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ANTICOAGULANT AND FIBRINO(GENO)LYTIC PROTEASE FROM *LEUCAS ASPERA*

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ABSTRACT: *Leucas aspera* is the medicinal plant commonly known as 'Thumbai' found all over India. It is being used in traditional medicine to treat cough, cold, diarrhea, inflammatory diseases, and paste of the plant that has been used to treat the wound to enhance the wound healing process. The plant is reported to have anti-inflammatory, analgesic, antidiarrheal, antimicrobial, antioxidant, and insecticidal activities. The current study has been taken up to evaluate the aqueous extract of *L. aspera* leaves (LaLE) for its proteolytic activity and its role in the blood coagulation cascade. The concentration of protein in LaLE was estimated to be 1 mg/ml. The proteolytic activity was determined with a specific activity of 2.40 U/mg/ml. The protein banding profile was analyzed on SDS-PAGE. LaLE hydrolyzed fibrinogen, fibrin, and collagen (I and IV) in a dose-dependent manner. LaLE increased the clotting time in prothrombin time (PT) and recalcification time (RT) by 3.87- and 5.43-folds, respectively, indicating its potentiality as an anticoagulant protease. LaLE inhibited ADP-induced platelet aggregation by 2.38-folds, suggesting its anti-platelet aggregation property. Further studies for the identification and characterization of the active principle from LaLE might lead to the discovery of new protease(s) of pharmacological significance.

INTRODUCTION: Cardiovascular diseases (CVD) such as ischemia and myocardial infarction is one of the leading causes of death in developing countries. CVDs are caused due to the blockage of arteries and veins with thrombi or blood clots. Thrombus formation is caused by the activation of blood coagulation factors during vascular injuries or imbalance in endogenous anticoagulants.

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The major causative agents for the aggregation of prothrombotic molecules in blood are oral contraceptives, heavy smoking, surgery, trauma, infection, and other chronic inflammatory disorders [1-3]. Generally, the intrinsic/contact phase pathway of blood coagulation is triggered by the exposure of tissue factors during vascular injury. Factor VIIa binds to the tissue factor and thereby activates factor X and factor IX. Factor Xa converts the inactive prothrombin (factor II) to active thrombin (IIa). Thrombin activates both the platelet-mediated primary hemostasis and clotting factor-mediated secondary hemostasis. The activated platelets also activate the secondary hemostasis leading to the formation of a blood clot or thrombus.

Anticoagulant drugs are used for prophylaxis and to treat venous thromboembolism (VTE), pulmonary embolism (PE), atrial fibrillation (AF), and other thrombolytic disorders. Anticoagulants directly or indirectly interact with the coagulation factors to inhibit thrombogenesis. The currently used anticoagulants in pharmacological and medical applications are unfractionated heparin (UFH), low molecular weight heparin (LMWH), fondaparinux, hirudin, and argatroban [4-7]. The limitation of the existing anticoagulants is spontaneous hemorrhage and osteoporosis. There is a need to identify an anticoagulant molecule with fewer or no side effects. Medicinal plants were mainly used to treat different ailments by traditional medicinal practitioners [8, 9]. Currently, these plants were studied for the presence of therapeutically important molecules [10].

Leucas aspera is commonly known as 'Thumbai', is used traditionally to treat malaria, rheumatism, psoriasis, and other chronic skin eruptions. Decoctions of leaves are applied locally to treat snake bites [11]. Earlier studies report the presence of phytochemicals such as glycosides, terpenes, terpenoids, sterols, alkaloids, flavonoids, fatty compounds, and others [12]. The plant extracts were known to possess antimicrobial, antioxidant, anti-cancer, antivenom, hepatoprotective, antiinflammatory, anti-analgesic, antinociceptive, antiulcer, antimalarial, antipyretic, and antidiabetic activities [12, 13]. In this study, we are reporting the protease associated with fibrino(geno)lytic and anti-platelet aggregating property from the aqueous extract of *L. aspera* leaves.

