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COMPARATIVE STUDIES ON ANTICOAGULANT ENZYME FROM PLANT LATEX SAMPLES

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ABSTRACT

Plant latex is composed of organic, inorganic and hydrolytical enzymes and is a source of proteases which belongs to cysteine or serine super family. It also exhibits many pharmacological properties used in folk medicines and are involved in haemostasis. In present study, latex collected from eleven plant samples such as *Calotropis gigantea, Croton bonplandianum, Ficus benghalensis, Ficus carica, Synadenium umbellatum, Nerium oleander, Artocarpus heterophyllus, Manilkara zapota, Ficus religiosa, Carica papaya* and *Plumeria acutifolia* to evaluate the best source for anticoagulant enzyme. Among the eleven samples three samples gave the highest anticoagulant activity confirmed by blood clotting and milk clotting activity. These three samples were subjected for purification by 3 step method such as salt precipitation & dialysis, ion exchange chromatography and gel filtration chromatography. The fold purification and percentage yield of plants are 6.33 and 34.04 for *Croton banplandianum,* 54.83 and 86.69 for *Carica papaya,* 22.35 and 66.77 for *plumeria acutifolia* respectively. These enzymes show optimum pH at 7, Temperature at 40°C, Incubation time for 10 min and Substrate concentration activity increases till 9 mM. The molecular weight of purified protease enzyme of *Croton bonplandianum, Carica papaya* and *Plumeria acutifolia* are ~35kDa, ~25kDa and ~34kDa respectively detected by SDS PAGE. Purified enzymes subjected for immobolization, the stability of enzymes of *Croton bonplandiaum* and *Plumeria acutifolia* found to be high in Polyurethane method and that of *Carica papaya* found to be high in Polyacrylamide method. In APTT test, clotting time of citrated plasma for control is 32±2 sec and there was no clotting for *Croton bonplandiaum*, *Carica papaya* and *Plumeria acutifolia*.

KEYWORDS: Plant latex, Anticoagulant, Chromatography, SDS PAGE, Blood clotting.

1. INTRODUCTION

Plant latex is composed of organic, inorganic compounds,[1] and many hydrolytic enzymes,[2] such as protease, esterases and lipases which act as anticoagulant enzymes.[3,4] One of the reason behind plants resistance towards infectious diseases is presence of hydrolytic enzymes especially protease.[5] Multiple proteases are present in plant latex which belongs to cysteine or serine super family.[6,7] Plant latex also exhibit many pharmacological properties used in folk medicine,[8] and are involved in haemostasis.[9,10] Anticoagulant enzymes are used strokes, transient ischaemic attacks (TIAs), heart attack, deep vein thrombosis (DVT), pulmonary embolism etc.[11]

Proteases in mammals involved in the activation of fibrinolysis,[12] degrade unwanted proteins,[13] zymogens activation,[14] angiogenesis, tissue proliferation, wound healing, atherosclerosis,[15] and myocardial infarction.[16] Proteases effecting fibrinolysis are present in snake venoms,[17,18] leeches,[19] insects,[20] algae,[21] and plants latex of *Carica papaya* has also shown the anticoagulant factor.[22]

There are many plant sources for anticoagulant enzymes such as *Calotropis gigantea, Croton bonplandianum, Ficus benghalensis, Ficus carica, Synadenium umbellatum, Nerium oleander, Artocarpus heterophyllus, Manilkara zapota, Ficus religiosa, Carica papaya* and *Plumeria acutifolia*. Plant latex with anticoagulant property are having high importance in comparison to currently present drugs as they do not have any side effects.[23] There is more research on anticoagulant enzyme from microbes and animal tissue but some of the reviews says that the plant latex also produces the anticoagulant enzyme. Less work has been done on latex anticoagulant enzymes but many plants produce the latex, so for that reason the present study is to evaluate the anticoagulant enzyme by purification and characterization from 11 plant latex samples for the best plant species.

2. MATERIALS AND METHODS

Plant Latex Samples

The latex of plants, *Calotropis gigantea, Croton bonplandianum, Ficus benghalensis, Ficus carica, Synadenium umbellatum, Nerium oleander, Artocarpus heterophyllus, Manilkara zapota, Ficus religiosa, Carica papaya* and *Plumeria acutifolia*, collected from Gandhi Krishi Vignana Kendra Bangalore, Karnataka.

