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Original Article

IN VITRO STUDY ON PROTEASE AND THROMBOLYTIC ACTIVITY OF AQUEOUS EXTRACT FROM *LEUCAS ASPERA* (L.) LEAVES

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ABSTRACT

Objective: The current study is an attempt to screen for the *in vitro* clot lysis and proteolytic activity of aqueous extract of *Leucas aspera* leaves.

Methods: Thrombolytic activity and protease activity of the crude enzyme obtained by ammonium sulfate precipitation and dialysis were assayed using blood clot and casein as substrates respectively. Native PAGE and gel documentation studies were performed to calculate the molecular weight of the enzyme.

Results: In the study, 40% salt fractioned crude enzyme sample exhibited significant thrombolytic and caseinolytic activity. Further dose-dependent increased activity was observed with the maximum lytic activity of 52.11±1.04 % at 1 mg/ml of the sample when compared to the reference drug streptokinase (71.39±0.32%). Also, 68.72±0.62 U/hr of caseinolytic activity was observed for 1 mg/ml of the sample fraction.

Conclusion: The study highlights and validates the efficacy of *Leucas aspera* leaves extract for thrombolytic and proteolytic actions. Enzyme with an approximate molecular weight, 19.89 KDa could be responsible for the significant lytic activity.

Keywords: *Leucas aspera*, Thrombolytic activity, Proteolytic activity, Native PAGE

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INTRODUCTION

Blood clot (thrombus) developed in the circulatory system due to the failure of homeostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases [1-3]. Thromboembolic disorders such as myocardial infarction, cerebrovascular thrombosis, pulmonary embolism, and venous thrombolism are life-threatening for human beings [4]. Thrombolytics are used to dissolve the fibrin of blood clots, which are potentially life-threatening, especially those in the arteries of the heart and lungs. It is also used against the clot formed in shunts during kidney dialysis and multiple pulmonary emboli. Commonly used thrombolytic agents are Alteplase, Anistreplase, Streptokinase, Urokinase, and tissue plasminogen activator (TPA) to dissolve clots [5].

All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity. Though streptokinase and urokinase are widely used due to their lower cost, as compared to other thrombolytic drugs, these are dangerous because they might cause serious bleeding complications along with reocclusion and reinfarction [6]. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs [7-9]. Day by day the context, concept, and methods of the use of natural products in the treatment of humans have undergone remarkable changes. Such changes occurred since natural medicine or traditional medicine made a revolutionary come-back with renewed strength and vigor to play a more significant role in the management of human health [10].

Some plants or plant parts showing thrombolytic activity have also been reported. For example, plant extracts of Ageratum conyzoides L, Clausena suffruticosa, Leea indica and peel extract of Punica granatum showed a significant percentage of clot lysis compared to reference drug streptokinase [11-13]. Leucas aspera, belonging to the family of Labiatae, is a common aromatic herb. Traditionally, the whole plant is used for analgesic, antipyretic, antirheumatic, anti-inflammatory, and antibacterial treatment. Its anti-inflammatory activity has been shown in animal models through prostaglandin inhibition [14-17]. The current study aims at determining the thrombolytic and proteolytic activities of Leucas aspera leaves extract.

MATERIALS AND METHODS

Chemicals and reagents

Streptokinase and trypsin (RM612) were purchased from Sigma Aldrich, St Louis, MO, USA. Casein, Bovine Serum Albumin, TEMED and PAGE chemicals from HI–MEDIA, Mumbai, India. All the chemicals and reagents used were of analytical grade. Fresh human blood samples were collected from healthy volunteers after obtaining their consent.

Plant material

Fresh leaves of *Leucas aspera* were collected during the year 2017–18 from Saranda chiria forest, Paschimi Singhbhum district of Jharkhand, India and taxonomically authenticated by Prof. Hari Shankar, Plant taxonomist, Jharkhand Biodiversity Board, Ranchi, India. The sample specimen was submitted to the Botanical survey of India, Kolkata (Herbarium voucher number–86206).

Preparation of extract

Leucas aspera leaves sample was cleaned, shade dried, pulverized, and stored in an airtight container.

50 gm of leaf powder was homogenized with distilled water using mortar and pestle. The extract was filtered and centrifuged at 5000 RPM for 15 min. The supernatant was subjected to protein precipitation for 40% ammonium sulphate fractionation. After ammonium sulphate precipitation, the sample was subjected to centrifugation for 10 min at 10,000g. The precipitated pellet was dissolved in 10 mmol phosphate buffer and dialyzed against the same buffer to remove ammonium sulphate. Protein concentration of the fraction was measured at 540 *nm* using Biuret reagent.