MATERIALS AND METHODS:

Materials: Casein, gelatin, and human fibrinogen were purchased from Sigma-Aldrich Chemicals, St. Louis, MO, USA. Uniplastin was procured from Tulip Diagnostics Pvt. Ltd, Goa, India. All other reagents were of analytical grade.

Sample Collection: The *Leucas aspera* plants were collected in Tumakuru. The plant leaves were collected, separated, and washed under the running tap water to remove dust and sand particles and later rinsed with distilled water. Then they were shade dried for two to three days at room temperature. The dried leaves were ground into a fine powder and used for extraction.

Preparation of Extract: The powdered *L. aspera* sample (10 g) was dispersed in a clean beaker containing 150 ml of distilled water and extracted on a magnetic stirrer at 500 rpm for about 24 h at room temperature. The extract was centrifuged at 10,000 rpm at 15 ºC for 15 min. The supernatant was carefully collected and stored at -20 ºC until further use. This extract is called as *Leucas aspera* Leaf Extract (LaLE).

Electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli, 1970 [14]. Briefly, LaLE (100 µg) was treated with a non-reducing sample buffer and incubated in a boiling water bath for 3 min. The sample was then loaded onto 12% SDS-PAGE along with standard molecular weight markers ranging from 7 to 175 kDa, and electrophoresis was performed. The gel was stained with 0.25% Coomassie brilliant blue R-250 to visualize the protein bands.

Zymography: Zymography was carried out according to the method of Nagaraju *et al.*, 2006 [15]. Briefly, the 12% gel was incorporated with 0.2% casein and gelatin separately as a substrate for the detection of proteolytic activity. The LaLE (40 µg) was incubated with a non-reducing sample buffer at 37 °C for 30 min, and electrophoresis was carried out. After electrophoresis, the gels were washed with 2.5% of Triton X-100 for 1 h to remove SDS. The gels were incubated overnight in incubation buffer containing Tris–HCl (50 mM, pH 7.6, 10 mM $CaCl_2$, and 150 mM NaCl). The gels were then stained with 0.25% Coomassie brilliant blue R-250 to observe the activity bands.

Protease Activity: Proteolytic activity was performed as described by Nagaraju *et al*., 2007 [16], using casein (2% in 200 mM Tris – HCl buffer, pH 7.0) as substrate. Briefly, 0.4 ml of casein was incubated with different concentrations of LaLE (0 – 40 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37 ºC for 2.5 h. About 1.5 ml of TCA (0.44 M) was added to terminate the reaction and allowed to stand for 30 min at room temperature. The mixture was centrifuged at 3,000 rpm for 5 min, and the supernatant (1 ml) was mixed with 0.4 M sodium carbonate (2.5 ml) and 1:2 diluted Folin reagent (0.5 ml). The colour developed was read at 660 nm. One unit of enzyme activity was defined

as the amount of enzyme required to increase the absorbance of 0.01 at 660 nm. Protease activity was expressed as units/min/mg.

Fibrinogenolytic Activity: The fibrinogenolytic activity was performed as described by Gubbi-veeranna *et al*., 2019 [17]. Briefly, human fibrinogen (50 µg) was treated with different concentration of LaLE (0 - 30 µg) in 25 µl of 10 mM sodium phosphate buffer (pH 7.0) and incubated at 37 °C for 2.5 h. Reducing sample buffer (10 µl) containing 1M urea, 4% SDS, and 4% β-mercaptoethanol was added to terminate the reaction and kept in a boiling water bath for 3 min. The hydrolyzed products were analyzed in 12% SDS-PAGE and visualized by staining with Coomassie brilliant blue R-250.