Screening of Proteolytic and Anticoagulant Activity

Screening of proteolytic activity done by adding a drop of fresh latex sample on a thin layer of milk. The zone formation was observed and compared with the control plate (drop of water). This is to find out the proteolysis of casein by the plant latex. The blood coagulation time of two adult volunteer was noted by adding 50µL of whole latex sample into 100µL of fresh blood to determine the anticoagulant activity.

Protease Assay

Enzyme activity of plant latex determined by protease assay.[24] Casein solution (0.65g in 100mL of 50mM phosphate buffer) incubated for 5min. After incubation 0.5mL of the sample added to 5mL of casein solution and incubated at 37°C for 10min. The solution treated with 5mL of TCA and incubated at 37°C for 30min. Filtrate (2mL) was taken and added 5mL of 0.5M Na_2CO_3 along with 1mL of FC reagent. Incubated at 37°C for 30min. The absorbance of filtrate read at 660nm. To calculate the specific activity protein was estimated by Lowry's method.[25]

Purification of Anticoagulant Enzymes

Salt precipitation: Salt precipitation was carried out.[26] Dried sample dissolved in 10mM tris HCl and centrifuged at 6000rpm for 15min to remove the impurities. Supernatant was taken and saturated to 70% by adding ammonium sulphate in ice cold condition by stirring. Incubated at 4°C for 1hour. After incubation centrifuged at 10000rpm for 10min and pellet was dissolved in 10mM Tris HCl (pH 7).

Dialysis: Cellulose acetate membrane with a molecular weight cut off 8kDa was used for the dialysis. To activate the dialysis membrane boiled in a 100mL water for 10min and replaced with Na_2HCO_3 and boil again for 10min. The membrane transferred to another beaker and boiled for 15-20min then allowed to cool down. Tied the membrane from one side and the sample was added. Other side of the membrane was tied without any leakage. Incubated overnight in ice cold condition. The process was continued with changing 5mM buffer for three times in half an hour interval and samples were taken for ion-exchange chromatography.

Ion exchange chromatography: Activated Anion exchanger matrix DEAE cellulose poured into the column and allowed to settle. Six elution buffer of different concentration such as 25mM, 50mM, 75mM, 100mM, 125mM and 150mM was prepared using NaCl and 25mM

tris HCl. Volume made up to 5mL with distilled water. Dialyzed samples were poured into the column and eluted by using elution buffer.

Gel filtration chromatography: Eluted sample with highest activity from ion-exchange chromatography was used in gel filtration chromatography.[27] The column was filled with SEPHADEX G75 pore size and allowed to settle. Sample was loaded onto SEPHADEX G75 column and eluted 1mL per 5min by using 100mM phosphate buffer. Protease activity was determined for each elution and protein elution was monitored at 280nm using spectrophotometer to calculate specific activity.

Enzyme Kinetics

Enzyme kinetics was carried out for pH, substrate concentration, incubation time and temperature.[28] The optimum pH was determined by protease activity at different pH range such as 4, 5, 6, 7, 8, 9 and 10. Acetate buffer of pH 4, 5 and 6 was prepared by adding 50mL of 0.2M acetic acid to 50mL of 0.2M sodium acetate. For pH 7 and 8, phosphate buffer was prepared by mixing 50mL of 0.2M KH_2PO_4 and 50mL of 0.2M K_2HPO_4 and for pH 9 and 10, glycine buffer was prepared by mixing 50mL of 0.2M glycine and 50mL of 0.2M NaOH. Protease activity was determined for different substrate concentration such as 1mM, 3mM, 5mM, 7mM, 9mM, 11mM, 13mM and 15mM. The optimum incubation time was determined at different time period such as 5, 10, 15, 20 and 25min and optimum temperature was determined at different temperature such as 20°C, 30°C, 40°C, and 50°C.

Molecular Weight Determination

SDS-PAGE was carried,[29] by using 12% separating gel and 4% stacking gel. Gel was loaded with mixture of 50µL sample and 30µL loading dye. The gel was run about 70% and bands were visualized by staining with Coomassie brilliant blue solution. The molecular weight was determined by comparing protein bands with Novex* Sharp Pre Gel protein standard.

Immobilization

Entrapment by sodium alginate method

Immobilization by alginate method carried out,[30] with slight modification. Mix 5mL of 3% sodium alginate with 0.5mL of enzyme, beads were made by using 0.2M calcium chloride. The protease activity of immobilized enzyme was calculated. Beads without enzyme was used as blank. The immobilized enzyme was stored for about ten days and the assay was repeated again to determine the stability of immobilized enzymes.

