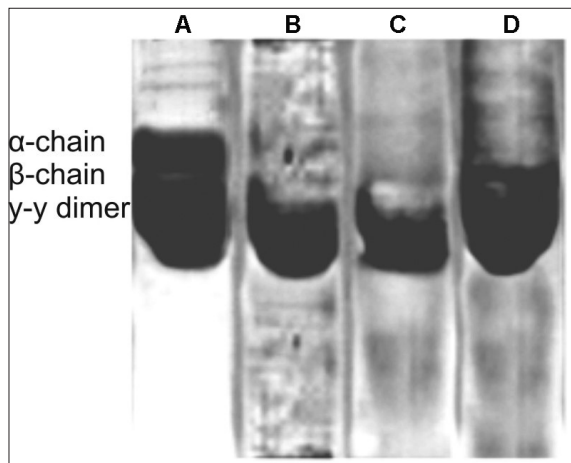
Figure 4: Electrophoresis pattern of fibrinogen by extracts. Reaction mixture of volume 40 µL contained 50 µg fibrinogen incubated with 1 mg of MOL and MOR in 10 mM Tris-Hcl buffer, pH 7.4, at 37°C for 30 min. Hydrolyzing pattern of fibrinogen by 10% SDS-PAGE under reduced condition. (A) 50 µg fibrinogen, (B) 50 µg fibrinogen + 0.2 units thrombin, (C) 50 µg fibrinogen + 1 mg MOL and (D) 50 µg fibrinogen + 1 mg MOR
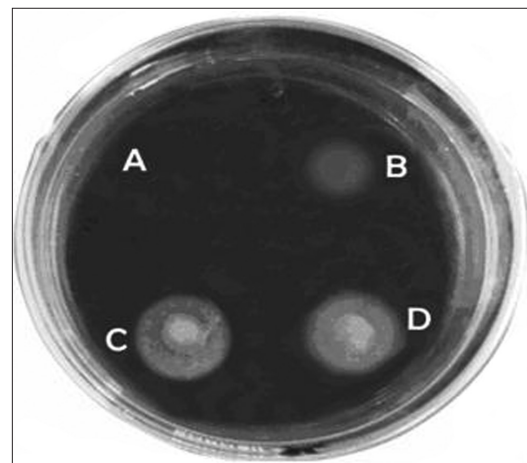

Figure 5: Fibrinogen–agarose plate assay to determine thrombin like activity of MOL and MOR. (A) Buffer alone, (B) buffer + thrombin (0.2 units), (C) buffer + 1 mg MOL, (D) buffer + 1 mg MOR

enzymes and evoked further investigation on its effect on pure fibrinogen. Both the extracts hydrolyze the Aα and Bβ chains of fibrinogen subunits, but not γ chain. Hydrolyzing pattern of fibrinogen was monitored using 10% SDS-PAGE under reduced conditions, along with thrombin-induced fibrin clot [Figure 4]. Thrombin like enzymes hydrolyze Aα and Bβ chains of fibrinogen at the N-terminal disulfide knot and from fibrin by releasing fibrinopeptide.[21] Further, the fibrinogenolytic like activity of MOL and MOR was compared with known units of thrombin enzyme, using fibrinogen agar plate. Figure 5 shows the polymerization of fibrinogen by both the extracts. At the concentration of 1 mg, the diameter of the zone of fibrin rings formed due to the polymerization