Fibrinolytic Activity: Fibrinolytic activity was carried out according to the method of Shivaiah and Kempaiah, 2011 [18]. Briefly, trisodium citrate (3.2%) treated blood in the ratio 1:9 was centrifuged at 3,000 rpm for 5-10 min. The supernatant obtained was separated and used as platelet-poor plasma (PPP). An equal volume of PPP (100 µl) and 25 mM $CaCl_2$ (100 µl) was incubated at 37ºC to get a fibrin clot. The clot formed was thoroughly washed with 10 mM sodium phosphate buffer (pH 7) 5–6 times. The washed fibrin clot was incubated with different concentrations of LaLE (0 to 40 µg) in a total reaction volume of 40 µl of 10 mM sodium phosphate buffer (pH 7) at 37ºC for 2.5 h. Reducing sample buffer (20 µl) containing 1M urea, 4% SDS, and 4% β-mercaptoethanol was added to terminate the reaction and kept in a boiling water bath for 3 min. An aliquot (20 µl) of the supernatant was subjected to 10% SDS-PAGE to analyze the fibrin hydrolyzing pattern.

Collagenolytic Activity: The collagenolytic activity was performed as described by Gubbi-veeranna *et al*., 2019 [19]. Briefly, collagen type I (10 µg) and collagen type IV (25 µg) were incubated separately with different concentration of LaLE (0 – 20 µg for Collagen I and 0 – 16 µg for Collagen IV) in 40 µL of 10 mM sodium phosphate buffer (pH 7.0) at 37 ºC for 2.5 h. Reducing sample buffer (10 µl) containing 1M urea, 4% SDS, and 4% β-mercaptoethanol was added to terminate the reaction and kept in a boiling water bath for 3 min. The sample was loaded onto 7% SDS-PAGE, and electrophoresis was carried out. The degradation pattern was analyzed by staining the gel with Coomassie brilliant blue R-250.

Prothrombin Time (PT): Prothrombin time was determined according to the method described by Tulip Diagnostics (P) Ltd., India. Briefly, PPP (100 µl) was pre-warmed to 37ºC before use and incubated with different concentrations of LaLE (0 – 150 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37ºC for 5 min. Later, 200 µl of uniplastin reagent was added, and the clotting time was recorded. The 10 mM sodium phosphate buffer (pH 7.0) alone without LaLE was considered as a negative control.

Recalcification Time (RT): Plasma recalcification time was determined according to the method described by Gubbiveeranna *et al*., 2019 [17]. Briefly, PPP (100 µl) was pre-warmed to 37 ºC before use and incubated with different concentrations of LaLE (0 – 90 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37 °C for 5 min. Later, 100 µl of 25 mM $CaCl_2$ was added, and the clotting time was recorded. The 10 mM sodium phosphate buffer (pH 7.0) alone without LaLE was considered as a negative control.

Platelet Aggregation Studies: Platelet aggregation was monitored by analyzing light transmission in a CHRONOLOG Model 700–2D aggregometer (ChronoLog Corp). Platelet-rich plasma (PRP) was prepared by centrifuging trisodium citrate (3.2%) treated blood at 800 rpm for 15 min. The supernatant obtained was separated and used as PRP. The remaining blood was centrifuged at 3,000 rpm for 20 min to obtain PPP as the supernatant. PRP (0.25 mL) was transferred to a siliconized glass cuvette containing a Teflon–coated stir bar.

The sample was maintained at 37°C and allowed to reach equilibrium by constant stirring at 600 rpm. The instrument was calibrated, and aggregation was initiated by adding 10 µM adenosine diphosphate (ADP). The effect of LaLE was evaluated by preincubating PRP with different concentrations of LaLE (10, 20, and 30 µg) for 5 min before the addition of ADP. The extent of aggregation was constantly monitored for 6 min by using an optical method for identifying the change

in turbidity with PRP and PPP representing 0% and 100% transmittance, respectively.

Protein Estimation: The protein concentration was estimated as described by Lowry *et al*., 1951 [20]. Briefly, bovine serum albumin (BSA) was used as standard, and the protein concentration of LaLE was determined by comparing it with the known concentration of BSA.

