

Hemostatic potential of latex proteases from *Tabernaemontana divaricata* (L.) R. Br. ex. Roem. and Schult. and *Artocarpus altilis* (Parkinson ex. F.A. Zorn) Forsberg

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Abstract Pharmacological properties exhibited by latex of plants are due to various biologically active compounds including several proteolytic enzymes. Present study evaluates hemostatic potential of *Tabernaemontana divaricata* and *Artocarpus altilis* from Apocynaceae and Moraceae families respectively. The latex of these plants were initially subjected to dialysis and crude extracts were estimated for proteolytic activity using casein as the substrate. Mean caseinolytic activity for 100 µg of latex protein was found to be 56.16 ± 0.57 and 45 ± 0.3 U/h for *T. divaricata* and *A. altilis* respectively. Caseinolytic activity by both the plant extracts was higher than standard proteases, papain and trypsin. However the difference was significant ($p < 0.05$) with papain alone. Crude enzymes (CE) from both plants exhibited coagulant activity on human platelet poor plasma by recalcification time. A significant reduction in clotting time was exhibited by *T. divaricata* compared to *A. altilis* ($p < 0.05$). These results were further substantiated with fibrinogen agarose plate assay. Crude enzyme of both plants also hydrolyzed blood clot. Mean % of thrombolysis by *T. divaricata* was 80.75 ± 1.2 and that of *A. altilis* was 70.24 ± 1.52 . Inhibition studies confirmed cysteine protease nature of CE. Comparative analysis revealed *T. divaricata* to be the best among the two for its hemostatic potential. This study scientifically validates the use of latex from these plants in the management of fresh cuts or wounds.

Keywords Latex · Proteases · Procoagulant · Thrombolysis

Introduction

The presence of latex is one of the characteristic features of plants belonging to the families Euphorbiaceae, Asclepiadaceae, Moraceae and Apocyanaceae [1]. *Tabernaemontana divaricata*, commonly known as crape jasmine, is a medicinally important latex producing plant belonging to family Apocynaceae. *Tabernaemontana* is one of the genera that were used in Chinese, Ayurvedic and Thai traditional medicine for the treatment of fever, pain and dysentery [2–4]. It is a common shrub in India, Thailand and Bangladesh. Diverse chemical constituents from *T. divaricata* leaves, stems and roots have been reported previously and found effective in antimicrobial, antiparasitic, anti-inflammatory and anti-tumor activities [5–7]. The most common medicinal use of crude *T. divaricata* extract involves its antimicrobial action against infectious diseases such as syphilis, leprosy and gonorrhoea, as well as its antiparasitic action against worms, dysentery, diarrhoea and malaria [7]. Moraceae, which is often called mulberry family, comprises of 40 genera and 1,000 species, nearly all with milk sap. *Artocarpus altilis*, a moraceae member commonly known as breadfruit, is cultivated throughout the tropics. This plant has long been an important staple crop and a primary component of traditional agro forestry systems. Its latex, leaves, roots and bark are used to treat skin ailments, fungal diseases, broken bones and as an analgesic [8–10].

Wounds are major concern for the patients and clinicians alike. Chronic wounds affect a large number of patients and seriously reduce their quality of life [11]. In

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rural areas of developing countries wounds and dermatological conditions constitute one of the five most common reasons for people seeking medical care [11, 12]. Topical application of *Tabernaemontana* and *Artocarpus* plant species have been used in healing wounds in folk medicine [7, 13]. However, the exact mechanism of the healing process of wound by these plants is not clearly understood.

Coagulation and fibrinolysis are two important proteolytic events of wound healing process. Many proteolytic enzymes are involved in these processes in vivo. Proteolytic activity found in the latex of many medicinally important plants was shown to be associated with blood coagulation. Proteases interfering in blood coagulation and fibrin hydrolysis were isolated and characterized from several plant lattices [14, 15]. However, there are no reports available on the hemostatic role of *T. divaricata* and *A. altilis* latex proteases till date. Aim of the present study was to decipher the molecular mechanism behind the procoagulant and subsequent clot hydrolyzing activity of latex proteases from *T. divaricata* and *A. altilis*.

