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RESEARCH ARTICLE

The hemostatic activity of *Manilkara zapota* (L.) P. Royen latex associated with fibrinogenolytic activity

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ABSTRACT

Manilkara zapota (L.) P. Royen (Sapotaceae), is widely used in traditional medicine for various ailments like, diarrhea, pulmonary diseases, piles, ulcers and to treat wounds. The present study evaluates the role of *M. zapota* latex in hemostasis. The processed latex named as *M. zapota* natant latex (MzNL), has proteins at the concentration of 8 mg/ml and showed protein bands in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteolytic activity of MzNL was evaluated using casein in comparison with trypsin. The phenylmethylsulfonyl fluoride (PMSF) inhibited the protease activity indicating the possible presence of serine protease. The effect of temperature, pH and metal ions on proteolytic activity was evaluated. MzNL exhibited fibrinogenolytic activity by hydrolysing Aα and Bβ subunits of fibrinogen. However, γ subunit remained resistant for hydrolysis. MzNL hydrolyzed all the subunits of collagen type I and IV at the concentration of 8 µg and 25 µg in 20 µl each respectively. MzNL showed procoagulant activity and is devoid of hemolytic activity. Fibrinogenolytic activity and procoagulant nature of MzNL suggests its possible role in blood coagulation that in turn restores hemostasis.

Introduction

Hemostasis is a complex process to avoid loss of blood during injury. It is regulated by interactions of blood vasculatures, platelets, cytokine mediators, coagulation factors and fibrinolytic proteins. During injury to the blood vessel, platelets and immune cells enters injured site to initiate blood coagulation and wound healing. Simultaneously the exposed sub-endothelial collagen triggers the activation of intrinsic pathway and tissue factor initiates extrinsic pathway of blood coagulation cascade. Both the pathways activate coagulation proteins in cascade manner and enters common pathway by activating Factor X. This in turn activates thrombin which forms fibrin mesh from the fibrinogen, avoiding blood loss (1). The cascade of activation of coagulation factors is represented in Fig. 1.

Several hemostatic components have been reported from different sources such as venoms, microbes and plants latex (2–4). Plant latex has been extensively used to stop bleeding from fresh cuts and to

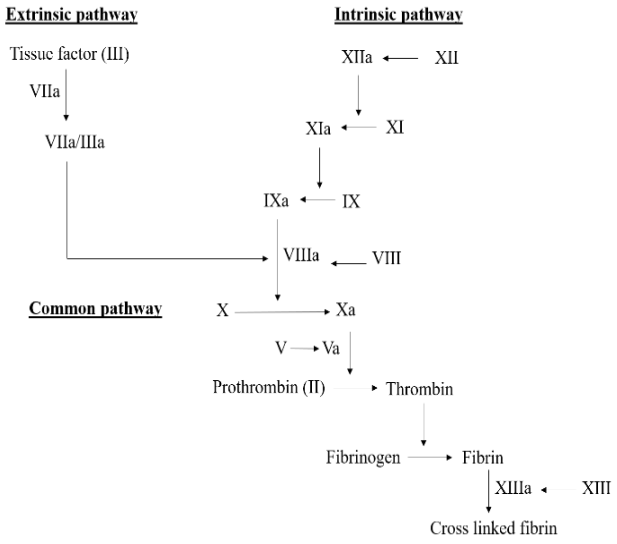

Fig. 1. Schematic representation of blood coagulation cascade

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enhance the wound healing process in folklore medication (5). Proteases in plant latex are reported to be the causative agent for hemostatic effect. Latex proteases interfere in coagulation pathway majorly at the common pathway (5, 6). Some latex proteases mimic the activity of thrombin, these thrombin-like enzymes convert fibrinogen to fibrin (7).