Statistical Analysis: The experiments were performed in triplicates, and the data obtained from the experiments were expressed as mean ± standard error of the mean (SEM). The results were statistically analyzed using one–way ANOVA followed by Tukey's Multiple Comparison Test. The data were considered significant at $p < 0.05$.

RESULTS:

Electrophoresis: LaLE (100 µg) was subjected to 12% SDS-PAGE under non-reducing conditions. The LaLE showed protein bands in the higher molecular weight range between 165 and 145 kDa **Fig. 1**.

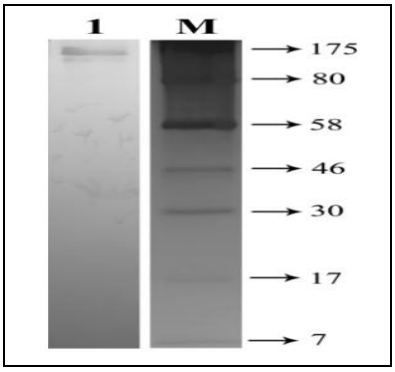

FIG. 1: PROTEIN BANDING PROFILE OF LaLE. The LaLE (100 µg) was treated with a non-reducing sample buffer and incubated in a boiling water bath for 3 min. The sample was then loaded onto 12% SDS-PAGE under non-reducing conditions. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 to visualize the protein bands. Lane 1: LaLE (100 µg); M: molecular weight markers.

Proteolytic Activity and Zymography: LaLE was evaluated for proteolytic activity using casein as a substrate. LaLE showed a specific activity of 2.40 U/mg/ml. For further detection of the protease activity, the zymographic technique was performed by incorporating 0.2% casein and gelatin separately into the 12% SDS-PAGE gels. Translucent activity bands were observed in the higher molecular weight region between 125 and 80 kDa for caseinolytic activity and between 160 and 70 kDa for gelatinolytic activity **Fig. 2**.

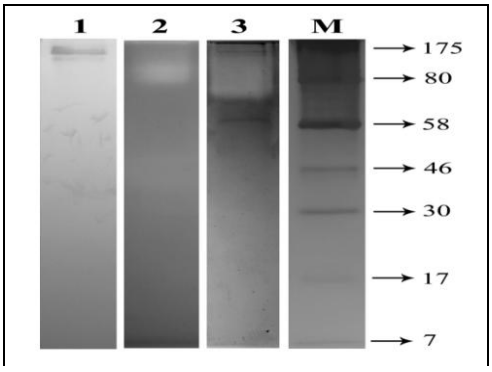

FIG. 2: ZYMOGRAPHY. Casein (0.2%) and gelatin (0.2%) were copolymerized with the polyacrylamide gel separately for the detection of proteolytic activity. LaLE (40 µg) was incubated with a non-reducing sample buffer at 37 ºC for 30 min and loaded onto 12% SDS-PAGE under non-reducing conditions. After electrophoresis, gels were washed with 2.5% of Triton X-100 for 1 h to remove SDS. The gels were incubated overnight in incubation buffer containing Tris–HCl (50 mM, pH 7.6, 10 mM $CaCl_2$, and 150 mM NaCl). Gels were then stained with 0.25% Coomassie brilliant blue R-250 to visualize the activity bands. Lane 1: LaLE (100 µg); lane 2: caseinolytic zymogram; lane 3: gelatinolytic zymogram; M: molecular weight markers.

Fibrinogenolytic Activity: LaLE was studied for fibrinogenolytic activity, and the hydrolyzed products were examined using SDS-PAGE. LaLE degraded all the chains (Aα, Bβ, and γ) of fibrinogen in a dose-dependent manner. Aα subunit was most susceptible to hydrolysis compared to other subunits. Aα subunit was hydrolyzed at a concentration of 5 µg of LaLE. Bβ and γ subunits were resistant and degraded at a concentration of 30 µg of LaLE **Fig. 3**.