Caseinolytic activity

Caseinolytic activity was assayed as per the method of Murata *et al.* [18]. Briefly, 0.4 ml casein (2% in 0.2 M Tris–HCl buffer. pH 8.5) was

incubated with different concentrations (250- 1000 µg) of 40% fraction and trypsin at 37 °C separately for 2 h. The reaction was stopped by adding 1.5 ml of the 0.44M TCA and allowed to stand for 30 min followed by centrifugation at 1500g for 15 min. An aliquot (1 ml) of the supernatant was mixed with 2.5 ml of the 0.4M sodium carbonate and 0.5 ml of FC reagent (1:2) followed by reading absorbance at 660 nm. One unit of enzyme activity was defined as the amount of the enzyme required to increase in absorbance of 0.01 at 660 *n*m/h at 37 °C. Enzyme activity was expressed as U/h at 37 °C.

Thrombolytic activity

In vitro thrombolytic activity in terms of clot lysis was carried out. Venous blood drawn from healthy volunteers was transferred in different pre-weighed sterile microcentrifuge tubes (500 µl/tube) and incubated at 37 °C for 45 min. After clot formation, serum was completely removed and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube-weight of the tube alone). Each micro-centrifuge tube containing the clot was properly labeled and various concentrations of the crude enzyme were added to the tubes (20-100 µg). Distilled water and streptokinase were added separately to the tubes containing the clot to serve as a negative control and positive control respectively. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. Fluid obtained after incubation was removed and tubes were again weighed to observe the difference in weight after clot disruption. The difference obtained in the weight before and after clot lysis was expressed as the percentage of clot lysis.

$$\text{Percentage of clot lysis (\% of clot lysis)} = \frac{\text{Wt of released clot}}{\text{Wt of total clot}} \text{ X } 100$$

Electrophoresis

Native PAGE (12%) was carried out for 40% dialyzed fraction and the bands were visualized by staining with Coomassie Brilliant Blue R250. Gel imaging and documentation were done using the Bio-Rad Gel Doc EZ system.

Statistical analysis

The analysis was done using Microsoft excel. All the experiments were conducted in triplicates. One-way ANOVA and post hoc tests were conducted and P values less than 0.05 were considered as significantly different. Values were represented as mean±SEM.

RESULTS AND DISCUSSION

Proteases constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market. Recent years have envisaged a surge in enzyme market growth due to diverse key factors including cost-effectiveness and productivity. Plant proteases have been implicated in the design and synthesis of therapeutic agents [19]. Herein we report on the potent protease and clot lysis activities of the enzyme from *L. aspera* leaves extract. 40% dialyzed fraction contained 1.6 mg/ml of crude protein. Native PAGE under non-reducing conditions revealed a dense single banding for the crude enzyme of 40% dialysis fraction (DF) from *L. aspera* (fig. 1a). Proteolytic activity of the crude enzyme from 40% dialysis fraction was examined using casein as a substrate. DF exhibited potential caseinolytic activity when 2 % of casein was used as a substrate. The activity was progressively increased with an increase in the concentration of the enzyme fraction. The protein concentration ranging from 250µg/ml to 1000µg/ml exhibited a mean activity of 19.63±0.19 U/hr to 68.72±0.62 U/hr respectively (fig. 2). Trypsin and distilled water (DW) used as a positive and negative control showed activities of 84.2±1.18 U/hr and 2.56±0.09 U/hr respectively (table 1). Furthermore, purification and characterization of protease(s) are in progress to categorize the protease for two varied classes viz., cysteine proteases or serine proteases.

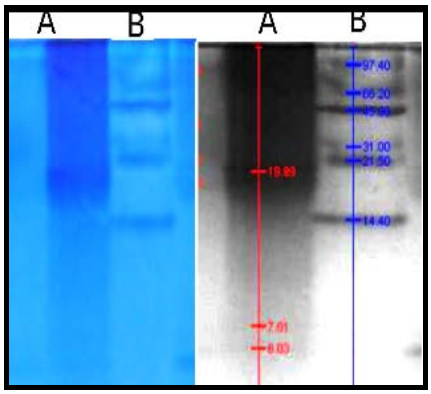

Fig. 1: Native PAGE of 40% DF A–sample, B–marker protein

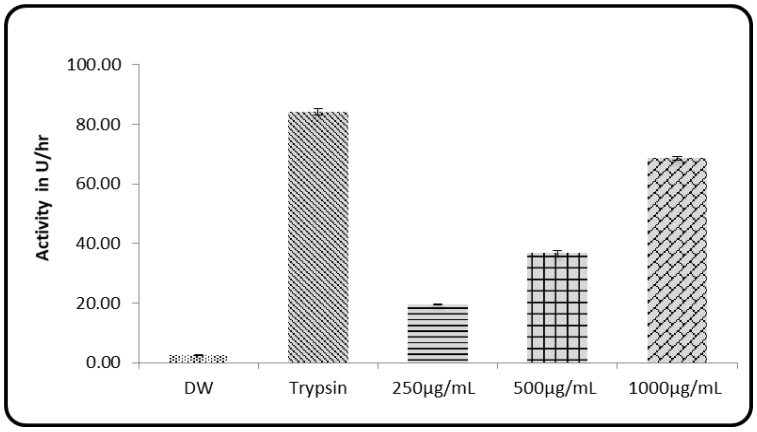

Fig. 2: Protease activity of 40% dialysed fraction, values are mean±SEM, n=3, significant at ap<0.05