The *M. zapota* is one such latex producing evergreen tree belonging to the family Sapotaceae. The plant is native to Southern Mexico, north-eastern Guatemala and Northern Bleize. The plant has been cultivated abundantly in Central America, Caribbean islands and also in Asian countries like Malaysia, Bangladesh, India, Pakistan, Thailand, Cambodia and Indonesia (8). The plant has named differently in different countries; Chickoosapote, Chickoozapote (Mexico, Hawai, California, southern Florida), Cheeku (Urdu). Chickoo, Sapota (Hindi), Sopeta, Sofeda (Bengali), Lamut farang (Thai), Sapoti (Brazil, Haiti), Ciku, Sawo manila (Malay), Nispero (Colombia, Venezuela) (9, 10). The image of plant and its fertile parts are represented in Fig. 2, (11).

Fig. 2. A. Habit of *M. zapota*, **B.** Fruit, **C.** Flower.

Traditionally most parts of *M. zapota* plant have been used for medicinal purposes; latex from *M. zapota* is coagulated to form chickle, used to prepare chewing gums (12). The fruits, leaves and bark are used to treat diarrhea and pulmonary diseases. Fruits have reported reducing inflammation and pain in gastritis (13). The paste prepared from seed used to reduce pain and inflammation caused by bites and stings (14). In folk medicine the juice prepared from the leaf of *M. zapota* is used to treat wounds and ulcers. There are reports on the significance of leaves extracts from *M. zapota* on corneal wound healing activity (15).

Based on the pharmacological properties and the traditional usage, an attempt has been made to evaluate the hemostatic property of *M. zapota* latex.

Materials and Methods

Chemicals

Casein, gelatin (from porcine skin), fibrinogen (from human plasma), collagen type I (from rat tail), collagen type IV (from the human placenta), E-64, PMSF, ethylenediaminetetraacetic acid (EDTA) and pepstatin A from Sigma Aldrich (St. Louis, MO, USA). Trypsin from HiMedia (Mumbai, Maharashtra, India), and all other reagents used were of analytical grade.

Latex collection and processing

The *M. zapota* latex was collected near Tumkur, Karnataka, India, in the month of March. The plant was identified by Dr. P. Sharanappa, Professor, Department of Studies and Research in Bioscience, Hemagangotri, University of Mysore, Hassan, Karnataka, India. The voucher specimen (TU16DOS-RBC004) for *M. zapota* plant is deposited in the herbarium of the Department of Studies and Research in Botany, Tumkur University, Tumkur, Karnataka, India.

Latex of *M. zapota* was collected in a clean container from freshly plucked half ripened fruit and freeze overnight at –20 °C. The latex thawed and processed in centrifuge (RemiC-24BL, Maharashtra, India) with speed 10000 rpm for 20 min; the supernatant was subjected to dialysis using cellulose membrane having molecular cut off of 12 kDa against distilled water for 24 hr. The obtained clear protein-rich latex was named as MzNL.

Protease activity

Protease activity was analysed as explained as per the procedure mentioned (16). The casein (2%) dissolved in 200 mM Tris-HCl buffer (pH 7.0) was used as substrate. Various concentrations of MzNL (0–200 µg/20 µl) along with standard trypsin were incubated with 0.4 ml of casein separately at temperature 37 °C for 2 hrs 30 min. This reaction was terminated using 1.5 ml of trichloroacetic acid (0.44 M); After 30 min, centrifugation was done with the speed 3000 rpm for 5 min. To the 1 ml of supernatant 0.4 M sodium carbonate (2.5 ml) and 1:2 diluted Folin-Ciocalteu (FC) (0.5 ml) reagents were added and incubated at 37 °C for 20 min. The developed colour was analysed at 660 nm (BioSpectrometer Kinetic, Eppendorf, CA, USA). One unit of enzyme activity is stated as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm in 1 hr.

Effect of temperature, pH, inhibitors and metal ions on protease activity

Effect of temperature and pH on protease activity was tested by incubating 80 µg of MzNL at different temperatures (0 °C–80 °C) and pH (3–12) for 30 min. Later, the proteolytic activity was measured (16). For inhibition studies, the experiment was repeated with 30 min prior incubation of MzNL (80 µg) with 5 mM concentrations of standard protease inhibitors (E-64, EDTA, PMSF, pepstatin A). Effect of metal ions was evaluated by incubating 10 mM concentrations of different metal ions such as Ba^{2+}, K^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} with 80 µg of MzNL separately for 30 min. Further, proteolytic activity was evaluated as per procedure (16).

