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ORIGINAL ARTICLE

Synergistic Caseinolytic Activity and Differential Fibrinogenolytic Action of Multiple Proteases of *Maclura spinosa* (Roxb. ex Willd.) latex

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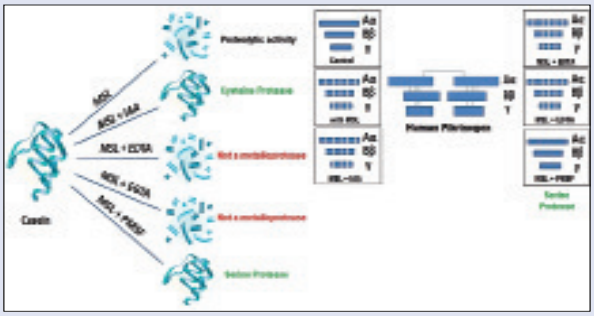
ABSTRACT

Background: Kollamalayaali tribes of South India use latex of *Maclura spinosa* for milk curdling. This action is implicated to proteases which exhibit strong pharmacological potential in retardation of blood flow and acceleration of wound healing. **Objective:** To validate the presence of a proteolytic enzyme(s) in *Maclura spinosa* latex (MSL), and to investigate their probable role in hemostasis. **Materials and Methods:** Processed latex was examined for proteolytic and hemostatic activity using casein and human fibrinogen as substrates, respectively. Caseinoltyic activity was compared with two standard proteases viz., trypsin I and trypsin II. Effect of various standard protease inhibitors viz., iodoacetic acid (IAA), phenylmethylsulfonyl fluoride (PMSF), ethylene glycol tetraacetic acid, and ethylenediaminetetraacetic acid on both caseinolytic and fibrinogenolytic activities were examined. Electrophoretogram of fibrinogenolytic assays were subjected to densitometric analysis. **Results:** Proteolytic action of MSL was found to be highly efficient over trypsin I and trypsin II in dose-dependent caseinolytic activity ($P < 0.05$; specific activity of 1,080 units/mg protein). The Aα and Bβ bands of human fibrinogen were readily cleaved by MSL (for 1 µg crude protein and 30 min of incubation time). Furthermore, MSL cleaved γ subunit in dose- and time-dependent manner. Quantitative correlation of these results was obtained by densitometric analysis. The caseinolytic activity of MSL was inhibited by IAA, PMSF. While, only PMSF inhibited fibrinogenolytic activity. **Conclusions:** MSL contains proteolytic enzymes belonging to two distinct superfamilies viz., serine protease and cysteine proteases. The fibrinogenolytic activity of MSL is restricted to serine proteases only. The study extrapolates the use of *M. spinosa* latex from milk curdling to hemostasis.

Key words: Cysteine protease, hemostasis, *Maclura spinosa* latex, moraceae, plecospermum spinosum, serine protease

SUMMARY

- Proteolytic enzymes present in latex of *Maclura spinosa* can be assigned to two different protease superfamilies viz., serine protease and cysteine protease as revealed by the inhibitory studies of caseinolytic activity. Among them, only serine protease can be considered as hemostatically significant as inhibition of fibrinogenolytic action of *Maclura spinosa* latex protease is shown only by PMSF, a serine protease-specific inhibitor.



Abbreviations used: MSL: *Maclura spinos* Latex, IAA: Iodo Acetic Acid, EDTA: Ethylene Diamine Tetra Acetic Acid, EGTA: Ethylene glycol tetra acetic acid, PMSF: Phenyl methyl sulphonyl fluoride.