Thrombolytic activity

Fibrinolytic enzymes dissolve fibrin, the main component of blood clots. Accumulation of fibrin in the blood vessels results in thrombosis, leading to myocardial infarction, and other heart diseases [20]. Several anticoagulants, anti-platelet, and thrombolytic medications are used for the treatment of thrombotic disorders. Anticoagulants and anti-platelet agents prevent the formation of blood clots but do not dissolve existing clots, whereas thrombolytic agents can dissolve a clot but emboli can form even after successful treatment. Thus, none of them provide a permanent and complete solution [21]. Thrombolytic drugs dissolve blood clots by activating plasminogen, which forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, thereby dissolving the blood clots.

The addition of the DF with enzyme aliquots varying from 250 to 1000µg/ml to the preformed blood clots and subsequent incubation for 90 min at 37 °C demonstrated a dose-dependent response with maximum lysis of 52.11±1.04 % (table 1). 100µl of streptokinase

taken as a positive control (35000 IU) showed 71 % clot lysis activity and distilled water used as negative control exhibited 6.04 % of lysis action (fig. 3). Gel documentation study for the 40% DF resolved under nonreducing native PAGE divulged the approximate molecular weight of the crude enzyme as 19.89 KDa (fig. 1b) which is attributed to the lysis of clot releasing the fibrin monomers.

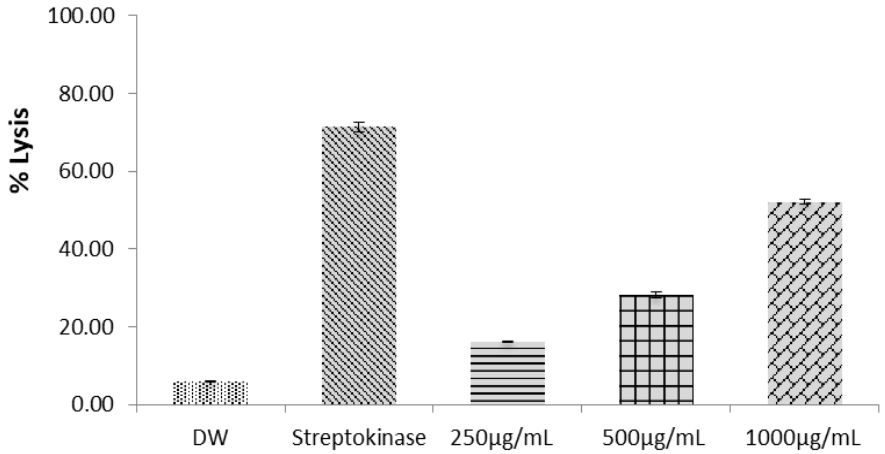

Fig. 3: Clot lysis activity of crude enzyme from dialyzed fraction, values are mean±SEM, n=3, significant at ap<0.05

Table 1: Protease and thrombolytic activities of crude enzyme

Sample	Protease activity (U/h)	Thrombolytic activity (% lysis)
DW	2.56±0.09	6.04±0.21
Trypsin	84.20±1.18	-
Streptokinase	-	71.39±0.32
DF (250µg/ml)	19.63±0.19a	16.18±0.26a
DF (500µg/ml)	36.79±0.86a	28.17±0.59a
DF (1000µg/ml)	68.72±0.62a	52.11±1.04a

DF–Dialyzed fraction from 40% salt precipitation, values are means±SEM, n = 3, significant at ap<0.05

CONCLUSION

In the context of the above result and discussion, it can be concluded that the enzyme extract of *L. aspera* leaves possesses significant protease and thrombolytic activity compared to the standard enzyme trypsin and streptokinase respectively. However, further studies are in progress to purify and characterize the enzyme from the crude dialyzed sample. Also cytotoxic studies have to be taken up further to explore the medical and pharmaceutical potentiality of the plant.

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AUTHORS CONTRIBUTIONS

All authors contributed to the study's conception and design. Nagamani JE and Usha sah performed the experiment and analysis. The first draft of the manuscript was written by Nagamani JE and the critical revision of the manuscript was done by Ravindranath H A. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

This statement is to declare that all authors involved in this manuscript have no conflict of interest.

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