Electrophoresis and zymography

SDS-PAGE was performed as explained in Laemmli (17). MzNL (80 µg) was treated with a non-reducing sample buffer and boiled for 3 min. SDS-PAGE (10%) was performed for MzNL and molecular weight standards ranging between 14.2–97.0 kDa. The protein bands obtained were detected using 0.25% Coomassie brilliant blue R-250 (CBBR-250).

Periodic acid-Schiff base (PAS) staining was performed (18). SDS-PAGE was performed for 80 µg of MzNL and 50 µg of fibrinogen. The gel was fixed in 7.5% acetic acid at temperature 37 °C for one hr, and then washed with nitric acid. Further, the gel was kept at 4 °C in aqueous periodic acid (0.2%) for 45 min. This gel was stored overnight at 4 °C in the Schiff reagent. To visualize the reddish-pink bands the gel was de-stained with 10% acetic acid.

Zymography was performed (17). The 10% SDS-PAGE was performed for 80 µg of MzNL, where the resolving gel was incorporated with 0.2% casein/gelatin separately. After electrophoresis, the SDS was removed by washing the gel using Triton X-100 (2.5%). Further, the gel was incubated in Tris-HCl (50 mM; pH 7.0) buffer containing $CaCl_2$ (10 mM) and NaCl (150 mM). The gel was stained using CBBR-250 (0.25%) to observe activity bands.

Fibrinogenolytic activity

The fibrinogenolytic activity was evaluated (3). 50 µg of human fibrinogen was treated with various concentrations (0–15 µg/20 µl) of MzNL at 37 °C for 2 hrs 30 min. Reducing sample buffer (10 µl) was used to terminate the reaction. The hydrolysed products were analysed in SDS-PAGE (12%) and stained with 0.25% of CBBR-250 to visualize bands.

Recalcification time

Recalcification time assay was carried out (19). Different concentration (50–150 µg/20 µl) of MzNL was treated with 100 µl of citrated plasma from goat at 37 °C for 5 min. Further, 25 mM $CaCl_2$ (100 µl) was added and recorded the time until the formation of a clot.

Collagenolytic activity

The collagenolytic activity was identified (20). Type I (10 µg) and IV (25 µg) collagens were treated with different concentrations of MzNL (2–8 µg/20 µl for type I and 2–25 µg/20 µl for type IV) and incubated at 37 °C for 2 hrs 30 min. Reducing sample buffer (5 µl) was used to stop the reaction. Hydrolysed products were analysed in SDS-PAGE (7%) and stained with 0.25% CBBR-250.

Hemolytic activity

The assay was carried out (21). Blood from a healthy goat was treated with 3.2% of trisodium citrate in the ratio 1:9 and centrifuged for 5 min at speed 3000 rpm to obtain red blood cell (RBC) pellet. The pellet was washed and re-suspended in normal saline (0.9% NaCl) to obtain 2% erythrocyte suspension. The MzNL with the concentration ranging from 0–500 µg/50 µl were treated with 0.5 ml erythrocyte suspension in a reaction volume of 1 ml made with saline and incubated at temperature 37 °C for 30 min. Further, normal saline (2 ml) was pipette to the mixture and this mixture was subjected to centrifugation at speed 1500 rpm for 2–3 min. The amount of free hemoglobin was measured from 1 ml of the supernatant at 540 nm. The absorbance obtained while using normal saline was considered as 0% and water as 100%.

Protein estimation

The protein estimation was performed (22). The protein measured with the Folin's phenol reagent, where bovine serum albumin was used as standard.

Statistical analysis

Results were represented as mean ± standard error. One-way analysis of variance was used for statistical significance of intergroup differences and the Tukey test was used for comparison of means. The Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA) was used to perform all analysis.

