Anti-Coagulant and Fibrino(Geno)Lytic Protease from Aqueous Extract of Leucus As per a leaves

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Abstract- *Leucas aspera is the medicinal plant commonly known as "Thumbai" is found all over India. It is being used for many years to treat several diseases such as cough, cold, diarrhoea and inflammatory diseases. The plant is reported to have anti-inflammatory, analgesic, antidiarrheal, antimicrobial, antioxidant and insecticidal activities. L. aspera have been used in traditional medicine for treating wounds for fastening wound healing process. Current study has been taken up to evaluate the L. aspera leaves aqueous extract for its proteolytic activity and role of these proteases in blood coagulation pathway. The concentration of protein in the leaves extract was estimated to be 1 mg/ml. Protein banding profile was analysed on SDS-PAGE. The proteolytic activity was determined with a specific activity of 2.40 U/mg/ml. Extract cleave fibrinogen, fibrin and collagen (I and IV) in dose dependent manner. The extract increased the clotting time in prothrombin time (PT) and recalcification time (RT) indicating that it acts as an anti-coagulant nature of proteases.*

Keywords- Leucas aspera, prothrombin time, recalcification time, SDS-PAGE, Protein banding

I. INTRODUCTION

Leucas aspera (Willd.) Linn. (Family: Lamiaceae) commonly known as 'Thumbai' is distributed throughout India from the Himalayas down to Ceylon. The plant is used traditionally as an antipyretic and insecticide. Flowers are valued as stimulant, expectorant, aperient, diaphoretic and insecticide. Leaves are considered useful in chronic rheumatism, psoriasis and other chronic skin eruptions. Bruised leaves are applied locally in snake bites (Rai et al, 2005).*Leucas aspera* is an annual, branched, herb erecting to a height of 15-60 cm with stout and hispid acutely quadrangular stem and branches (Figure 1). Leaves are sub-sessile or shortly petiolate, linear or linearly lanceolate, obtuse, pubescent up to 8.0 cm long and 1.25 cm broad, with entire or crenate margin; petiole 2.5-6 mm long; flowers white, sessile small, in dense terminal or axillary whorls; bracts 6 mm long, linear, acute, bristle-tipped, ciliate with long slender hairs; calyx variable, tubular, 8-13 mm long; tube curved, contracted above the nutlets, the lower half usually glabrous and membranous, the upper half ribbed and hispid; mouth small, very oblique, not villous, the upper part produced forward; teeth small, triangular, bristle-tipped, ciliate, the upper tooth being the largest. Corolla 1 cm long; tube 5 mm long and pubescent above, annulate in the middle; upper lip 3 mm long, densely white-woolly; lower lip about twiceas long, the middle lobe obviate, rounded, the lateral lobes small, subacute. Fruit nutlets, 2.5 mm long, oblong, brown, smooth, inner face angular and outer face rounded (Hooker, 1985).

1.1 Phytochemical studies:

Preliminary chemical examination of *L. aspera* revealed presence of triterpenoids in entire plant. Whole plant is reported to contain oleanolic acid, ursolic acid and 3-sitosterol. Aerial parts are reported to contain nicotine, sterols, two new alkaloids (compound A m.p. 61-2°, α-sitosterol and β-sitosterol) (m.p. 183-4°), reducing sugars (galactose), glucoside (230-1°), diterpenes (leucasperones A and B, leucasperols A and B, isopimarane glycosides (leucasperosides A, B and C), together with other compounds like asperphenamate, maslinic acid, (-)-isololiolide, linifolioside, nectandrin B, meso-dihydroguaiaretic acid, macelignan, acacetin, apigenin 7-O-[6'-O-(p-coumaroyl)-3-D-glucoside], chrysoeriol, apigenin, erythro-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol, myristargenol B, and machilin C, (-)-chicanine, (7R,8R)- and (75,85)-licarin A. Among the 25 compounds identified from the leaf volatiles, u-farnesene (26.4%), x-thujene (12.6%) and menthol (11.3%) were the major constituents. The flower is reported to contain 10 compounds; among them amyl propionate (15.2%) and isoamyl propionate (14.4%) were dominant. Seed is reported to contain palmitic acid (6.25%), stearic acid (2.84%), oleic acid (42.07%), linoleic acid (48.11%), and linolenic acid (0.65%). The unsaponifiable fraction contained 3-sitosterol and ceryl alcohol. Shoot contained novel phenolic compounds (4-(24-hydroxy-1-oxo-5-n-propyltetracosanyl)-phenol), aliphatic ketols (28-hydroxypentatriacontan-7-one, 7-hydroxydotriacontan-2-one), long-chain compounds (1-hydroxytetratriacontan-4-one, 32-methyltetratriacontan-8-ol), nonatriacontane, 5-acetoxytriacontane, β-sitosterol and dotriacontanol. Leucolactone (I), isolated from the root of *L. aspera* have been