Materials and methods

Materials

Human fibrinogen and thrombin were obtained from Sigma Aldrich, USA. All other chemicals and reagents purchased were of analytical grade. Plant latex was collected from in and around Bangalore, India. Voucher specimens for the plants were deposited at the National Institute of Ayurveda and Dietetics, Jayanagar, Bangalore for identification and authentication (*T. divaricata* (L.) R. Br. ex. Roem. and Schult. RRCBI-Mus/09 and *A. altilis* (Parkinson ex. F.A. Zorn) Forsberg RRCBI-Mus/09). Fresh human blood samples were collected from healthy volunteers after obtaining their consent.

Plant material and its processing

The latex was collected in clean glass beaker by breaking tender parts of *T. divaricata*, and *A. altilis*. Latex was diluted with equal volume of 10 mM phosphate buffer (pH 7.0) and kept overnight at 4 °C. The supernatant was decanted and centrifuged at 12,000×g for 20 min at 4 °C. The clear supernatant was decanted and dialyzed against 10 mM phosphate buffer (pH 7.0). The supernatants were subjected to protein precipitation by 80 % ammonium sulphate. The samples were then subjected to centrifugation for 10 min at 10,000×g. The precipitated pellets were dissolved in 10 mM phosphate buffer and dialyzed against the same buffer to remove ammonium sulphate [16]. The protein concentration of crude enzyme (CE) was measured at 660 nm using Folin's reagent [17].

Caseinolytic activity

Caseinolytic activity was assayed by the method of Murata et al. [18]. Briefly, casein 0.4 ml was incubated with different concentration (20–100 µg) of CE, trypsin and papain at 37 °C separately for 2 h. The reaction was stopped by adding 1.5 ml of the 0.44 M TCA and allowed to stand for 30 min followed by centrifugation at 1,500×g for 15 min. An aliquot (1 ml) of the supernatant was mixed with 2.5 ml of the 0.4 M 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 nm/h at 37 °C. Activity was expressed as U/h at 37 °C. Inhibition studies were carried out after preincubating the latex enzyme fractions with or without specific protease inhibitors separately for 15 min. Further, the assay was carried out as described above.

Native PAGE

Native PAGE was carried out according to the method of Walker et al. [19] for CE using Tris–HCl buffer. The bands were visualized by staining with Coomassie brilliant blue R-250.

Coagulant activity

Re-calcification time was determined according to procedure described by Condrea et al. [20]. Fresh human blood was mixed with 0.11 M tri-sodium citrate in the ratio of 9:1 and centrifuged for 15 min at 500×g. The supernatant was used as platelet poor plasma (PPP). To 300 µl of pre warmed (37 °C) PPP, different concentrations of crude latex extracts in 0.01 M Tris–HCl buffer (pH 7.4) was added and incubated for 1 min. The clot formation was initiated by adding 30 µl of 0.25 M calcium chloride. The time taken for visible clot to appear from the time of addition of CaCl₂ was recorded. Tris–HCl buffer alone was added instead of the enzyme source in control experiments.

Fibrinogen agarose plate assay

The assay was carried out by the method of Shivaprasad et al. [21]. 3 ml of 1.2 % agarose warmed at 50 °C was mixed with 3 ml of 0.4 % (w/v) human fibrinogen dissolved in 0.1 M Tris–HCl; pH 7.4 also at 50 °C. Fibrinogen agarose mixture was poured into a petriplate and allowed to solidify for 1 h at room temperature. Wells of about 3 mm diameter were made onto the plate and 10 µl of buffer containing 10 µg of *T. divaricata* and *A. altilis* latex CE was loaded into the wells and moist incubated at 37 °C for 2 h. Following incubation period, the diameter of

the precipitation zones formed around the wells due to polymerization of fibrin was measured. 0.2 U of human thrombin and buffer served as positive and negative controls respectively.