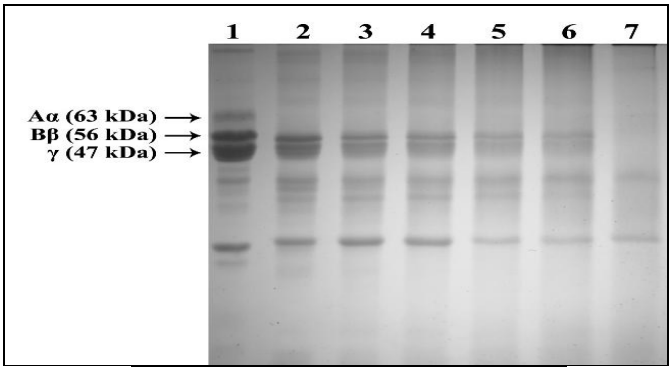

FIG. 3: CONCENTRATION-DEPENDENT FIBRINO-GENOLYTIC ACTIVITY BY LaLE. The fibrinogen (50 µg) was incubated with different concentration of LaLE (5 µg to 30 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37 °C for 2.5 h. The reaction was terminated by adding denaturing sample buffer containing 1M urea, 4% SDS, and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. SDS-PAGE (12%) was performed in reducing conditions to visualize degradation patterns. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R-250. Lane 1: fibrinogen (50 µg); lane 2–7: fibrinogen incubated with 5, 10, 15, 20, 25, and 30 µg of LaLE, respectively

Fibrinolytic Activity: LaLE was studied for fibrinolytic activity, and the hydrolyzed products were examined using SDS-PAGE. LaLE degraded fibrin in a dose-dependent manner. The γ-dimer and α-chain subunits of the fibrin were degraded at a concentration of 40 µg of LaLE. The α-polymer and β-chain were resistant for hydrolysis up to the concentration of 40 µg of LaLE **Fig. 4**.

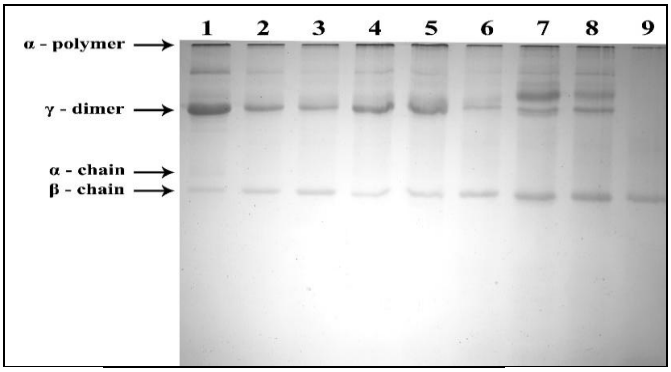

FIG. 4: CONCENTRATION-DEPENDENT FIBRINOLYTIC ACTIVITY BY LaLE. Equal volumes of PPP (100 µl) and 25 mM $CaCl_2$ (100 µl) were mixed and incubated to get fibrin clot. The fibrin clot was washed in 10 mM sodium phosphate buffer (pH 7.0) and incubated with different concentration of LaLE (5 µg to 40 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37 °C for 2.5 hours. The reaction was terminated by adding denaturing sample buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The sample was loaded onto 10% SDS-PAGE in reducing condition. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 to visualize the bands. Lane 1: control (fibrin clot); lane 2 – 9: fibrin clot incubated with 5, 10, 15, 20, 25, 30, 35 and 40 µg of LaLE, respectively.