characterized as 3,3,16c-dihydroxyoleanan-28-1,3-olide (Mangathayaru et al., 2006; Sadhu et al., 2006).

1.2 Pharmacological Studies:

1.2.1 Antifungal activity:

In vitro study of chloroform and ether extracts of *L. aspera* revealed its antifungal activity against *Trichophyton* and *Microsporumgypseum*. The minimum inhibitory concentration was found to be 5 mg/mL.*Leucas aspera* had both fungistatic and fungicidal actions (Thakur et al., 1987).

1.2.2 Prostaglandin inhibitory and antioxidant activities:

Leucas aspera was tested for its prostaglandin (PG) inhibitory and antioxidant activities. The extract of the plant showed both activities, that is, inhibition at 3-4 g/mL against PGE1- and PGE2-induced contractions in guinea pig ileum and a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect. Phytochemical investigation suggested the presence of nectandrin B, meso-dihydroguaiaretic acid, macelignan, acacetin, apigenin 7-O-[6'-O-(p-coumaroyl)-3-D-glucoside], chrysoeriol, apigenin, erythro-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol, myristargenol B and machilin C, (-)-chicanine, (7R,8R)- and (75,85)-licarin A.Toxicity evaluation of herbal smoke and synthetic mosquito mat on *Culex quinquefasciatus* was performed. The smoke of leaves of *Vitex negundo* and *L. aspera* are more toxic to the filarial vector mosquito, *Culexquinquefasciatus*, than the synthetic mosquito mats, which contain 4% d-allethrin (Sadhu et al., 2003).

1.2.3 Antimicrobial activity of *Leucas aspera* flowers:

The methanol extract of *L. aspera* flowers, its fractions, the alkaloidal residue and the expressed flower juice showed good antibacterial activity for methanol extract and methanol fraction with maximum activity for the alkaloidal residue (Mangathayaru et al., 2005).

1.2.4 Antimicrobial action of some essential oils:

The essential oils from *L. aspera* possessed bacteriostatic activity against *Staphylococcus aureus*, *Vibrio cholerae*, *Salmonella typhi*, *Klebsiella aerogenes*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas pyocyanea* and *Dys. Flexneri* (Rao et al., 1971).

1.2.5 Antinociceptive, antioxidant and cytotoxic activities of *Leucas aspera* root:

The ethanolic extract was subjected to acetic acid induced writhing inhibition, 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging assay and brine shrimp lethality bioassay for screening of antinociceptive, antioxidant and cytotoxic activity, respectively. The ethanolic extract of *L. aspera* root produced significant inhibition in acetic acid induced writhing in mice at the doses of 250 and 500 mg/kg. The extract showed a significant free radical scavenging activity with an IC50 of 8 µg/ml. The extract showed significant lethality to brine shrimp (Rahman et al., 2007).

II. METHODOLOGY

2.1 Materials:

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

2.2 Sample collection:

The *Leucas aspera* plants were collected in the local areas of Tumakuru. The plant leaves were separated and washed under the running tap water to remove the dust and sand particles and later rinsed with distilled water. Then they were shade dried for two to three days at room temperature and later dried leaves were grinded into fine powder (Figures2 and 3).