Blood clot lysis assay

Venous blood drawn from healthy volunteers was transferred in different pre weighed sterile micro centrifuge tube (500 µl/tube) and incubated at 37 °C for 45 min. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube –weight of tube alone). Each tube containing clot was properly labeled and various concentrations of crude enzymes were added to it. Water served as the negative control. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, fluids obtained were removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference in weight before and after clot lysis was expressed as percentage of clot lysis [22].

Statistical analysis

The data obtained from five independent experiments were analyzed using Graph Pad Prism (CA92037, USA). Each value represents the mean of five independent experiments performed in triplicates, with average standard deviation of <5 %. The data were analyzed by two tailed paired *t* test, wherever applicable and *p* values of <0.05 were considered statistically significant.

Results

The milky latex collected from *T. divaricata* and *A. altilis* was processed via dilution, overnight incubation, centrifugation and subsequent dialysis. The dialyzed samples were further precipitated for protein with 80 % ammonium sulphate followed by a second dialysis to remove ammonium sulphate to finally procure the CE. The CE exhibited proteolytic activity with casein as substrate. The activity was progressively increasing with increase in concentration of the latex protein. 20–100 µg of latex protein concentration gave a mean activity of 14 ± 0.3 to 56 ± 0.2 and 11 ± 0.2 to 45 ± 0.3 U/h for *T. divaricata*, and *A. altilis* respectively. In comparison with standard proteases, papain and trypsin both the plant extracts exhibited higher caseinolytic activity. However, the difference was significant (*p* < 0.05) with papain alone (Fig. 1).

The crude enzymes on native gel electrophoresis resolved into three major bands corresponding to different

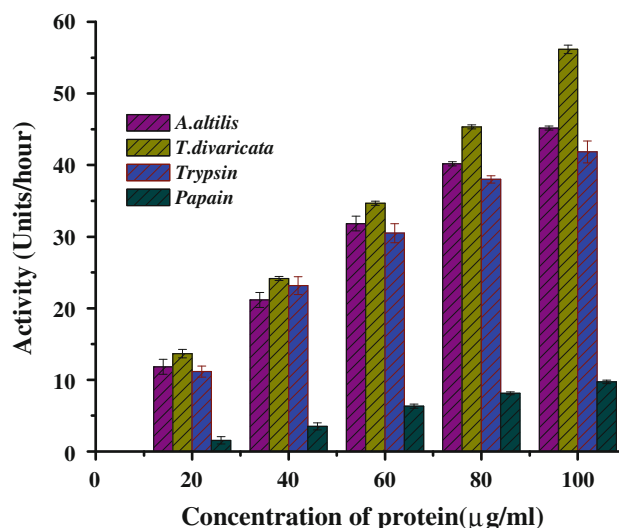
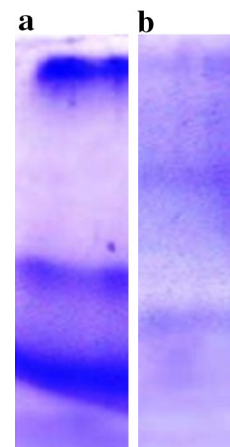


Fig. 1 Caseinolytic activity of crude latex enzyme, papain and trypsin. Reaction mixture, 1 ml contained 0.4 ml of 2 % casein in 0.2 M Tris–HCl buffer (pH 8.5), incubated with different concentrations (ranging from 20 to 100 µg protein) of crude latex enzymes of *T. divaricata* (hatched) and *A. altilis* (diagonal lines), papain (solid black) and trypsin (solid blue) for 2 h at 37 °C showing progressive increase in proteolytic activity with increase in protein concentration. Values represent mean ± SD (*n* = 5)