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INTRODUCTION

Laticiferous cells, possessed by plants belonging to more than 40 families of Angiosperms, upon natural or artificial incision, exude cytoplasmic ingredients in the form of a sticky white fluid called latex.[1] This sticky emulsion comprises numerous compounds with heterogeneous chemistry ranging from highly hydrophobic resins, gums, oils, and tannins to contrastingly hydrophilic sugars, starch, alkaloids, and hydrolytic enzymes. Most of these compounds are reported to bear toxic properties like insecticidal, anti-microbial, etc., and latex thus serves as a potential defense tool for the host plant.[2] Among the hydrolytic enzymes, proteases exhibit remarkable protective role in host-pathogen interaction. Papain, a commercially well-known proteolytic enzyme purified from *Carica papaya* (*Caricaceae*) has been recently reported to show immense toxicity against herbivores insect *Samia ricini* (*Saturniidae*); two ill-famed pests, *Mamestra brassicae* (*Noctuidae*) and *Spodoptera litura* (*Noctuidae*);[3] and pathogenic fungi *Fusarium solani*.[4] Apart from serving the host, these proteases also favor innumerable human needs and accordingly find stupendous applications in food, leather, detergent, and pharmaceutical industries.[5] Further, proteases are widely employed to fulfill various requirements of biological research like peptide synthesis, nucleic acid purification, cell culturing, preparation of recombinant antibody fragments, structure-function relationship studies, peptide sequencing, etc.[6]

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Hemostasis accomplished by the formation of the insoluble fibrin clot is an important aspect of wound healing. Recently, the traditional practice of applying plant lattices on fresh wounds to stop bleeding has been scientifically validated proclaiming the involvement of proteolytic enzymes that act via activation of different zymogens involved in various steps of the coagulation cascade. For example, Ficin obtained from *Ficus carica* activates human factor Xa,[7] Pergularain eI purified from *Pergularia extensa* displays fibrinogenolytic activity.[8] Proteolytic enzymes of lattices thus serve as potential therapeutic tools that find applicability in inducing hemostasis and enhancing wound healing.

Maclura spinosa is a large woody, straggling, armed shrub belonging to family Moraceae widely found in Indian sub-continent.[9] The yellow resinous latex of the plant is reported to be used for curdling of milk by Kollamalayali tribes native to Tamil Nadu, South India.[10] The curdling activity is attributed to proteolytic enzymes, and these enzymes are known to possess vast therapeutic potentials.[11] The current study was taken up to examine whether the proteolytic enzyme(s) of the latex involved in milk curdling possess hemostatic property. Here, we report the presence of multiple proteases in the latex that belong to two different protein superfamilies viz. serine protease and cysteine protease. Further, the human fibrinogen degradation role observed is restricted to serine proteases of the latex.

MATERIALS AND METHODS

Materials

Trypsin I, human fibrinogen, specific protease inhibitors viz., iodoacetic acid (IAA) and phenylmethylsulfonyl fluoride (PMSF) were procured from Sigma-Aldrich Corporation (St. Louis, MO). Trypsin II was purchased from Hi-media Laboratories Pvt. Ltd., (Mumbai, India). All the chemicals like casein, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), ammonium persulfate etc., were from Sisco Research Laboratories Pvt. Ltd.(Mumbai, India).

Plant material

M. spinosa was collected from outskirts near Hemagangothri campus, University of Mysore, Hassan which is under the Forest Department, Government of Karnataka and was identified by Dr. P. Sharanappa, Assistant Professor, Department of Studies in Biosciences, Hemagangothri, A voucher specimen of the plant (PS/55/19.02.2012) was deposited at the herbarium of the Department of Studies in Biosciences, Hemagangothri, Hassan, India.

PREPARATION OF *MACLURA SPINOSA* LATEX CRUDE ENZYME EXTRACT

Freshly collected 5 mL of latex was diluted with equal volume of 10 mM phosphate buffer pH 7.0 and was subjected to repeated freezing (−20°C) and thawing followed by multiple centrifugation at 12,000 g for 20 min at 4°C. The clear aqueous solution thus obtained was dialyzed (MWCO 3 kDa) against same buffer overnight, to remove small components such as inorganic ions and phenolic compounds. This rules out the possible influence of metal ions such as Ca^{2+} and Mg^{2+} on enzyme activity and also sets aside the interference of phenolic compounds in protein estimation. The resultant 6.5 mL clear solution of latex extract (*Maclura spinosa* latex [MSL]) thus processed was used as an enzyme source for further assays.