Entrapment by polyacrylamide gel

This method was carried out,[31] with 15% polyacrylamide gel, prepared by dissolving 7.5g acrylamide, 0.5g bisacrylamide and 50mg ammonium per sulphate in 25ml of phosphate buffer (pH-6.8). Mix 2.5ml of polyacrylamide gel and 1ml of enzyme, layered it on the Petri plate and was cut into pieces. The protease activity of immobilized enzyme was calculated. Polyacrylamide gel without enzyme used as blank. The acrylamide immobilized enzyme was preserved for ten days and stability was determined.

Entrapment by polyurethane

This method was carried out,[32] with slight modification. Pre-cooled 2g of urethane polymer was mixed with 2ml chilled phosphate buffer (pH-5) along with 1ml of enzyme and incubated at room temperature. After polymerization and formation of foam the gel was rinsed with the buffer. The gel was cut into small pieces and used as immobilized enzyme samples and the protease activity was determined. The immobilized enzyme was preserved for ten days and assay was performed again to determine the stability.

Confirmatory Test by Activated Partial Thromboplastin time (APTT)

The modified method,[33] was used for the APTT tests. The partial thromboplastin with activator and calcium chloride (0.02M) were pre-warmed at 37°C separately in a water bath. 50µL of plasma was placed in a test tube and incubated for 3min in the water bath. 50µL of partial thromboplastin was added and the contents were mixed rapidly. The mixture was then incubated for another 3minutes, after which 50µL of PBS along with 50µL of the pre-warmed calcium chloride solution was added while simultaneously starting a timer. The tube was then allowed to remain in the water bath while gently tilting the test tube every 5sec. At the end of 20sec, the test tube was removed from the water and gently tilted back and forth until a clot was seen and the time was recorded. For the test samples, 50µL of the material was added to the contents of the test tube just prior to the addition of calcium chloride, and the time was recorded.

3. RESULTS AND DISCUSSION

Screening of Proteolytic and Anticoagulant Activity

Anticoagulation depends on the proteolysis of coagulant factors,[34] as in fibrinolysis.[12] Proteolytic activity of plant latex samples was determined by proteolysis of casein protein, as casein is a suitable substrate for hydrolytic enzyme like protease,[35] where zone formation in thin layer of milk indicated the degradation of casein protein by hydrolytic enzyme and blood

clotting time was noted for all samples by using 50µL of whole latex in 100µL of blood to determine the anticoagulant property, while for control 50 µL of phosphate buffer was used in blood. *Calotropis gigantia, Croton bonplandianum, Ficus benghalensis, Ficus carica, Ficus religiosa, Artocarpus heterophyllus, Carica papaya and Plumeria acutifolia* showed the positive result in proteolytic activity whereas only *Croton bonplandianum, Carica papaya* and *Plumeria acutifolia* showed high anticoagulant activity with blood clotting time of more than 24hours for *C. bonplandianum* and *C. papaya*, while *P. acutifolia* blood clotting time was 12hours ±30min. Blood clotting time of adult volunteers without latex was 6min 23sec ±2sec. Rest of the plant latex samples did not show any significant anticoagulant activity.

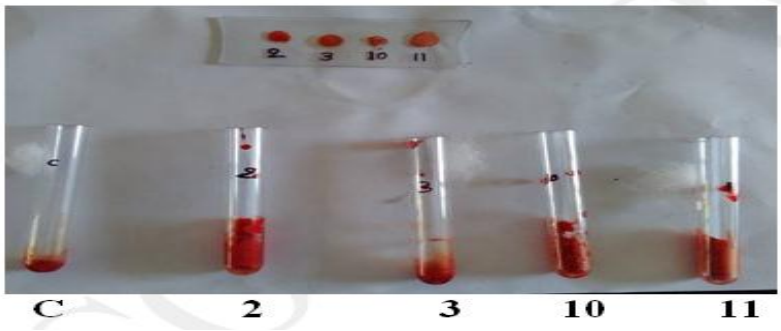


Fig. 1: Anticoagulant activity by samples 2(*Croton bonplandianum*), 10(*Carica papaya*) and 11 (*Plumeria acutifolia*) against C (Control).