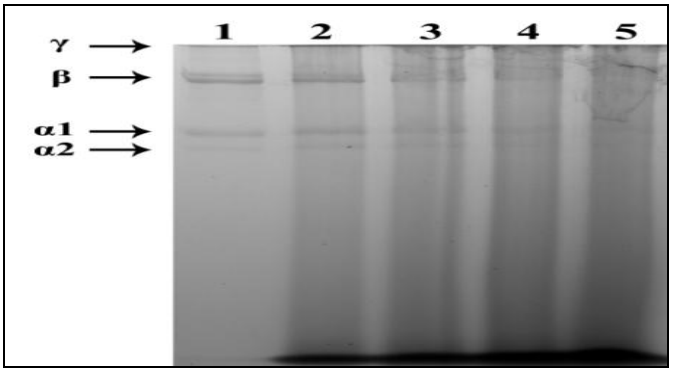

FIG. 5: CONCENTRATION-DEPENDENT DEGRADATION OF COLLAGEN TYPE I BY LaLE. Collagen type I (10 µg) was incubated with different concentration of LaLE (0, 5, 10, 15 and 20 µg) in 40 µL of 10 mM sodium phosphate buffer (pH 7.0) at 37 ºC for 2.5 h. The reaction was terminated by adding a reducing buffer containing 1M urea, 4% SDS, and 4% β-mercaptoethanol and kept in a boiling water bath for 3 min. The sample was loaded onto 7% SDS-PAGE, and electrophoresis was carried out. The degradation pattern was analyzed by staining the gel with Coomassie brilliant blue R-250. Lane 1 - 5: collagen type I (10 µg) with 0, 5, 10, 15, and 20 µg of LaLE, respectively.

Collagenolytic Activity: LaLE was studied for collagenolytic activity, and the hydrolyzed products were examined using SDS-PAGE. LaLE degraded all the bands of both collagen type I **Fig. 5** and collagen type IV **Fig. 6** at a concentration of 20 µg and 16 µg, respectively.

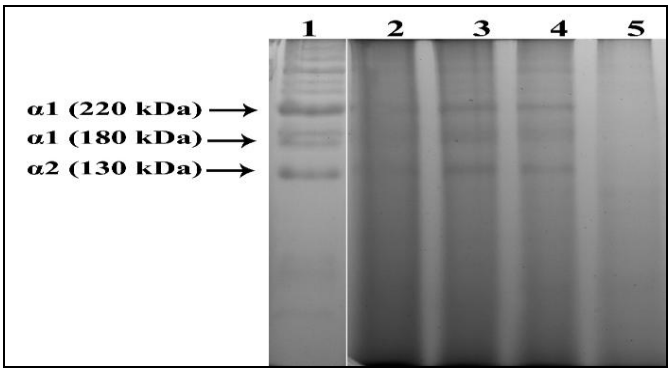

FIG. 6: CONCENTRATION-DEPENDENT DEGRADATION OF COLLAGEN TYPE IV BY LaLE. Collagen type IV (25 µg) was incubated with different concentration of LaLE (0, 4, 8, 12 and 16 µg) in 40 µL of 10 mM sodium phosphate buffer (pH 7.0) at 37 ºC for 2.5 h. The reaction was terminated by adding a reducing sample buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in a boiling water bath for 3 min. The sample was loaded onto 7% SDS-PAGE, and electrophoresis was carried out. The degradation pattern was analyzed by staining the gel with Coomassie brilliant blue R-250. Lane 1 - 5: collagen type IV (25 µg) with 0, 4, 8, 12, and 16 µg of LaLE, respectively.

Coagulation Studies: LaLE showed anticoagulant activity by increasing the plasma clotting time for both Prothrombin time (PT) and Recalcification time (RT) in a dose-dependent manner. LaLE at a concentration of 150 µg increased the PT from control 15 seconds to 58 seconds. This shows that LaLE increases PT by ~4 folds **Fig. 7**.