of fibrinogen was similar to the zone area produced by 0.2 unit of thrombin, suggesting that both the extracts have fibrinogenolytic activity. The proteolytic activity that hydrolyzes fibrinogen can either induce clot or prolong clot formation. The clot inducing or prolonging ability of these extracts was studied by determining the recalcification time of human citrated plasma. The extracts promoted clot formation after addition of $CaCl_2$ to human citrated plasma. The induction of clot was from 180 ± 10 sec to 119 ± 8 sec in MOL, while MOR decreased the clotting time to 143 ± 10 sec at a concentration of 2.5 mg/mL. Hydrolysis of fibrinogen by both the extracts results in the formation of fibrin clot (fibrinogenolytic activity). This study very clearly suggested that both MOL and MOR extracts exhibited procoagulant activity after addition of $CaCl_2$ to human citrated plasma, and reduced the recalcification time in clot formation by activating the factors or by precipitating the co-factors involved in the blood coagulation cascade.
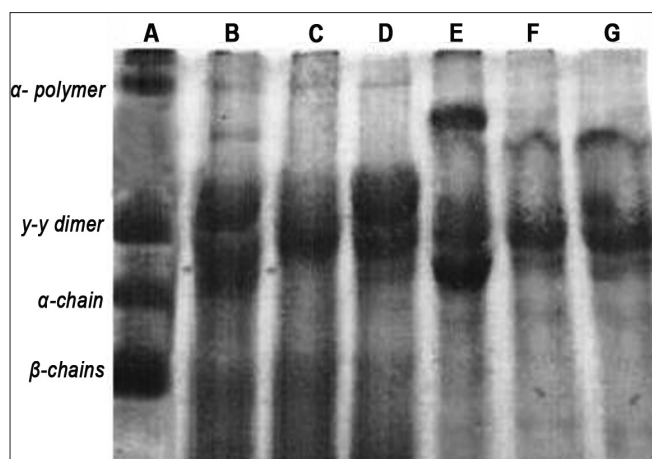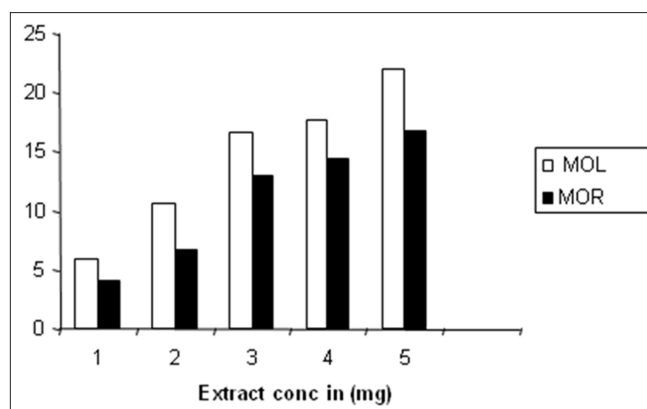
Figure 6: Dose-dependent hydrolysis of plasma clot by MOL and MOR. The washed plasma clot was incubated separately with the different concentrations of extract for 12 h in 40 µL of 10 mM Tris-HCl buffer, pH 7.4, at 37°C. The samples included (A) plasma clot alone, (B) plasma clot treated with MOL l mg, (C) plasma clot with MOL 2 mg, (D) plasma clot treated with MOL 3 mg, (E) plasma clot treated with MOR l mg, (F) plasma clot treated with MOR 2 mg and (G) plasma clot treated with MOR 3 mg
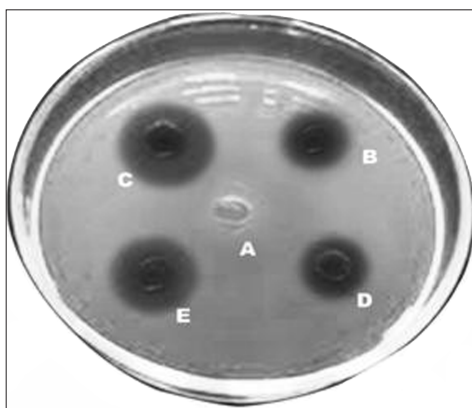
Figure 8: Fibrinolytic activity of MOL and MOR. The samples in 10 µL of 10 mM Tris-HCl buffer, pH 7.4, were separately placed on the surface of the gel and incubated for 12 h at 27°C. (A) Buffer alone, (B) MOL 1 mg, (C) MOL 2 mg, (D) MOR 1 mg, (E) MOR 2 mg

The observed procoagulant activity/fibrinogenolytic activity might be the basis for the use of extracts to stop bleeding by traditional healers.

Besides fibrinogenolytic activity, the extracts exhibited plasma clot hydrolyzing properties on prolonged incubation, resulting in degradation of the formed fibrin clot, which suggests fibrinolytic like activity by the hydrolysis of only few subunits of α-polymer, α-chains, β-chains of crude fibrin clot in a dose-dependent manner. To substantiate these clot hydrolyzing properties, the hydrolyzed products of fibrin clots were analyzed on SDS-PAGE 7.5% [Figure 6] under reducing condition, colorimetric method

Figure 7: Concentration-dependent hydrolysis of human plasma clot. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h

and plate method [Figures 7 and 8]. Eventually, blood clots are reorganized and resorbed by a process termed fibrinolysis. The main enzyme responsible for this process (plasmin) is regulated by various activators and inhibitors. Plasmin is generated by proteolytic cleavage of plasminogen, a plasma protein synthesized in the liver. This cleavage is catalyzed by tissue plasminogen activator (t-PA), which is synthesized and secreted by endothelium. Plasmin proteolytically cleaves fibrin into fibrin degradation products that inhibit excessive fibrin formation and hemostatic plug, facilitating wound healing.[22] However, extracts of leaf (MOL) and root (MOR) exhibited a mixture of both fibrinogenolytic and fibrinolytic activities. Present data suggested the dual action of crude extracts, i.e. both fibrinogenolytic and fibrinolytic activities, might be due to the presence of thrombin and plasmin like enzymes or a single enzyme responsible for the dual action.

CONCLUSION

The study provides scientific validation for the use of *M. oleifera* in traditional medicine as an anticoagulant. The results suggest the involvement/presence of thrombin and plasmin like enzymes. Further studies are needed to understand the mechanism of action. Hence, attempts are underway to purify both thrombin and plasmin like proteases from *M. oleifera* leaf and root to promote their pharmacological utilization.

ACKNOWLEDGMENT

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