Figure 1. *Leucas aspera* plant

Figure 2. Dry leaves

2.3 Preparation of extract:

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

2.4 Electrophoresis:

SDS-PAGE (Sodium Dodecyl Sulfate - Poly Acrylamide Gel Electrophoresis) was carried out according to the method of Laemmli, et al.,1970. Briefly, LaLE (20 µg) was loaded onto 12% SDS-PAGE and electrophoresis was done. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R-250.

2.5 Zymography:

Zymogram was carried out according to the method of Laemmli, et al., 1970. The 12% gel was incorporated with 0.2% casein and 0.2% gelatin separately as a substrate for the detection of protease activity. TheLaLE was incubated with SDS-PAGE sample buffer at 37º C for 1.5 h and electrophoresis was done. 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% Commassive brilliant blue R-250 to observe the activity bands.

2.6 Protease activity:

Protease activity was assayed according to themethod of Satake,et al.,1960, using casein (2% in200 mM Tris – HCl buffer, PH 8.0) as substrate. Briefly, 0.4 ml of casein was incubated with LaLE at 37 ºC for 2.5 h. The reaction was stopped by adding 1.5 ml of TCA (0.44 M) and allowed to stand for30 min. The mixture was centrifuged at 3000 rpm for 5 min. An aliquot (1 ml) of the supernatant was mixed with 2.5 mlof 0.4 M sodium carbonate and 0.5 ml of 1:2 diluted Folinreagent and the colour developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase in the absorbance of 0.01 at 660 nm. Protease activity was expressed as units/min/mg.

2.7 Fibrinogenolytic activity:

Fibrinogenolytic activity was carried out according to the method of Rajesh, et al., 2005. Briefly, human fibrinogen (50 µg) was treated with different concentrations (0 – 30 µg) of LaLE in 25 µl of 10 mM sodium phosphate buffer (pH 7.0). This mixture was incubated at 37º C for 2.5 hours. The reaction was terminated by adding 10 µl of reducing sample buffer. The degraded products were analyzed in 12% SDS-PAGE and visualized by staining with Commassive brilliant blue R-250.

2.8 ibrinolytic activity:

Fibrinolytic activity was carried out according to the method of Rajesh, et al., 2005. Briefly, sodium citrate (3.2%) treated blood in the ratio 1:9 was centrifuged for 5-10 min at 3000 rpm to separate plasma. Equal volume of Plasma (100 µl) and 25 mM $CaCl_2$ (100 µl) was incubated at 37 ºC to get fibrin clot. The clot formed was thoroughly washed with 10 mM PBS (pH 7) for 5–6 times. The washed fibrin clot was incubated with different concentrations of LaLE (0 to 40 µg) separately in a total reaction volume of 40 µl of 10 mM sodium phosphate buffer, pH 7 at 37 ºC for 2.5 h. The reaction was terminated by adding 20 µl of reducing sample buffer. An aliquot (20 µl) of the supernatant was subjected to 10% SDS-PAGE to analyze fibrin hydrolyzing pattern.

2.9 Collagenolytic activity:

The collagenolytic activity assay was performed according to the method of Misook Kim et al., 2007. Briefly, collagen type I (10 µg) and collagen type IV (25 µg) were incubated separately with varying concentrations of LaLE (0 – 20 µg for Collagen I and 0 – 16 µg for Collagen IV) in 40 µL sodium phosphate buffer (10 mM, pH 7.0) at 37 ºC for 3 h. The reaction was terminated by adding denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min and loaded onto 7% SDS-PAGE and electrophoresis was performed at 90 V. The degradation pattern was analysed by staining the gel with Coomassie brilliant blue R-250.

2.10 Prothrombin time (PT):

Prothrombin time was determined according to the method described by Tulip Diagnostics (P) Ltd., India. Briefly, fresh human blood was mixed with 3.2% trisodium citrate (1:9 v/v) and centrifuged for 5 min at 3000 rpm. The human plasma samples were incubated with LaLE in different concentrations (0 – 150 µg) and incubated for 5 min at 37 ºC. Later, 200 µl of uniplastin reagent was added and the clotting time was recorded. Neagative control was performed without the addition of the sample.