Caseinolytic activity

Caseinolytic activity was assayed according to the method of Murata *et al.*[12] Casein 0.4 mL (2% in 0.2 M Tris-HCl buffer, pH 8.5) was incubated with different concentration (10–100 µg) of MSL, trypsin I and trypsin II at 37°C separately for 2.5 h. The reaction was stopped by adding 1.5 mL of 0.44 M trichloroacetic acid and allowed to stand for 30 min. The mixture was centrifuged at 1,500 g for 15 min. An aliquot of 1 mL of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of 1:2 diluted Folin reagent and the color 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/h at 37°C. Activity was expressed as units/h at 37°C.

Effect of specific protease inhibitors

The effect of different specific protease inhibitors viz., PMSF, IAA, EGTA, and EDTA on the activity of MSL was investigated using the same method as mentioned above with slight modification wherein the MSL was preincubated with specific protease inhibitors. The results were compared with a positive control that is an activity without the presence of inhibitors.

Human fibrinogenolytic activity

Fibrinogenolytic activity was measured according to the method of Ouyang and Teng.[13] The reaction mixture 40 µL contained 50 µg of human fibrinogen, 10 mM Tris-HCl buffer (pH 7.6) was incubated at 37°C with different concentrations of crude latex extract and for incubation time. The reaction was terminated by adding 20 mL of denaturing buffer containing 1M urea, 4% SDS, and 4% β-mercaptoethanol. The hydrolyzed products were analyzed by 10% SDS-polyacrylamide gel electrophoresis and protein pattern was visualized by staining with Coomassie brilliant blue R-250.

Inhibition of fibrinogenolytic activity

Inhibition studies were carried out by preincubating 5 µg of MSL (showing optimum activity) with effective concentrations of specific protease inhibitors. The further protocol for the fibrinogenolytic activity was same as mentioned above.

Densitometric analysis

The density of the bands obtained in all the resultant polyacrylamide gels of dose- and time-dependent fibrinogenolytic activity were analyzed using Image Lab software (version 2.0.1, Bio-Rad Laboratories). Graphs of the band percentage versus dose in µg/time in min were constructed.

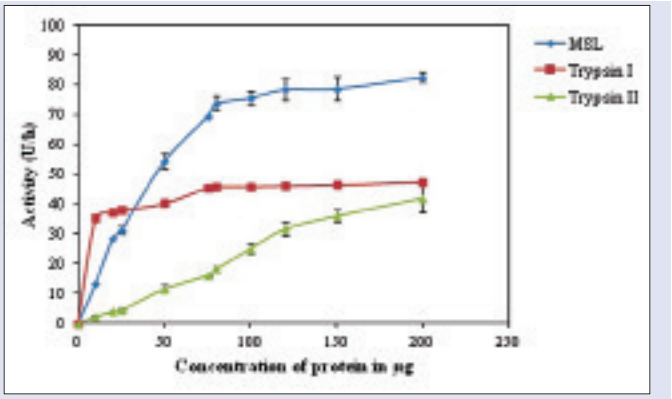

Figure 1: Caseinolytic activity of *Maclura spinosa* latex (blue) compared with trypsin I (red) and II (green) with increasing concentration from 0 to 250 µg. Activity was expressed as units/h at 37°C. All the values are mean ± standard deviation (n = 3). The comparison of the activities of *Maclura spinosa* latex, trypsin I, and trypsin II was statistically significant ($P < 0.05$)

Statistical analysis

Data were analyzed using GraphPad Prism 6.01 statistical software Prism 6 for Windows (version 6.01, GraphPad software). All the experiments were conducted in triplicates. All the values were represented as mean ± standard deviation. One-way ANOVA was conducted to analyze the significance of the data.