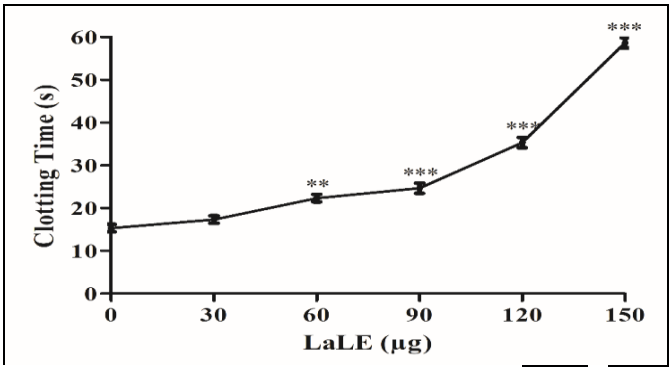

FIG. 7: PROTHROMBIN TIME (PT) OF LaLE. LaLE was incubated with 100 µl PPP at 37 ºC for 5 min. Then, 200 µl of uniplastin reagent was added and clotting was initiated. The time required for the appearance of the first clot was recorded. LaLE (30, 60, 90, 120 and 150 µg) increased the clotting time from control 15 seconds to 17, 22, 24, 36 and 58 seconds, respectively. Values are presented as means ± SEM. ***$p < 0.001$, analysed by one–way ANOVA, followed by Tukey multiple comparison testing.

While LaLE at a concentration of 90 µg increased the RT from control 120 seconds to 670 sec. Thus, increasing the clotting time by ~6 folds **Fig. 8**.

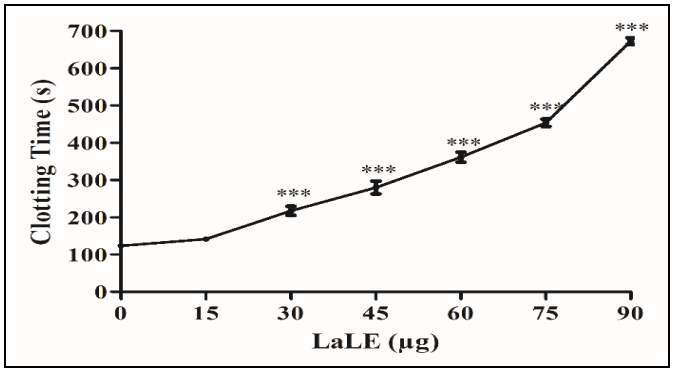

FIG. 8: RECALCIFICATION TIME (RT) OF LaLE. LaLE was incubated with 100 µl PPP at 37 ºC for 5 min. Then, 100 µl of 25 mM CaCl2 was added to initiate clotting and the clotting time was recorded. LaLE (15, 30, 45, 60, 75 and 90 µg) increased the clotting time from control 124 seconds to 141, 217, 280, 361, 453 and 673 seconds, respectively. Values are presented as means ± SEM. ***p < 0.001, analyzed byone–way ANOVA, followed by Tukey multiple comparison testing.

Platelet Aggregation Studies: The effect of LaLE on ADP-induced platelet aggregation was studied using PRP. The LaLE inhibited the ADP-induced platelet aggregation in a dose-dependent manner.

LaLE at a concentration of 30 µg decreased the platelet aggregation from control 100% to 42%. This shows that LaLE inhibits ADP-induced platelet aggregation by ~2.5 folds **Fig. 9**.