2.11 Recalcification time (RT):

Recalcification time was determined according to the procedure described by Condrea et al., 1983. Briefly, fresh human blood was mixed with 0.1 mM trisodium citrate (1:9 v/v). The mixture was centrifuged for 5 min at 3000 rpm and the supernatant obtained is used as platelet poor plasma (PPP). PPP was pre-warmed to 37 ºC before use. PPP (100 µl) was incubated with different concentration of LaLE (0 – 90 µg) in normal saline buffer (pH 7.0) and incubated for 5 min. The clot formation was initiated by adding 100 µl of 25 mM $CaCl_2$. The time taken for the appearance of first visible clot was recorded. Negative control was performed without the addition of the sample.

2.12 Protein estimation:

The protein concentration of LaLE was estimated according to the method of Lowry et al.,1940, and it was estimated to be 1 mg/ml.

III. RESULTS

3.1 Electrophoresis:

LaLE (20 µg) was subjected to 12%SDS-PAGE to obtain protein banding pattern under non-reducing condition. In SDS-PAGE the *L. aspera* extract showed protein bands of higher molecular weight in the range of 145 - 165 kDa (Figure 4).

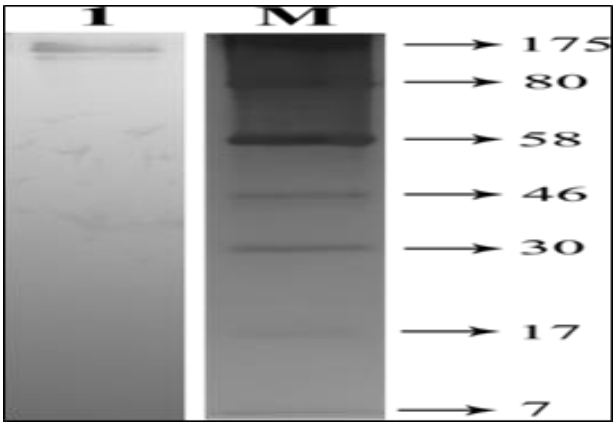

Fig 4: **Protein banding profile of LaLE.** The LaLE(20 µg) was loaded onto the 12% SDS-PAGE under non-reducing condition. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Lane 1: LaLE(20 µg); M: molecular weight marker.

3.2 Proteolytic activity and Zymography:

The LaLEwas evaluated for proteolytic activity using 2% casein as substrate. It showed a specific activity of 2.40 U/mg/ml. For further detection of the protease activity zymographic technique was performed by incorporating 0.2% casein and 0.2% gelatin separately into the 12% SDS-PAGE gels. The gels were stained after electrophoresis and translucent activity bands were obtained (Figure 5).

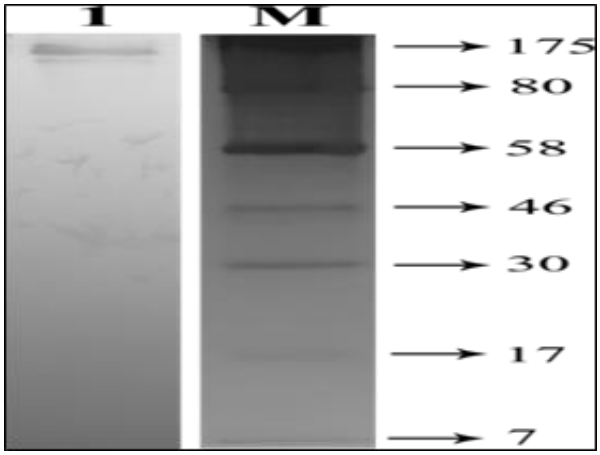

Fig 5: **Zymogram of** LaLE**.** Gelatin (0.2%) and casein (0.2%) were copolymerized with the polyacrylamide gel separately for the detection of proteolytic activity. The LaLE(20 µg) was incubated with SDS-PAGE sample buffer at 37 ºC for 20-30 minutes and loaded onto 12% SDS-PAGE under non-reducing condition. 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% Coommassive brilliant blue R-250 to visualize the activity bands. Lane 1: Protein banding profile; lane 2: casienolytic activity; lane 3: gelatinolytic activity; M: molecular weight marker.