RESULTS

MSL hydrolyzed casein in a dose-dependent manner. The caseinolytic activity of MSL was compared with two standard proteolytic enzymes trypsin I and trypsin II. MSL showed very high efficiency of proteolytic action than both trypsin I (Sigma-Aldrich) and trypsin II (Hi-media) [Figure 1]. The specific activity of crude was found to be 1,080 units/mg protein. Inhibition of caseinolytic activity of MSL was observed with two specific protease inhibitors IAA and PMSF [Table 1]. MSL was inhibited up to 96.01 ± 0.40% by IAA and up to 75.76 ± 2.55% by PMSF. EDTA and EGTA had no inhibitory effect on MSL. Final concentrations of all inhibitors were maintained to 5 μM in the reaction mixture. Human fibrinogen was hydrolyzed by MSL, which was observed using poly acryl amide gel electrophoresis under denaturing conditions. Aα and Bβ bands of human fibrinogen were cleaved by MSL by concentration as low as 1 μg of crude protein. Cleavage of γ subunit of human fibrinogen by MSL was observed in dose- and time-dependent manner [Figures 2a and 3a]. Densitometric analysis of γ subunit of human fibrinogen revealed a decrease in band percentage from 42.2% to 0% for increasing concentrations 0–5 μg [Figure 2b]. With increase in time, the band percentage of γ subunit was decreased from 25.8% to 5.1% for incubation time between 0–3 h [Figure 3b]. As Aα and Bβ bands were not visible, they were not subjected to densitometric analysis. Fibrinogenolytic activity was inhibited by PMSF (5 μM) and not by IAA (100 μM), EDTA (5 μM), EGTA (5 μM). As the inhibition was evident in the gel [Figure 4], the inhibitory studies were not subjected to densitometric analysis.

Table 1: Effects of specific inhibitors on caseinolytic activity of MSL

Inhibitors	Percentage of inhibition
W/O inhibitor	0
EDTA	0
EGTA	0
IAA	96.01±0.40
PMSF	75.76±2.55

EGTA: Ethylene glycol tetraacetic acid; EDTA: Ethylenediaminetetraacetic acid; IAA: Iodoacetic acid; PMSF: Phenylmethylsulfonyl fluoride; MSL: *Maclura spinosa* latex

DISCUSSION

More than 25,000 species of the plants belonging to 40 different families exude natural latex. Among these, the hemostatic roles of the plants belonging to families Apocynaceae, Asclepiadaceae, and Euphorbiaceae are well-studied.[14] Remote plant species, especially those which are not familiar in traditional knowledge stay away from the focus of researchers. Hence, such a plant *M. spinosa* belonging to family Moraceae was chosen for the study.

Proteolytic activities of latex were examined using casein as a substrate. Since the *in vitro* caseinolytic assay is carried out in aqueous media the