Results and Discussion

In this study, we have evaluated the MzNL for proteolytic activity and its effect on hemostasis. The MzNL has a protein concentration of 8 mg/ml. Analysis of MzNL by SDS-PAGE showed protein bands distributed between 14–65 kDa (Fig. 3A). PAS staining showed the presence of glycosylated proteins among them (Fig. 3B). The plants latex is known to contain a wide range of proteases which function in its physiology and their defence mechanism (7). MzNL was evaluated for proteolytic activity in comparison with trypsin using casein as a substrate, where, MzNL showed less proteolytic activity compared to trypsin (Fig. 4). Further, MzNL in zymogram assay (using casein and gelatin as substrate) exhibited translucent activity bands, confirming the presence of protease(s) in the sample (Fig. 3C). Proteases from plant latex have broad substrate specificity. They can hydrolyse casein, gelatin and extracellular matrix (3, 23).

MzNL showed optimal proteolytic activity at temperature 27 °C (Fig. 5A) and pH 7.0 (Fig. 5B). To evaluate the type of protease in MzNL sample, standard inhibitors for protease was used to estimate the proteolytic activity. PMSF maximally inhibited the proteolytic activity of MzNL, indicating the presence of the serine type of protease (Fig. 5C). The metal ions effect on the proteolytic activity of MzNL was carried out by treating 10 mM concentration of various salts containing monovalent (K^+, Na^+) and divalent ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+}) with MzNL for 30 min. Among the ions, Mn^{2+} and Cu^{2+} showed an inhibitory effect of up to 47% and 25% respectively (Fig. 5D).

In mammalian blood coagulation system, direct involvement of serine proteases is observed. The extrinsic and intrinsic pathways culminate in common pathway of blood coagulation and converts fibrinogen to fibrin. Fibrinogen is hydrolysed by thrombin and polymerizes to insoluble fibrin threads. Cleaving at specific sites of fibrinogen by thrombin forms fibrinopeptides A and B (1, 24). Studies show that many

plant latexes showed thrombin-like activity by hydrolyzing fibrinogen and they were pro-coagulant in nature (5, 6). MzNL was checked for its action on fibrinogen by incubated 50 µg of pure fibrinogen with different concentration of MzNL 0–15 µg/20 µl for 2 hrs 30 min and analysed on SDS-PAGE under reduced

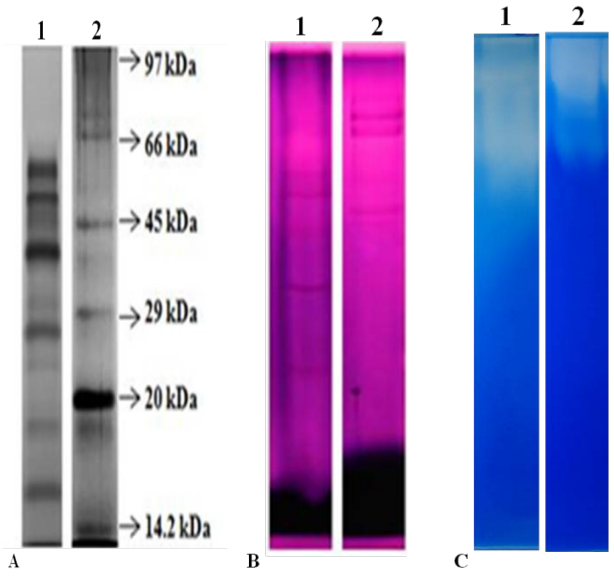

Fig. 3. Electrophoresis and zymography A. SDS-PAGE (10%) was performed under non-reducing condition and stained with 0.25% CBBR-250. Lane 1: MzNL (80 µg); lane 2: molecular-weight markers. **B.** The SDS-PAGE performed was subjected to PAS staining for glycoprotein observation. Lane 1: MzNL (80 µg); lane 2: fibrinogen (50 µg); positive control. **C.** Zymogram of MzNL (80 µg). Lane 1: gelatinolytic zymogram; lane 2: caseinolytic zymogram. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBBR-250, coomassie brilliant blue R-250; MzNL, *M. zapota* natant latex; PAS, periodic acid-Schiff base.