Ficus benghalensis, Ficus carica, Ficus religiosa and Artocarpus heterophyllus has degraded the casein protein due to the presence of protease in their latex, this is in accordance with result reported by.[36] Proteolytic activity by *Carica papaya, Ficus carica and Calotropis gigantea* has also reported by several researchers.[37,38,39,40] *Plumeria acutifolia* have shown proteolytic activity in accordance with the result reported by.[41] Less work has been done on *Croton bonplandianum* proteolytic activity, while in present study it has shown the casein degradation in milk layer which evince the presence of protease in its latex. *Carica papaya* have shown the high anticoagulant activity as hydrolytic enzyme (Protease) degraded the coagulant factor in blood and similar result was also reported by.[22] Whereas for *Plumeria acutifolia* not much attention has given for anticoagulant activity, however another species of same genus i.e. *Plumeria rubra* has shown the protease effect on clotting factors.[41] *Croton bonplandianum* have shown the wound healing property which is in accordance with the result reported by,[42] although in present study it has shown anticoagulant activity. The concentration of latex used might be the reason of appearance of both properties as it has reported in case of *Jatropa carcus*.[43]

Protease Activity of Anticoagulant Enzymes

Protease activity and protein estimation was done by using dried latex samples. Standard values were estimated to calculate the protein content and the enzyme activity for the evaluation of active enzyme content present in the latex samples. Protease activity is the enzymatic hydrolysis of peptide bond which is preferred over chemical method as it avoids the L-amino acid degradation and formation of toxic substances.[44,45]

Croton bonplandianum with the protease activity 305.08UmL^{-1} showed the presence of high active enzyme content in its latex in comparison to highest activity by *Carica papaya* (312.26UmL^{-1}). *Carica papaya, Ficus carica and Calotropis gigantea* has shown the high protease activity as well, which was also reported by several researchers.[37,38,39,40] *Ficus benghalensis, Ficus religiosa and Artocarpus heterophyllus* did not show considerable amount of protease activity because casein is not ideal for these latex as explained by.[36] Whereas *Ficus benghalensis* have shown protease activity in acidic condition according to,[36] but in the present study assay was carried out at pH 7. *Synadenium umbellatum, Nerium oleander* and *Manilkara zapota* did not show any significant amount of protease activity. *Plumeria acutifolia* also have not shown considerable amount of protease activity in comparison to *Carica papaya* but due to the presence of protease activity as well as anticoagulant activity, it has taken further for purification. Based on the results of anticoagulant activity and protease activity, three samples were selected namely, *Carica papaya, Croton bonplandianum* and *Plumeria acutifolia* for further study.

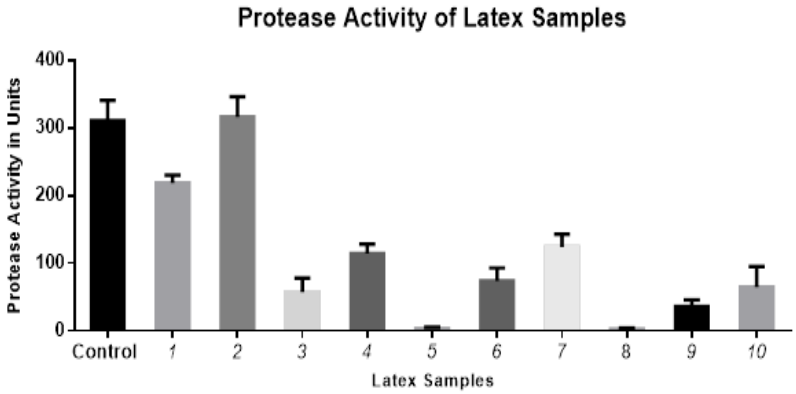


Fig. 2: Protease activity of 11 plant latex samples.

Note: Control-*Carica papaya,* 1-*Calotropis gigantea,* 2-*Croton bonplandianum,* 3-*Ficus benghalensis,* 4-*Ficus carica,* 5-*Synadenium umbellatum,* 6-*Nerium oleander,* 7-*Artocarpus heterophyllus,* 8-*Manilkara zapota,* 9-*Ficus religiosa,* and 10-*Plumeria acutifolia*.

Purification of Anticoagulant Enzymes

Latex samples of *Croton bonplandianum, Carica papaya* and *Plumeria acutifolia* were taken for purification. Purification was done by four steps method i.e. Salt precipitation, Dialysis, DEAE cellulose anion exchange chromatography and sephadex G-75 column gel filtration to remove the unwanted proteins or enzymes from the latex samples and to obtain the purified protease enzyme based on their charge and size.