Fig. 2 Native PAGE of crude latex enzyme. Latex protein was loaded onto 10 % polyacrylamide gel and electrophoresis was performed in a constant current under basic conditions using Tris- HCl buffer (a *T. divaricata*, b *A. altilis*). CE protein resolved into three major bands



groups of proteins present in the latex (Fig. 2). CE from both plants exhibited coagulant activity on human PPP (Fig. 3). There was a dose dependent clot formation (190 to 43 ± 2 and 190 to 71 ± 0 s for *T. divaricata* and *A. altilis* respectively). A significant reduction in clotting time was exhibited by *T. divaricata* compared to *A. altilis* (*p* < 0.05) (Fig. 3). The observation of procoagulant potential was substantiated with a fibrinogen agarose plate assay. A zone of precipitation having approximate diameter of 1.1 and 0.7 cm was given by CE (10 µg of protein) of *T. divaricata* and *A. altilis* respectively (Fig. 4). 0.2 U of thrombin gave a precipitation zone of diameter 0.9 cm (picture not shown). There was absolutely no change around control well, confirming the thrombin like activity associated with

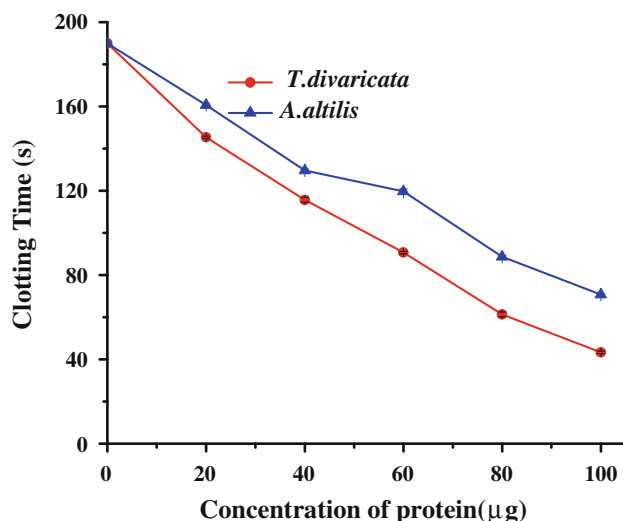


Fig. 3 Effect of crude latex enzyme on recalcification time of human plasma. CE protein of *T. divaricata*. (—●—) and *A. altilis* (—▲—) ranging from 20 to 100 μg was preincubated with 300 μl of human plasma in presence of 30 μl Tris-HCl buffer (10 mM; pH 7.4) for 1 min at 37 °C. Thirty microlitres of CaCl₂ (0.25 M) was added to the pre-incubated mixture. The time for clot formation was recorded. In the control experiments, Tris-HCl buffer was used instead of enzyme source. Clot formation time reduced progressively with increase in enzyme concentration

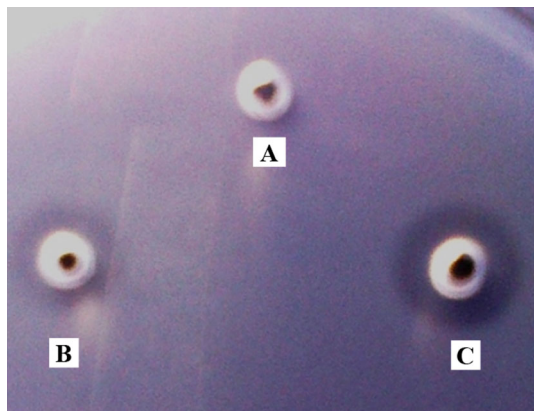


Fig. 4 Fibrinogen agarose plate assay showing zone of clearance. Thrombin like activity of latex enzyme fraction observed as clear zone in fibrinogen agarose plate. A Buffer alone B CE of *A. altilis* and C CE of *T. divaricata*

the latex of these plants. CE of both plants lysed the blood clot (Fig. 5a). *T. divaricata* exhibited higher mean % clot lysis (80.75 ± 2.1) than that of *A. altilis* (70.24 ± 1.52). Figure 5b is a representative picture of clot lysis by *T. divaricata* CE.

Inhibition studies were carried out using several specific protease inhibitors to understand the nature of proteases in the crude latex enzymes. The proteolytic effect was completely inhibited by IAA, a specific cysteine protease inhibitor. There was no inhibition observed with any of the other specific protease inhibitors (Table 1).