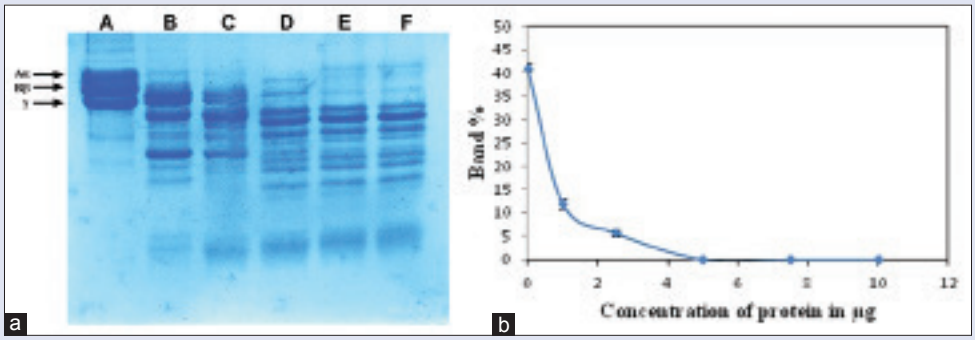

Figure 2: (a) Dose-dependent fibrinogenolytic activity of *Maclura spinosa* latex crude extract. Different protein concentrations of *Maclura spinosa* latex ranging from 1 to 10 μg were incubated with 50 μg of human fibrinogen at 37°C for 3 h in the presence of 10 mM Tris-HCl buffer (pH 7.6). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) was performed to visualize the fibrinogen degradation pattern. (A) Control (B) 1 μg (C) 2.5 μg (D) 5 μg (E) 7.5 μg (F) 10 μg of *Maclura spinosa* latex. (b) Densitometric analysis of γ bands obtained in electrophoretogram of the dose-dependent fibrinogenolytic assay. Band percentage versus concentration of *M. spinosa* latex was done. All the values represent mean ± standard deviation ($n = 3$)

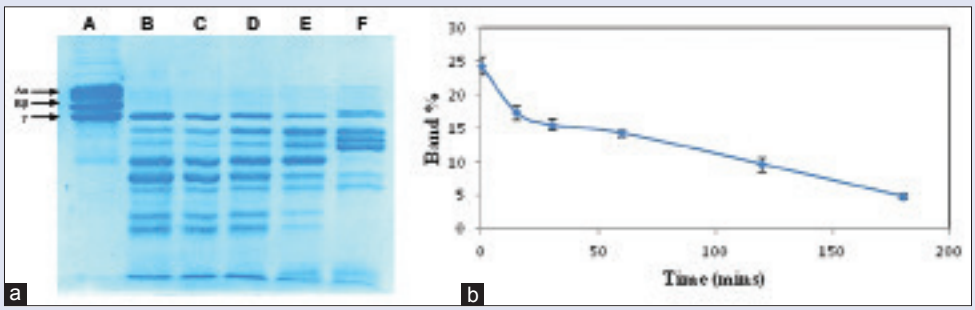

Figure 3: (a) Time-dependent fibrinogenolytic activity of *Maclura spinosa* latex crude extract. *Maclura spinosa* latex (5 μg) was incubated with 50 μg of human fibrinogen at 37°C for different incubation time ranging from 0 to 3 h in the presence of 10 mM Tris-HCl buffer (pH 7.6). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) was performed to visualize the fibrinogen degradation pattern. (A) Control (B) 15 min (C) 30 min (D) 1 h (E) 2 h (F) 3 h. (b) Densitometric analysis of γ bands obtained in electrophoretogram of time-dependent fibrinogenolytic assay. Band percentage versus time (min) was done. All the values represent mean ± standard deviation ($n = 3$)