The elution profile of the dialysed fraction from DEAE-cellulose column by linear gradient of NaCl (25-150mM) shows good recovery of activity yield in elution buffer 3 for *Croton bonplandianum* and *Plumeria acutifolia* while in elution buffer 2 for *Carica papaya*. elution fraction showed enzyme activity. The samples were eluted in Sephadex G-75 column at the rate of 1mL/5min and the protease activity of the eluted samples exhibited peak in fraction 6 for *Croton bonplandianum*, fraction 14 for *Carica papaya* and fraction 5 for *Plumeria acutifolia* at 280nm absorbance, spectrophotometrically. Table 1 summarizes the results of the purification of protease from latex of the plant.

Table. 1: Purification table.

Samples	Fractions	Protease Activity (UmL^{-1})	Protein ($mgmL^{-1}$)	Specific Activity (Umg^{-1})	Fold Purification	Percentage Yield (%)
Croton bonplandianum	Crude	392.27	0.558	702.99	1	100
	Salt ppt.	28.01	0.432	64.83	0.092	7.14
	Dialysis	54.79	0.289	189.58	0.269	13.96
	*IEC	79.38	0.098	810	1.15	20.23
	*GFC	133.56	0.03	4452	6.33	34.04
Carica papaya	Crude	294.83	0.506	582.66	1	100
	Salt ppt.	239.10	0.383	624.28	1.07	81.09
	Dialysis	215.44	0.218	988.25	1.69	73.07
	*IEC	220.73	0.172	1283.31	2.2	74.86
	*GFC	255.60	0.008	31950	54.83	86.69
Plumeria acutifolia	Crude	63.2	0,269	234.94	1	100
	Salt ppt.	7.47	0.052	143.65	0.61	11.81
	Dialysis	35.8	0.047	761.7	3.24	56.64
	*IEC	38.6	0.024	1608.33	6.84	61.07
	*GFC	42.2	0.008	5252.5	22.35	66.77

Note: *IEC – Ion-exchange chromatography *GFC – Gel filtration chromatography

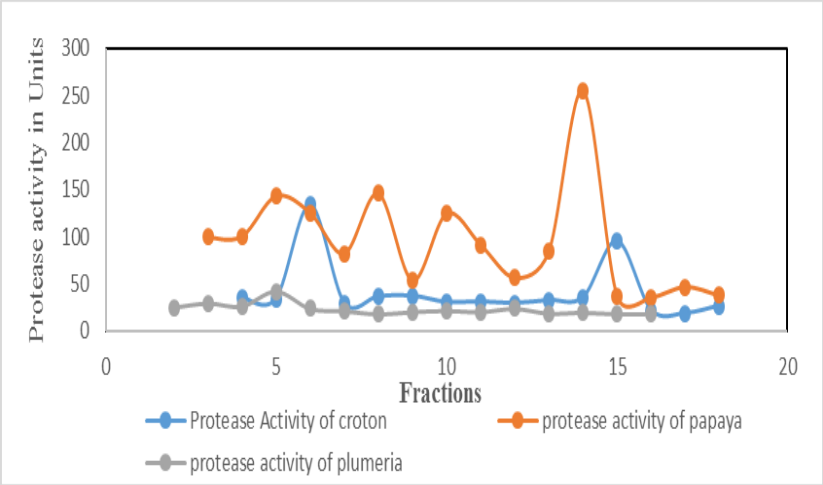


Fig. 3: Protease activity of G-75 column eluted purified enzyme samples.

The highest activity yield was given by *Carica papaya* purified enzyme sample as compared to *Croton bonplandianum* and *Plumeria acutifolia* whereas fold purification was highest in *Plumeria acutifolia* purified enzyme sample. The fold purification and percentage yield of enzyme (*Croton bonplandianum*) was found to be 6.33 and 34.04%, whereas for *Carica papaya* it was 5.48 and 86.69% respectively after gel filtration. The results are in accordance with the report for *Synadenium grantii* with fold purification of 4.6 and activity yield of 59.7%,[14] while another result reported for *Euphorbia hirta* with fold purification of 3.39 and activity yield of 38.4%.[46] Fold purification and percentage yield of enzyme (*Plumeria acutifolia*) was 22.35 and 66.77% respectively after gel filtration. The similar result was reported for *Codium latum* (green algae) with fold purification of 29 and activity yield of 51% for protease. [21] Another similar result of 31% of activity yield in *Synadenium grantii* was reported.[47] In contrast to our result, two similar results was reported by,[48,49] for different proteases.