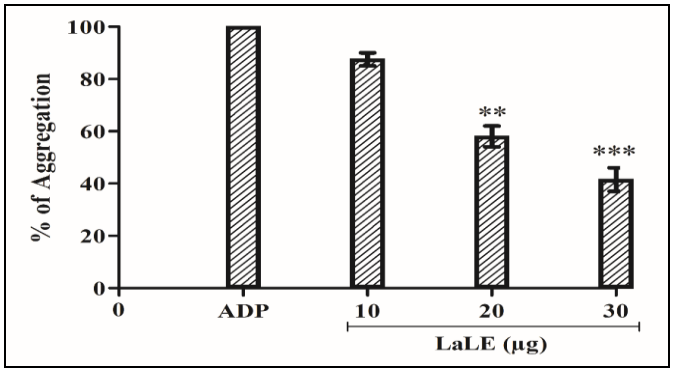

FIG. 9: PLATELET AGGREGATION ACTIVITY OF LaLE. Platelet aggregation was monitored by analyzing light transmission in a CHRONO–LOG Model 700–2D aggregometer. PRP (0.25 mL) was incubated with 10 µM adenosine diphosphate (ADP). The effect of LaLE was evaluated by preincubating PRP with different concentrations of LaLE (10, 20 and 30 µg) for 5 min before the addition of ADP. The extent of aggregation was constantly monitored for 6 min by using an optical method for identifying the change in turbidity with PRP and PPP representing 0% and 100% transmittance, respectively. Values are presented as means ± SEM. ** $p < 0.01$, *** $p < 0.001$, analysed by one–way ANOVA, followed by Tukey multiple comparison testing.

DISCUSSION: In this study, we have analyzed LaLE for the presence of protease and its effect on fibrinogen, fibrin, collagen, blood coagulation cascade, and platelet aggregation to study its anticoagulant property.

LaLE showed protein bands in high molecular weight region between 165 and 145 kDa as analyzed by SDS-PAGE. The protein concentration in LaLE was found to be 1 mg/ml. LaLE showed a specific activity of 2.40 U/mg/ml using casein as a substrate. The proteolytic activity was supported by zymography studies using casein and gelatin as substrates. In zymography studies, LaLE hydrolyzed casein and gelatin in high molecular weight regions between 125-80 kDa and 160-70 kDa, respectively.

LaLE showed fibrino(geno)lytic activity and degraded the subunits of fibrinogen and fibrin. The degradation is in a dose-dependent manner. LaLE hydrolyzed Aα, Bβ and γ chains of fibrinogen at a concentration of 30 µg while the fibrin subunits (γ-dimer and α-chain) were degraded at a concentration of 40 µg. α-polymer and β-chain of fibrin were resistant to hydrolysis.

Further, LaLE was analyzed for its specificity of action on extracellular matrix molecules such as collagen I and collagen IV. All the subunits of both the molecules were degraded in a dose-dependent manner. LaLE hydrolyzed both collagen type I and collagen type IV at a concentration of 20 µg and 16 µg, respectively.

LaLE interfered in the blood coagulation cascade as analyzed by PT and RT. LaLE, at a concentration of 150 µg, increased the clotting time in PT from control 15 seconds to 58 seconds, increasing the PT by 3.87-folds. Similarly, LaLE, at a concentration of 90 µg, prolonged the clotting time in RT from control 124 seconds to 673 seconds, increasing the RT by 5.43-folds. This indicates the anticoagulant nature associated with LaLE.

In platelet aggregation studies, LaLE inhibited the platelet aggregation induced by the agonist ADP from control 100% to 42%, decreasing the platelet aggregation by 2.38-folds at a concentration of 30 µg. This suggests the anti-platelet aggregation property of LaLE.

LaLE inhibited the platelet aggregation and blood coagulation cascade, indicating its interference in thrombus formation. Our study provides for the first time, the scientific evidence for the presence of a protease with anticoagulant property from the aqueous extract of *L. aspera* leaves (LaLE). This study provides an opportunity to identify, isolate and characterize the active principle from LaLE having clinical significance and decipher its molecular mechanism.

CONCLUSION: In conclusion, LaLE possesses protease with fibrinogenolytic, fibrinolytic, collagenolytic activities and is associated with anti-coagulant and anti-platelet aggregation properties. This suggests its possible role in inhibiting thrombus formation. Further isolation and characterization of the active principle could lead to the identification of a therapeutically significant molecule to treat the thrombolytic disorder.

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CONFLICTS OF INTEREST: There is no conflict of interest to disclose.

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