Both the plants were found to possess significant hemostatic potential. Statistical analysis reveals *T. divaricata* to be the best among the two for its haemostatic potential.

Discussion

The plants *T. divaricata*, and *A. altilis* gained importance because of their enormous application in traditional medicine [23]. The role of various plant and animal proteases in hemostasis has been studied extensively [24]. Presence of proteolytic enzymes in the latex of diverse medicinal plants exhibited varied potency and specificity to hydrolyze different protein substrates [16]. Application of plant latex on fresh cuts to stop bleeding has been the general tribal/rural practice globally [11]. Recent research in this field has given convincing evidence that this observed pharmacological activity is due to the presence of proteases or proteins [24]. Extracts of aerial and underground plant parts of a number of *Artocarpus* species have been applied in traditional medicine for wound healing [13]. *T. divaricata*, in addition to its antimicrobial action against infectious diseases, was reported to suppress mesangial cell proliferation via the reduction of IL-1, IL-6 and TNF- α expression suggesting its potential to be developed as new chemotherapeutic agents to prevent Immunoglobulin A nephropathy [25, 26]. A few *Tabernaemontana* species also has shown acetylcholine esterase (AChE) inhibiting activity in vitro targeting their therapeutic suitability for Alzheimer's disease [27, 28]. Accumulating data sheds light into the medicinal properties of apocyanaceae and moraceae family members including their wound healing effect. The protective effect of *T. divaricata* and *A. altilis* against wound healing has been mentioned in folk/traditional medicine. However the scientific validation of their hemostatic effect is unexplored. Current study is an attempt to understand their role in blood coagulation and subsequent thrombolysis considering the pressing need for suitable wound healing alternatives.

The crude extracts of latex hydrolyzed casein when used as a substrate. A progressive increase in caseinolytic activity was clearly observed with increase in concentration of protein. Caseinolytic activity of latex enzymes at different concentrations was compared with that of standard proteases, papain and trypsin. The ammonium sulphate latex enzyme fraction of both plants showed significantly higher caseinolytic activity when compared to that of standard cysteine protease, papain and trypsin. The proteolytic activities of CE of these two plants were inhibited by IAA supporting their cysteine protease nature. The results confirm the occurrence of enhanced protease activity associated with latex of these plants. The significantly high level of protease activity associated with some

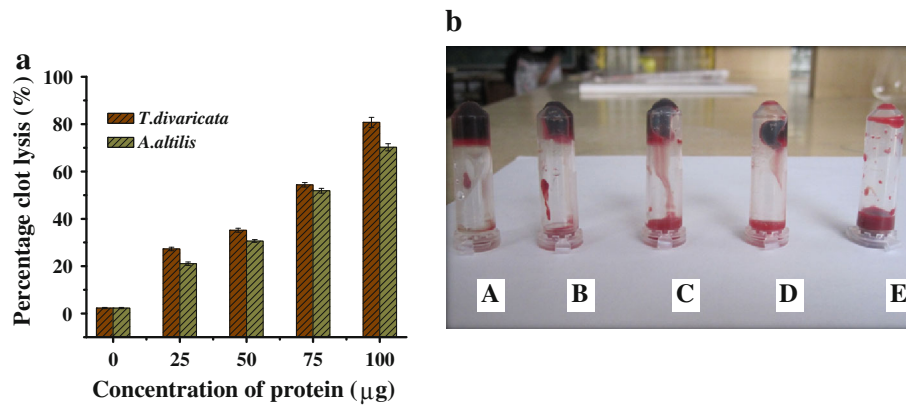


Fig. 5 a Clot lysis of blood samples by latex CE. Graph depicting percentage clot lysis by CE of *T. divaricata* (■), *A. altilis* (▨). **b** Representative picture of clot-lysis of blood samples by different concentrations of CE of *T. divaricata*. No clot lysis was observed in

tube A (control). Tube B–E (CE of *T. divaricata*) showing lysis by four different concentrations of CE (25–100 µg). After dissolution of the clots, tubes were inverted and fluid along with the remnants of clots could be clearly seen

Table 1 Effect of Inhibitors on proteolytic activity of CE of *T. divaricata* and *A. altilis*

Sl. no.	Inhibitor	Concentration (mM)	Inhibition (%)	
			<i>Tabernaemontana divaricata</i>	<i>Artocarpus altilis</i>
1	IAA	0.1	96	91
2	PMSF	5	Nil	Nil
3	EDTA	5	Nil	Nil
4	1,10-Phenanthroline	5	Nil	Nil

medicinally important latex producing plants was reported earlier [29–32].