Enzyme Kinetics

Enzyme kinetics was carried out for pH, substrate concentration, incubation time and temperature,[28] to examine the effect of different parameters on enzyme activity and to find out the optimum conditions under which enzymes acts with utmost efficiency.

Effect of pH: An important factor which effect enzyme activity significantly is pH. Protease assay of anticoagulant enzyme was carried out at different pH *viz.* 4, 5, 6, 7, 8, 9 and 10. Highest activity by protease enzyme of all the three samples was at pH 7. Lowest activity was shown at pH 4 and pH 10. The result signifies that protease enzymes are active at wide range

of pH (4 to 10). The results are in accordance with result reported in the case of *Euphorbia hirta*,[46] and in the case of *Synadenium grantii* with highest protease activity at pH 7.[14] Similar result was reported for *Plumeria rubra*.[50]

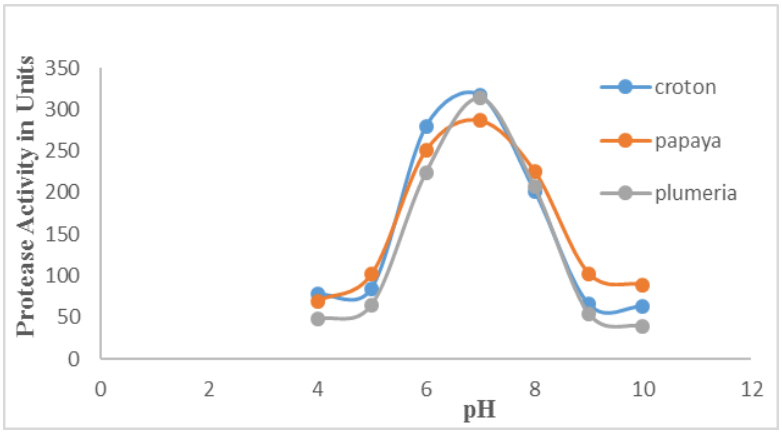


Fig. 4: Effect of pH on protease activity.

Effect of temperature: The enzyme assay was carried out at different temperature such as 20°C, 30°C, 40°C and 50°C. Highest activity by protease enzyme of each sample was at 40°C. Lowest activity was at 20°C and after 50°C enzyme activity decreases. Temperature is an important environmental factor for the normal action of an enzyme as it influences physiological changes in living cells.[51] The results are in accordance with result reported in the case of *Euphorbia hirta* with high protease activity at temperature ranging from 40°C to 55°C.[46] Similar result was reported for *Plumeria rubra*.[50]

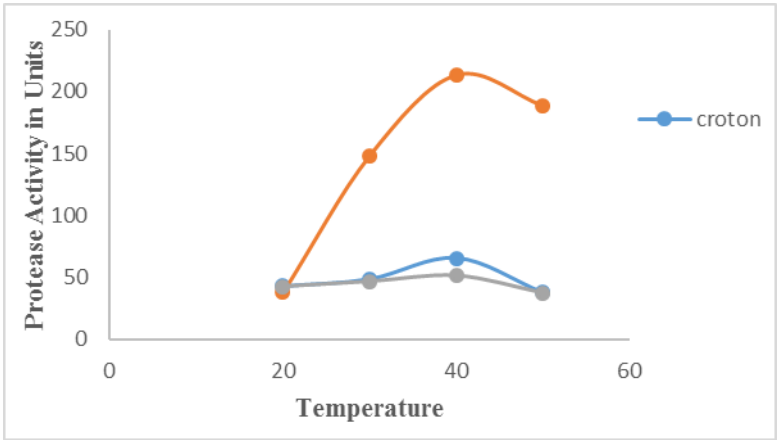


Fig. 5: Effect of temperature on protease activity.

Effect of incubation time: The enzyme activity of each sample was studied for 5min, 10min, 15min, 20min and 25min incubation time. There was activity increase from 5min to 15min of incubation, on further incubation the activity was decreased considerably. Highest activity

was at 10min of incubation. Decrease in the activity on further incubation is may be because of change in pH as product is formed.[52] The results are in accordance with result reported in the case of snake venom,[18] and in the case of *Synadenium grantii* with highest protease activity at 10 to 15min.[14]