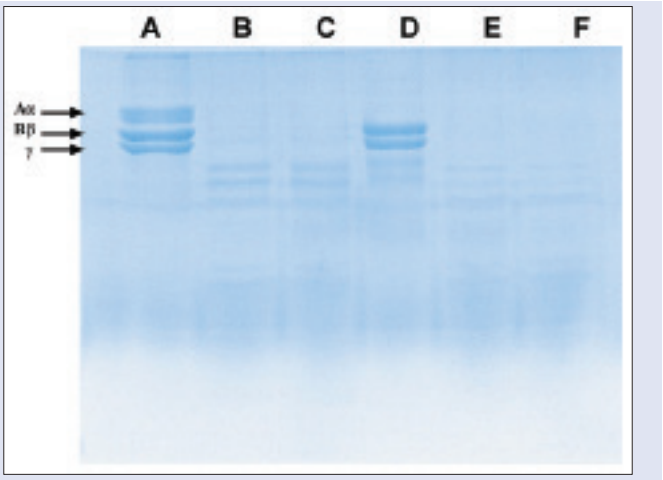

Figure 4: Inhibition of the human fibrinogenolytic activity of *Maclura spinosa* latex crude by specific protease inhibitors. *Maclura spinosa* latex 5 μg was preincubated with and without specific protease inhibitors for 15 min in the presence of 10 mM Tris-HCl buffer (pH 7.6) and the reaction was initiated by adding 50 μg of fibrinogen. After 3 h, the reaction was terminated by adding a denaturing buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) was performed to visualize the inhibition pattern of fibrinogen degradation. (A) Fifty micrograms of fibrinogen, (B) 50 μg fibrinogen + 5 μg *Maclura spinosa* latex, (C) 50 μg fibrinogen + 5 μg *Maclura spinosa* latex + 100 μM iodoacetic acid, (D) 50 μg fibrinogen + 5 μg *Maclura spinosa* latex + 5 mM phenylmethylsulfonyl fluoride, (E) 50 μg fibrinogen + 5 μg *Maclura spinosa* latex + 5 mM ethylenediaminetetraacetic acid, (F) 50 μg fibrinogen + 5 μg *Maclura spinosa* latex + 5 mM ethylene glycol tetraacetic acid

insoluble fraction of the latex had to be removed. The possible influence of metal ions such as Ca^{2+} and Mg^{2+} on enzyme activity and the interference of phenolic compounds in protein estimation were avoided by dialyzing the processed latex. MSL hydrolyzed casein in a dose-dependent manner which was found to be statistically significant. Crude MSL shows remarkably greater activity than purified proteases confirming the existence of efficient proteases with extremely promising applicability potential. Furthermore, purification and characterization of protease(s) are in progress. In order to categorize the protease(s), inhibition studies were conducted using various specific protease inhibitors viz., IAA, PMSF, EDTA, and EGTA. The caseinolytic activity of MSL was inhibited by two inhibitors IAA and PMSF while EDTA and EGTA had no effect. IAA inhibited up to 96.01 ± 0.40%, and PMSF showed 75.76 ± 2.55% inhibition compared to an activity control containing the same amount of crude protein. The data suggest the presence of protease(s) belonging to two varied classes viz., cysteine proteases and serine proteases.