CE of both plant latex exhibited pronounced and progressive increase in procoagulant activity of platelet poor plasma and induced fibrin clot formation. Similar to Shivaprasad et al.’s observation on selected asclepiadaceae members, the present study also could observe calcium independent fibrin clot induction by the CE. This observation points towards the possible bypassing of calcium requiring steps and direct action of these enzymes on the terminal step of blood coagulation cascade [30]. The thrombin like activity of CE was compared with known units of human thrombin using fibrinogen agarose plate. Direct incubation of crude latex enzyme with human fibrinogen, induced zone of precipitation which was comparable to the one given by the positive control (human thrombin). Similar observation was reported by Ramos et al., in latex proteases of *Calotropis procera* [24]. It may be inferred that the observed procoagulant activity triggered by these crude enzymes appears to be due to their thrombin like activity.

Apart from its usage to stop bleeding, plant latex have been used since time immemorial in folk medicine as remedy for wound healing [6]. Proteases from plant latices have been shown to exhibit blood clot dissolving properties

indicating their plasmin like behaviour. The dual activities by some latex proteases have projected them as good alternatives in the management of fresh cuts/wounds. Latex proteases efficiently hydrolyze the fibrin leading to its dissolution. Results from the present study show *T. divaricata* and *A. altilis* possess significantly high clot lysis potential additional to its procoagulant effect. Comparison of the present results with an earlier study which attempted to develop a new model system to study clot lysis in a simplified way using streptokinase shows the suitability of these plant candidates as a thrombolytic alternative [22]. It would be interesting to further study which is the predominant and important property among the observed procoagulant and clot lysis and whether there exists any temporal gap between these two events. Shivaprasad et al. [21] in their work on cysteine proteases from latex of Asclepiadaceae plants reported that latex enzyme fraction increased the absorbance of clear fibrinogen solution as a consequence of fibrin formation with time due to its thrombin like activity. However, prolonged incubation of latex enzyme resulted in decreased absorbance due to hydrolysis of formed fibrin clot suggesting its plasmin like activity [29]. Designing such studies, where formation of opaqueness and then clearing may be checked with a time factor taken into consideration, may help to assess the extent and progression

of these two activities. Several in vivo and in vitro studies with plants belonging to family Asclepiadaceae explain the involvement of cysteine proteases in both thrombin and plasmin like activities [21, 24, 30]. AMP48, a novel serine protease of jackfruit latex, with fibrino (geno)lytic activity was shown to be effective for the treatment of thromboembolic disorders [33]. Patel et al. [34] purified 34 kDa serine protease (hirtin) from *Euphorbia hirta*, studied its fibrinolytic activity and suggested its industrial and therapeutic applications. Observations from the present study suggest dual actions (procoagulant/clot inducing and thrombolytic) of latex crude enzyme/s supporting hemostatic potential associated with *T. divaricata* and *A. altilis* which may find therapeutic importance in management of fresh cuts/wounds.

Conclusion

The protective effect of *T. divaricata* and *A. altilis* against wound has been mentioned in folk/traditional medicine. However the scientific validation behind such an effect is lacking. The present study is the first attempt to understand the hemostatic potential associated with *T. divaricata* and *A. altilis*. Results of the study strongly support abundant hemostatic potential associated with crude latex enzyme/s of these plants, which may find potential therapeutic application in wound healing processes. Further studies on the purification and biochemical characterization of CE of these plants will give insight into the utility of the enzyme/s in the biotechnological industries and protein engineering.

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