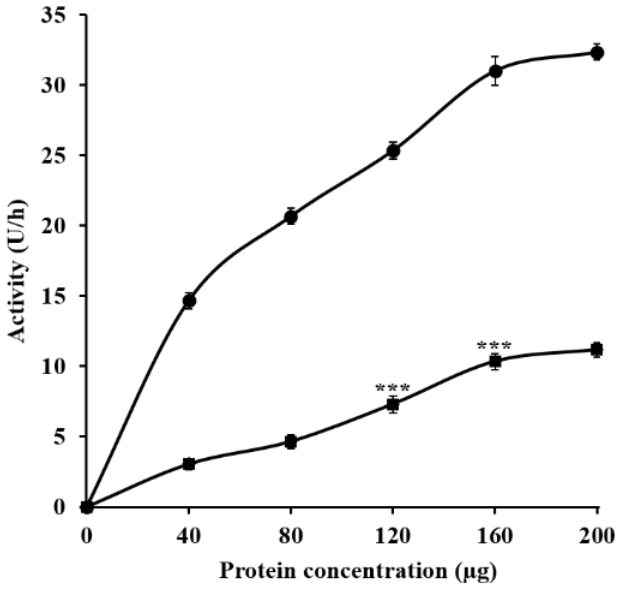

Fig. 4. Protease activity Different concentrations (0–200 µg/20 µl) of MzNL (■) and trypsin (●) were treated with 2% casein for 2 hours 30 min at 37 °C. The proteolytic activity has expressed in U/h. Values are presented as mean ± standard error of the mean (n=3). Statistically significant results are indicated by asterisks, *** – p < 0.001. MzNL, *M. zapota* natant latex.

condition. MzNL cleaved Aα and Bβ subunits of fibrinogen completely at concentrations 0.25 µg/20 µl