3.3 Fibrinogenolytic Activity:

Leucas aspera leaf extract (0-30 µg) degraded all the chains (Aα, Bβ and γ) of fibrinogen in a concentration dependent manner. The sample preferentially degraded the Aα, Bβ followed by γ chain of fibrinogen (Figure 6).

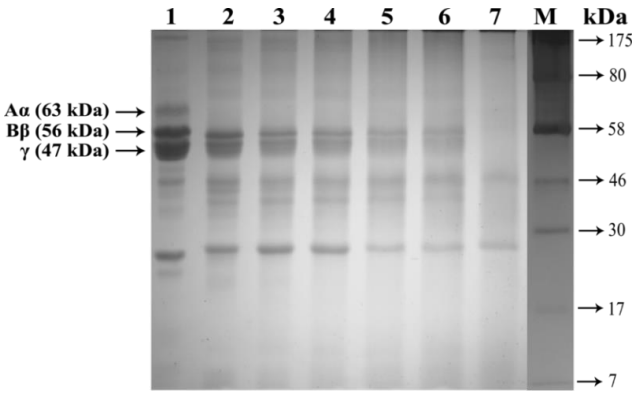

Fig 6: **Hydrolysis of human fibrinogen by LaLEin concentration dependent manner**. The fibrinogen (50 µg) was incubated with LaLEranging from 0 µg to 30 µg at 37° C for 4 h and SDS- PAGE (12%) was performed under reducing condition. After electrophoresis, the gel was stained with 0.25% Commassive brilliant blue R-250. Lane 1: fibrinogen (50 µg), lane 2–7: fibrinogen incubated with 5, 10, 15, 20, 25 and 30 µgof LaLErespectively, M: molecular weight marker.

3.4 Fibrinolytic Activity:

The LaLE (0-40 µg) degraded fibrin clots in a dose-dependent manner. The subunits of fibrin (α-polymer, γ-dimer, α-chain and β-chain) were degraded by the sample extract. To validate this data the hydrolyzed products of plasma clot were analyzed on 10% SDS-PAGE under reducing condition (Figure 7).

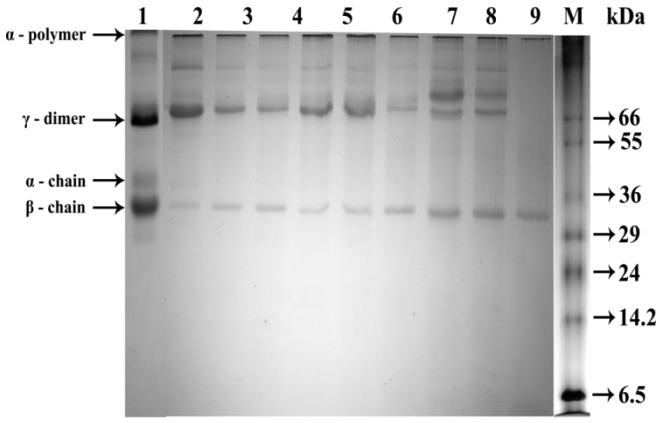

Fig 7:**Concentration dependent fibrinolytic activity by LaLE**. Equal volumes of plasma (100 µl) and 25 mM $CaCl_2$ (100 µl) were mixed and incubated to get fibrin hard clot. The fibrin clot was washed in 10 mM PBS (pH 7.0) and incubated with different concentrations of LaLE (5 µg to 40 µg) at 37 ºC for 2.5 hour. This 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 LaLErespectively; M: molecular weight marker.

3.5 Collagenolytic activity:

The LaLE(0 – 20 µg for Collagen I and 0 – 16 µg for Collagen IV) completely degrades all the bands of both type I (Fig. 8) and type IV (Fig. 9) collagen in a concentration dependent manner.