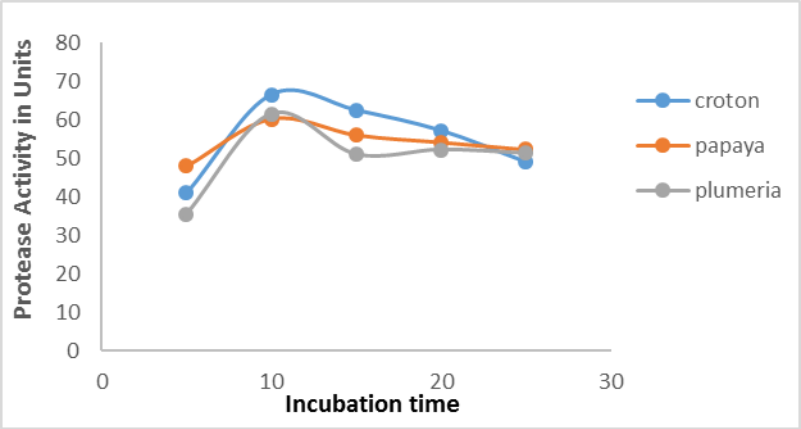


Fig. 6: Effect of incubation time on protease activity.

Effect of substrate concentration: Protease assay of protease enzyme was carried out in different substrate concentration of 1mM, 3mM, 5mM, 7mM, 9mM, 11mM, 13mM and 15mM. Enzyme activity of each sample increased with the increase in the substrate concentration till 9mM concentration, after that activity becomes constant. The result suggests that at an optimum concentration all enzymes are attached to their substrate due to which enzyme activity remains constant on further increase in the substrate concentration as there is no enzyme left to act on substrate. The results are in accordance with result reported in the case of *Synadenium grantii* where protease activity increases with the increase in substrate concentration.[14]

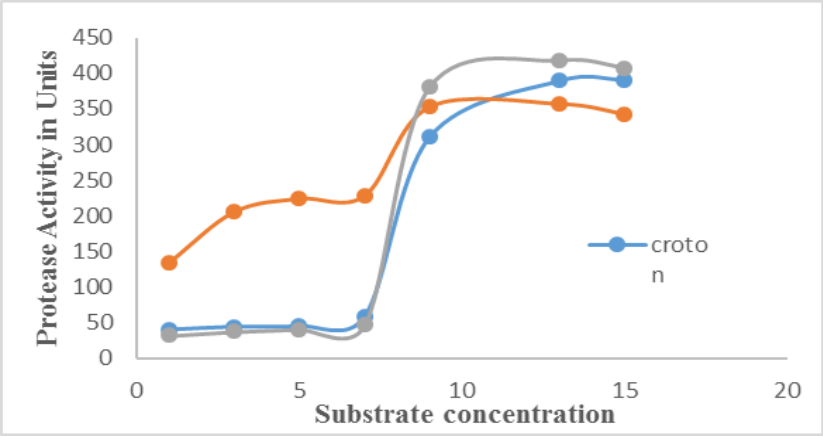


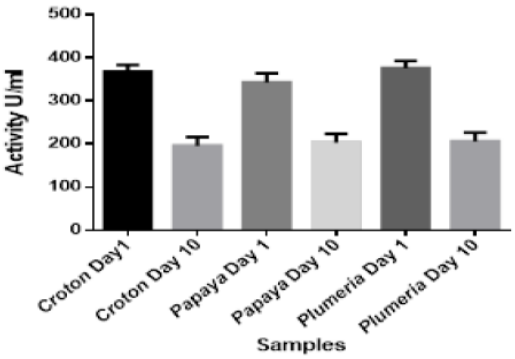
Fig. 7: Effect of substrate concentration on protease activity.

Molecular Weight Determination

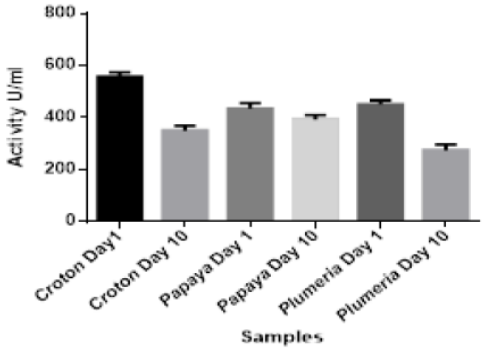
Molecular weight determination was done by SDS PAGE to identify the purified protein based on its molecular weight. The molecular weight of purified protease enzyme of *Croton bonplandianum*, *Carica papaya* and *Plumeria acutifolia* are ~35kDa, ~25kDa and ~34kDa respectively. Similar results were reported in the case of *Euphorbia hirta* with molecular weight of 34kDa for protease enzyme,[46] and with molecular weight of 34.4kDa for *Synadenium grantii*.[47] In previous studies, it was reported that some fibrinolytic proteases isolated from different sources represent molecular weight in the range of 23kDa to 43kDa and composed of single monomeric polypeptide like hirtin.[6,53,54]

Fig. 8: SDS PAGE – Molecular weight of anticoagulant enzymes.