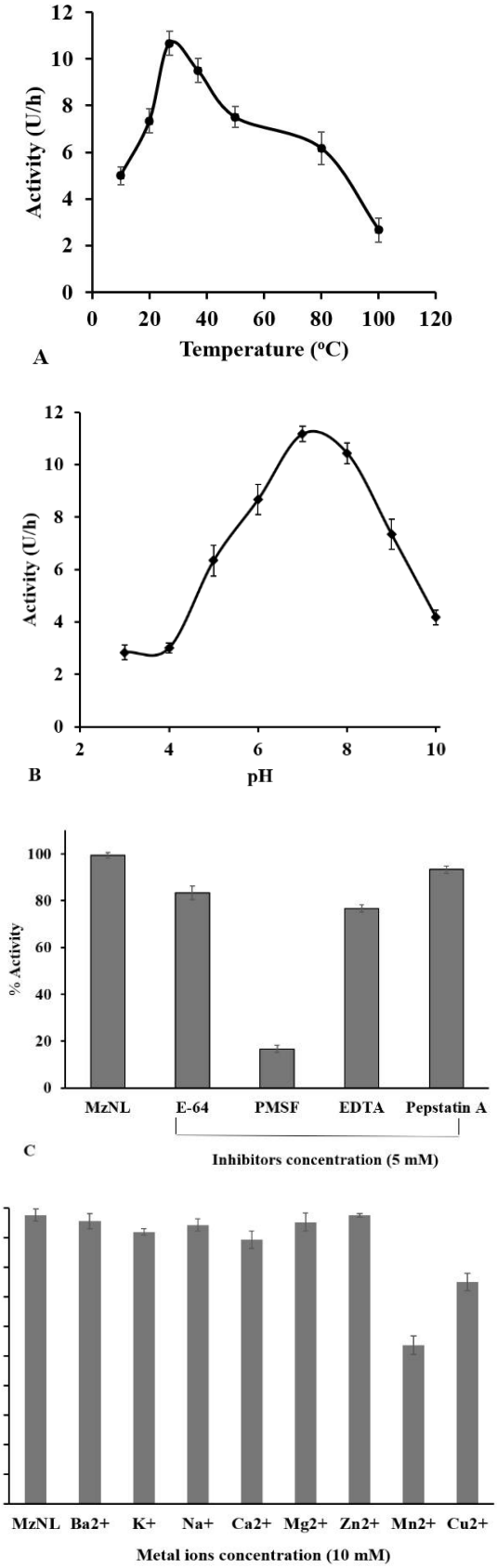

Fig. 5. Effect of temperature, pH, inhibitors and metal ions on protease activity of MzNL (80 µg/20 µl). **A.** Effect of temperature; optimum temperature found to be 27 °C. **B.** Effect of pH; optimum pH was 7.0.**C.** Effect of inhibitors; maximum inhibition observed with PMSF indicating the presence of serine protease. **D.** Effect of metal ions; Mn^{2+} and Cu^{2+} inhibited protease activity of MzNL by 47% and 25% respectively. MzNL, *M. zapota* natant latex; PMSF, phenylmethylsulfonyl fluoride.

and 4 µg/20 µl respectively. However, γ subunit was not hydrolysed even at 15 µg/20 µl of MzNL. Hydrolysis of fibrinogen by MzNL generated small molecular weight proteins and intensity of these proteins band were increased dose-dependently (Fig. 6). MzNL showed pro-coagulant effect in recalcification time. The clotting time of plasma was reduced from 143 s to 35 s with MzNL at a concentration of 150 µg/20 µl (Fig. 7).

Latex from many plants like *Calotropis gigantea, Wrightia tinctoria, Synadenium grantii, Cryptostegia grandiflora, Plumeria rubra, Pedilanthus tithymaloides* and *Euphorbia nivulia* (24–26) have showed procoagulant property. Few of them showed proteases are responsible for observed activity. Most of the proteases belongs to cysteine and serine class of proteases. To name few proteases, Pergularain e I, Hirtin and Heynein from the plants *Pergularia extensa, Euphorbia hirta* and *Ervatamia heyneana* respectively have been identified and purified (27–29).

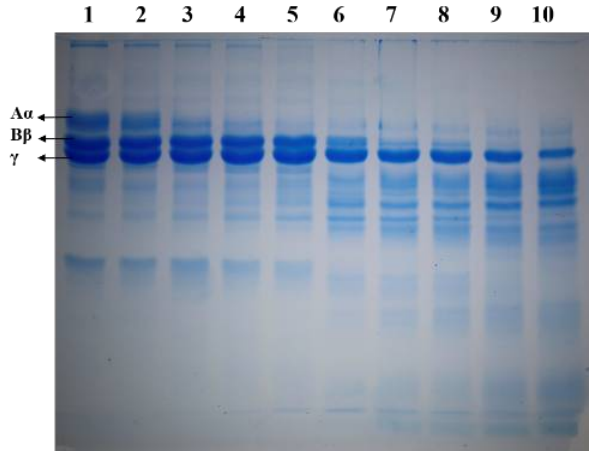

Fig. 6. Fibrinogenolytic activity. The 50 µg of human fibrinogen was incubated with various concentrations of MzNL (0–15 µg/20 µl) at 37 °C for 2 hrs 30 min. SDS-PAGE (12%) was performed in reducing condition. Lane 1: fibrinogen; lanes 2–10: fibrinogen incubated with 25.0 ng, 50.0 ng, 0.25 µg, 0.5 µg, 2.0 µg, 4.0 µg, 5.0 µg,10.0 µg and 15.0 µg of MzNL, respectively. MzNL, *M. zapota* natant latex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

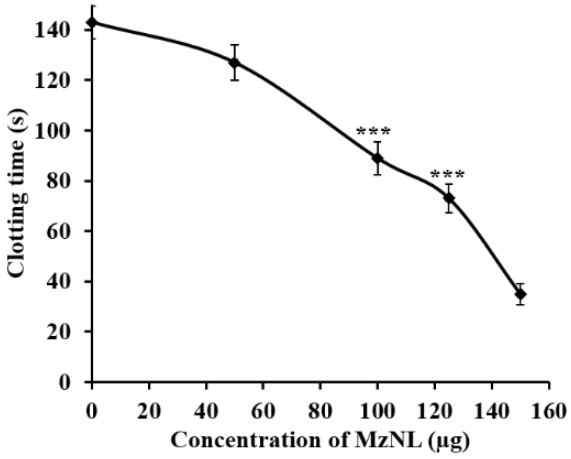

Fig. 7. Recalcification time. Different concentration of MzNL (50–150 µg/20 µl) was incubated with citrated goat plasma (100 µl) for 5 min. After incubation 25 mM $CaCl_2$ (100 µl) was pipette and clotting time was recorded. Values are presented as mean ± standard error of the mean (n=3). Statistically significant results are indicated by asterisks, *** – $p < 0.001$. MzNL, *M. zapota* natant latex.