Hemostasis is a complex physiological defense response shown by the mammalian system against vascular tissue damage. It is seen as a result of stepwise activation of inactive precursors into functional forms. Proteolytic enzymes play a crucial role at different steps of the coagulation cascade. The convergence of intrinsic and extrinsic pathways with Ca^{2+} ions leads to the formation of prothrombinase complex that activates prothrombin to thrombin. Fibrinogen, a heterodimer consisting of Aα, Bβ, and γ subunits acts as an ultimate substrate of the coagulation cascade, which is acted upon by thrombin to convert it into an insoluble fibrin clot.[15] This collective hemostatic process also acts as a key aspect in wound healing.[16] Hence, proteolytic enzymes with thrombin-like action attract keen interests because of their therapeutic potential in hemostasis and wound healing. Hence, to examine if the proteolytic enzymes of latex have hemostatic potential, its action on human fibrinogen was studied. MSL showed fibrinogenolytic activity both in dose dependent and time dependent manner. Aα and Bβ sub units were highly susceptible for the action of MSL. Degradation of Aα and Bβ was observed for concentrations as low 1 μg of crude protein and incubation time as less as 30 min. Although γ subunit showed resistance to some extent higher concentrations of MSL were successful in cleaving even the γ subunit. Furthermore, even the protein concentration of MSL as low as 5 μg upon prolonged incubation cleaved γ sub unit. To substantiate the fibrinogenolytic activity of MSL observed through visualization of bands in polyacrylamide gel electrophoresis, the bands were subjected to densitometric analysis. This gives a quantitative measure of the extent of hydrolysis of γ band by MSL. Band percentage of γ subunit was extensively decreased from 42.2% to 0% in dose-dependent assay and from 25.8% to 5.1% in time-dependent assay. Inhibition studies of the fibrinogenolytic activity of MSL revealed surprising results. Proteolytic enzymes belonging to only one class out of two classes identified through caseinolytic inhibition assay displayed fibrinogenolytic activity. Since PMSF exclusively inhibited the fibrinogenolytic activity of MSL, the underlying mechanism can be attributed to proteases belonging to serine protease super family. Cysteine proteases even though present in MSL do not possess fibrinogenolytic activity as suggested by the absence of inhibitory action by IAA. Thus, it can be concluded that two classes of proteases viz., cysteine protease(s) and serine protease(s) are present in MSL and both of these possess casienolytic activity. MSL also possess fibrinogenolytic activity which is restricted to serine protease(s). Our study explores the hemostatic potential of protease(s) of *M. spinosa* latex. The high efficiency of MSL compared to standard proteases compels us to address them with a biochemical and pharmacological perspective. To ascertain the application of MSL proteases in wound healing, further purification and characterization are under progress.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Lewinsohn TM. The geographical distribution of plant latex. Chemoecology 1991;2:64-8.
2. Ravi KU. Plant latex: A natural source of pharmaceuticals and pesticides. Int J Green Pharm 2011;5:169-80.
3. Konno K, Hirayama C, Nakamura M, Tateishi K, Tamura Y, Hattori M, *et al.* Papain protects papaya trees from herbivorous insects: Role of cysteine proteases in latex. Plant J 2004;37:370-8.
4. Souza DP, Freitas CD, Pereira DA, Nogueira FC, Silva FD, Salas CE, *et al.* Laticifer proteins play a defensive role against hemibiotrophic and necrotrophic phytopathogens. Planta 2011;234:183-93.
5. Tomar R, Kumar R, Jagannadham MV. A stable serine protease, wrightin, from the latex of the plant *Wrightia tinctoria* (Roxb.) R. Br: Purification and biochemical properties. J Agric Food Chem 2008;56:1479-87.
6. Mótyán JA, Tóth F, Tozsér J. Research applications of proteolytic enzymes in molecular biology. Biomolecules 2013;3:923-42.
7. Richter G, Schwarz HP, Dorner F, Turecek PL. Activation and inactivation of human factor X by proteases derived from *Ficus carica*. Br J Haematol 2002;119:1042-51.
8. Shivaprasad HV, Rajaiah R, Frey BM, Frey FJ, Vishwanath BS. 'Pergularain e I' – A plant cysteine protease with thrombin-like activity from *Pergularia extensa* latex. Thromb Res 2010;125:e100-5.

9. Rajamanoharan PR. An ethno botanical survey of medicinal plants in Sillalai, Jaffna, Northern Province, Sri Lanka. Int J Herb Med 2014;1:22-30.
10. Geetha S, Lakshmi G, Ranjithakani P. An ethnic method of milk curdling using plants. Anc Sci Life 1996;16:60-1.
11. Craik CS, Page MJ, Madison EL. Proteases as therapeutics. Biochem J 2011;435:1-16.
12. Murata J, Satake M, Suzuki T. Studies on snake venom. XII. Distribution of proteinase activities among Japanese and Formosan snake venoms. J Biochem 1963;53:431-43.
13. Ouyang C, Teng CM. Fibrinogenolytic enzymes of *Trimeresurus mucrosquamatus* venom. Biochim Biophys Acta 1976;420:298-308.
14. Rajesh R, Shivaprasad HV, Gowda CD, Nataraju A, Dhananjaya BL, Vishwanath BS. Comparative study on plant latex proteases and their involvement in hemostasis: A special emphasis on clot inducing and dissolving properties. Planta Med 2007;73:1061-7.
15. Kulman JD, Davie EW. Proteases in blood clotting. In: Lennarz WJ, Lane MD, editors Encyclopedia of Biological Chemistry. Vol. 3. Oxford: Elsevier; 2004. p. 457-63.
16. Yariswamy M, Shivaprasad HV, Joshi V, Nanjaraj Urs AN, Nataraju A, Vishwanath BS. Topical application of serine proteases from *Wrightia tinctoria* R. Br. (*Apocyanaceae*) latex augments healing of experimentally induced excision wound in mice. J Ethnopharmacol 2013;149:377-83.


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