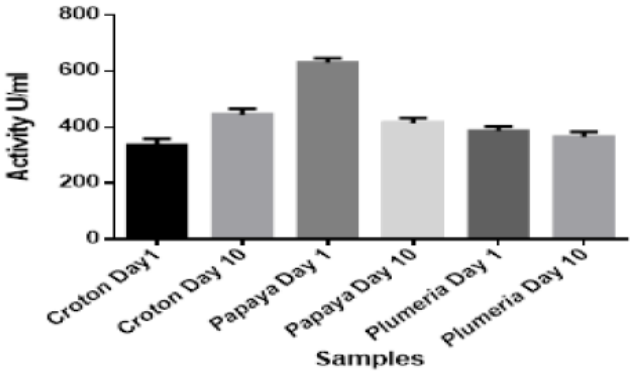
Immobilization: Immobilization is the process of entrapment of enzyme in a gel matrix used to examine the stability of the enzyme. Tightly cross-linked gels can inhibit the diffusion of macromolecules which can deactivate enzymes and thus immobilization protect enzymes from deactivation even in the aqueous organic solvent mixture.[55] Three methods were used for entrapment of protease enzymes from each sample *viz.* sodium alginate method, polyacrylamide method and polyurethane method. Stability was examined by activity estimation after 10 days of storage in Tris buffer at 4°C. When the stability was examined, activity decreased by ~50% in sodium alginate and polyacrylamide methods for *Croton bonplandiaum* and *Plumeria acutifolia*, whereas the stability was found to be high in polyurethane method as immobilized enzymes maintained ~100% of its activity. Activity of *Carica papaya* decreased by ~45% in sodium alginate and polyurethane methods, whereas the stability was found to be high in polyacrylamide method as immobilized enzymes maintained ~95% of its activity. Similar results of stability of enzymes within these matrices was reported earlier for polyurethane foam,[56] for alginate capsules,[30] and for polyacrylamide gel,[55] where immobilized enzymes have maintained 100% of its activity.



1. Sodium Alginate Method.



2. Polyacrylamide Method.



3. Polyurethane Method.

Fig. 9: Stability analysis in different immobilization methods.

Effect of Purified Enzymes on Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT)

APTT test and PT test was carried out as a confirmatory test for anticoagulant property of purified enzyme samples. Blood clotting is a very complex process, involving many factors found in the plasma and tissues in both the intrinsic and extrinsic pathways.[33,57] PT test and the APTT test are used for distinguishing between the effects of test agents on the extrinsic and intrinsic pathways, respectively.[33] The effect of the purified samples on APTT test from *Croton bonplandianum, carica papaya* and *Plumeria acutifolia* are recapitulated in Table 2. The prolongation of APTT evince that the purified enzymes samples have inhibited the intrinsic pathway that confirms the presence of anticoagulant enzymes in purified samples whereas they did not show any effect on PT test which evince that they do not inhibit extrinsic pathway. The results are in accordance with the report on *Jatropa carcus* latex,[43] along with the result reported on *Flaveria bidentis*.[58] Few more studies has reported similar results in the effect of protease on APTT test extracted from diffent sources.[59,60,34]

Table. 2: Effect of latex fractions on blood clotting time.

Sample	Sample (µL)	APTT Clotting Time (Sec)
Control	50 (PBS)	32 ±2
Croton bonplandianum	50	Not clotted
Carica papaya	50	Not clotted
Plumeria acutifolia	50	Not clotted

4. CONCLUSION

In the present study, three latex samples of *Croton bonplandianm*, *Carica papaya* and *Plumeria acutifolia* have shown anticoagulant property which is very significant in medical field. Anticoagulants are used in thrombotic disorders and these latexes can play an important role in treatments and can provide a very good alternative to those drugs which are high on side effects. *Carica papaya* latex showed highest anticoagulant property as it has also reported in earlier studies although, detailed studies on latex of *Croton bonplandianum* and *Plumeria acutifolia* can also provide alternatives in anticoagulant agents.

5. ACKNOWLEDGEMENT

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