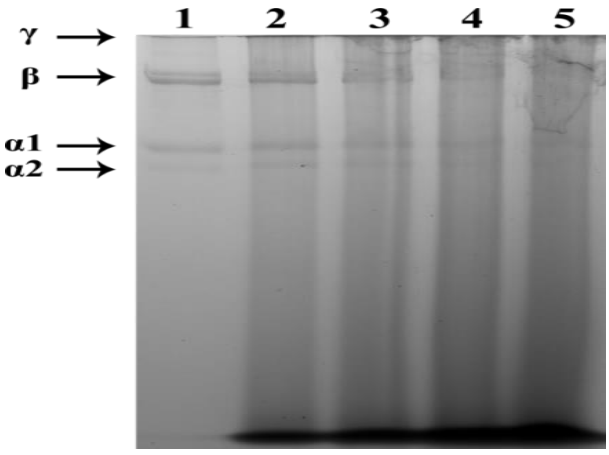

Fig 8:**Concentration dependent degradation of collagen I by LaLE.** Collagen type I (10 µg) was incubated with varying concentrations (0, 5, 10, 15 and 20 µg) of LaLE in 40 µL sodium phosphate buffer (10 mM, pH 7.0) at 37 ºC for 3 h. The reaction was terminated by adding denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min and loaded onto 7% SDS-PAGE and electrophoresis was performed at 90 V. The degradation pattern was analysed by staining the gel with Coomassie brilliant blue R-250. Lane 1 - 5: Collagen I (10 µg) with 0, 5, 10, 15 and 20 µg of LaLErespectively.

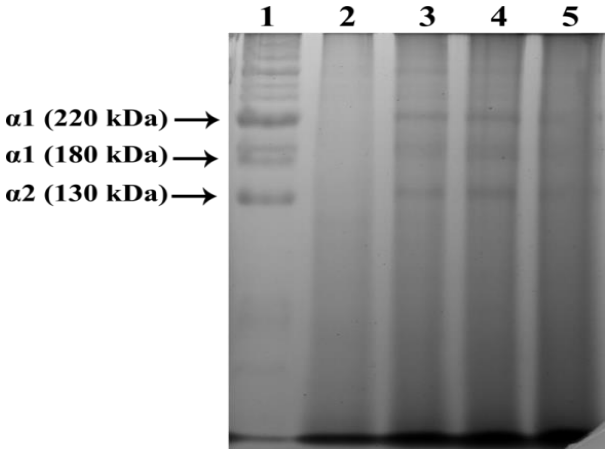

Fig 9:**Concentration dependent degradation of collagen I by LaLE.** Collagen type I (10 µg) was incubated with varying concentrations (0, 4, 8, 12 and 16 µg) of LaLEin 40 µL sodium phosphate buffer (10 mM, pH 7.0) at 37 ºC for 3 h. The reaction was terminated by adding denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min and loaded onto 7% SDS-PAGE and electrophoresis was performed at 90 V. The degradation pattern was analysed by staining the gel with Coomassie brilliant blue R-250. Lane 1 - 5: Collagen I (10 µg) with 0, 4, 8, 12 and 16 µg of LaLErespectively.

3.6 Coagulant activity:

The LaLE shows anti-coagulant activity for the Prothrombin time (PT) and Recalcification time (RT). The LaLE concentrations 30, 60, 90, 120 and 150 µg increased the clotting time from 15 seconds to 17, 22, 24, 36 and 58 seconds respectively for PT (Table 1) (Figure 10)and concentrations 15, 30, 45, 60, 75 and 90 µg increased the clotting time from 124 seconds to 141, 217, 280, 361, 453 and 673 seconds respectively for RT (Table 2)(Figure 11).

Table 1. Prothrombin time (PT) of LaLE. The LaLEwas incubated with 100 µl plasma for 5 minute at 37 ºC. Then 200 µl of uniplastin reagent was added and clotting was initiated. The time required for the appearance of the first clot was recorded. The LaLEconcentrations of 30, 60, 90, 120 and 150 µg increased the clotting time from 15 seconds to 17, 22, 24, 36 and 58 seconds respectively.

Serial number	Concentration of *L. aspera (µg)*	Time(sec)
1	0	15
2	30	17
3	60	22
4	90	24
5	120	36
6	150	58

Table 2. Recalcification time (RT) of LaLE. The LaLEwas incubated with 100 µl plasma for 5 minutes at 37 ºC. Then 100 µl of 25 mM $CaCl_2$ was added and the clotting time was recorded. The LaLE concentrations of 15, 30, 45, 60, 75 and 90 µg increased the clotting time from 124 seconds to 141, 217, 280, 361, 453 and 673 seconds respectively.