Plant latexes have been using in folklore medicine to treat bleeding from fresh cuts, skin diseases and wound healing (5). The observed procoagulant nature of plant latex supports the scientific reason behind folklore usage of plant latexes in first line of defence against bleeding from wounds. MzNL showed collagenase activity by cleaving collagen type I and IV at the concentrations 8 and 25 µg/20 µl respectively (Fig. 8). Collagenases have a significant role in wound care (30, 31). For instance, bacterial collagenases are used to treat scar tissue and to enhance the wound healing process (32). Hence, collagenolytic property of MzNL might also have role in wound healing along with hemostatic property. MzNL has not shown any hemolytic activity after treatment with RBC, indicating its nontoxic property.

Conclusion

MzNL has potent proteolytic activity associated with fibrinogenolytic activity and exhibit pro-coagulant property. Purification and characterization of the ac-

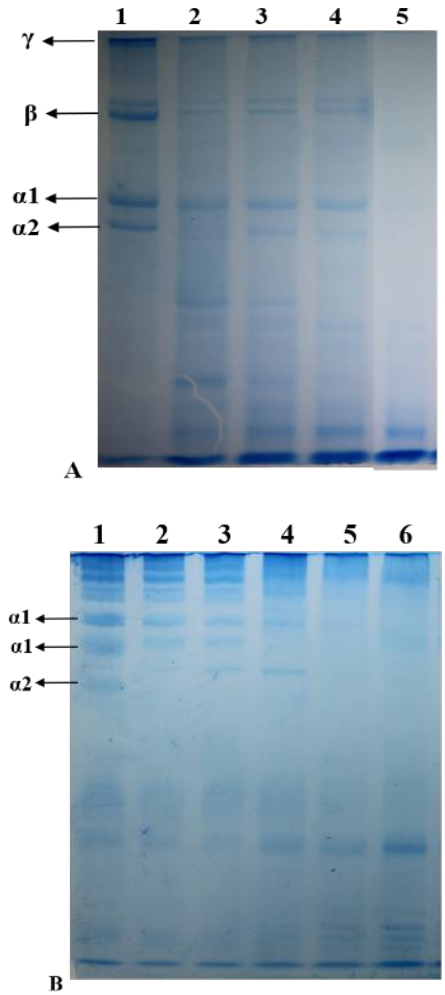

Fig. 8. Collagenolytic activity Different concentration of MzNL was treated with Type I (10 µg) and IV (25 µg) collagen at 37 °C for 2 hrs 30 min. SDS-PAGE (7%) was performed under reducing condition. **A.** Lane 1: 10 µg collagen I; lanes 2–5: 10 µg collagen I treated with 2, 4, 6 and 8 µg/20 µl of MzNL, respectively. **B.** Lane 1: 25 µg collagen IV; lanes 2–6: 25 µg collagen IV treated with 2.0, 5.0, 15.0, 20.0 and 25.0 µg/20 µl of MzNL, respectively. MzNL, *M. zapota* natant latex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

tive molecule(s) will be of great interest to decipher the pathway of blood coagulation by MzNL.

Authors' contributions

CGK & SN found the concept, involved in the experimental design, statistical analysis and data acquisition. CGK, VG, CKS, SB and HR involved in literature search, sample collection, experimental studies, manuscript preparation and review. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no competing interests

Ethical issues

The experiments involving animal blood (goat) were conducted in accordance with the Institutional Animal Ethical Committee, Sree Siddaganga College of Pharmacy, Tumkur with the sanctioned ethical approval number SSCPT/IAEC.clear/152A/2016–17.

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