Serial number	Concentration of *L. aspera* (µg)	Time(sec)
1	0	124
2	15	141
3	30	217
4	45	280
5	60	361
6	75	453
7	90	673

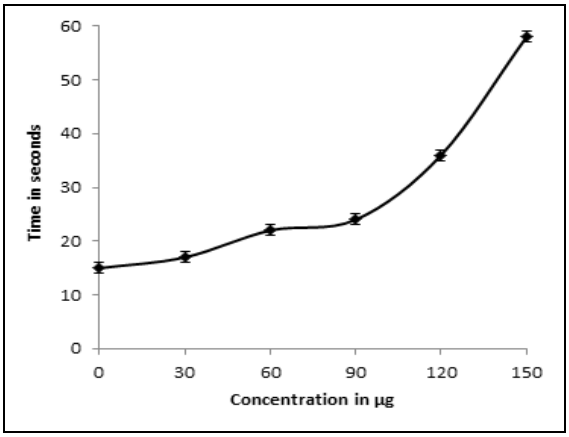

Figure 10. Prothrombin time (PT) of LaLE. The LaLE was incubated with 100 µl plasma for 5 minute at 37 ºC. Then 200 µl of uniplastin reagent was added and clotting was initiated. The time required for the appearance of the first clot was recorded. The LaLE concentrations of 30, 60, 90, 120 and 150 µg increased the clotting time from 15 seconds to 17, 22, 24, 36 and 58 seconds respectively.

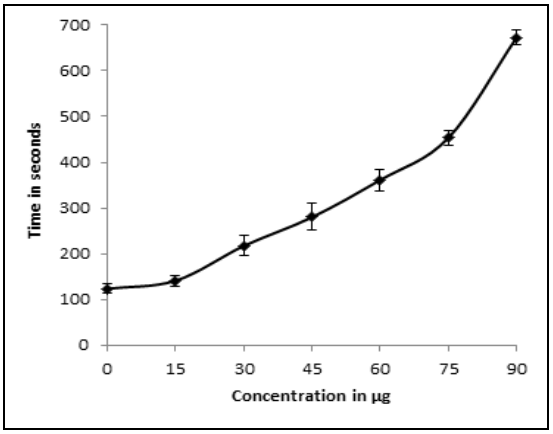

Figure 11. Recalcification time (RT) of LaLE. The LaLE was incubated with 100 µl plasma for 5 minutes at 37 ºC. Then 100 µl of 25 mM CaCl2 was added and the clotting time is recorded. The LaLE concentrations 15, 30, 45, 60, 75 and 90 µg increased the clotting time from 124 seconds to 141, 217, 280, 361, 453 and 673 seconds respectively.

IV. DISCUSSION AND CONCLUSION

In our project we have analyzed the LaLE for the presence of protease and further analysed for its fibrinogenolytic, fibrinolytic and role in blood coagulant. Protein concentration in the LaLE was found to be 1 mg/ml. The specific activity of protease was found to be 2.40 U/mg/ml using casein as substrate. This was supported by zymography using casein and gelatin as substrates. LaLE degraded fibrinogen and fibrin in concentration dependent

manner degrading all the chains. LaLE completely degraded Aα, Bβ followed by γ chain of fibrinogen at the sample concentration of 30 µg. Fibrin subunits (α-polymer, γ-dimer, α-chain, and β-chain) were degraded by the LaLEat the concentration of 40 µg. Further, LaLE was analyzed for its specificity of action on extracellular molecules such as collagen I and collagen IV in a dose dependent manner. LaLE degraded all the chains of both the molecules.LaLE analyzed for its interference in the blood coagulation pathways by PT and RT. LaLE prolonged the clotting time in PT by 3.87 fold and RT by 5.43 fold. This clearly indicates the anticoagulant nature of LaLE. This study provides the information that the LaLE possess the fibrinogenolytic, fibrinolytic, collagenolytic activity and is anti-coagulant in nature which might be the reason for its use in medicinal applications.

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