Original Research Article

Purification and Partial Characterization of Protease Enzyme from the Latex of *Jatropha gossypiifolia* Linn. 1753 and Study of Anticoagulation Potential on Human Blood.

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Abstract: The present study was aimed to purify, partially-characterize and to investigate the possible anti-coagulation effect of protease enzyme isolated from latex of *Jatropha gossypiifolia* Linn. on human blood. The latex of *Jatropha gossypiifolia* was obtained by nipping mature leaves near the stem or incision of the bark and branches of the plant, the fluid coming out was collected into a clean sterile test tube. The protease was purified from the latex of *Jatropha gossypiifolia* right through ammonium sulphate precipitation and dialysis. The latex enzyme (protease) was assayed for proteolytic activity using denatured casein as substrate and determined as 0.06 Umg^{-1}. The Milk clotting activity and enzyme optimization was performed. The milk clotting activity was found to be 8.88 Umg^{-1}. Effects of pH and temperature on the purified latex (protease) were determined. The purified protease remained active over a broad range of temperature but had optimum activity at 75 °C and pH 3.0 when casein was used as substrate. The suitability of purified latex as an anticoagulant for biochemical and haematological analyses was determined. Proteases from *Jatropha gossypiifolia* latex exhibited well-built anticoagulant action. The anticoagulant effect was found to be highest at a concentration of 1.00 ml per ml of blood. Thus, the purified latex of *Jatropha gossypiifolia* may be suitable as an anticoagulant for haematological analysis. However, further studies are necessary to identify the phytoconstitute with their mechanism of actions responsible for the observed pharmacological activities and any eventual toxic effects that could reduce its medicinal value.

Key words: Ammonium sulphate precipitation, Anticoagulation, *Jatropha gossypiifolia*, Plant latex, Proteases.

Introduction

India is one of the megadiversity hot spots with rich heritage of traditional knowledge of folk medicines (Sen *et al.,* 2013). Approximately 85% of traditional medicine preparations involve the use of plants or plants extracts (Vieira and Skorupa, 1993). Indian folk medicines comprises numerous prescription for therapeutic purposes such as healing of wounds, skin infections, inflammation, leprosy, diarrhoea, scabies, veneral disease, ulcers, snake bite etc. (Mukherjee *et al.,* 2000). Plant extracts are potentially curative (Sinha *et al.,* 2013). Various plant parts such as leaves, bark, fruits, roots and seeds are used in treatment of various diseases (Kumar *et al.,* 2013); the use of plant latex for medicinal purpose is gaining momentum. A large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin (Mahato *et al.,* 2013).

A number of herbs belonging to the genus Jatropha of Euphorbiaceae family are well-known for their medicinal benefits. *Jatropha gossypiifolia* Linn. 1753, member of the

Fig. 1. Twig of *Jatropha gossipifolia* fom sample collection region.

Euphorbiaceae family (synonym: *Adenoropium gossypiifolia* Pohl, *J. elegans*) is a perennial, short-lived, erect, bushy gregarious shrub, or small tree with leaves having a long petiole and about 1.8 meters high (Oduola *et al.,* 2005a) (Fig. 1). The name "*Jatropha*" is derived from the Greek words "jatros", which means "doctor" and "trophe," meaning "food," which is associated with its medicinal uses (Sabandar *et al*., 2013). The latex of *Jatropha gossypiifolia* wildly used almost throughout India. The latex contains active antimicrobial components (Ogundare, 2007) and the plants is traditionally used in treatment of dysentery (Uddin, *et al.,* 2006), leprosy, diarrhoea, anaemia, vertigo, malaria, toothache, as antidote for snake bite, antibiotics, insecticidal, blood purifier, purgative, stimulant, stomachic, febrifuge, emetic, emmenagogue, to treat wounds, sores, boils, carbuncles, eczema, ulcers, to reduce pain and to stop bleeding from skin and nose (Kirtikar and Basu, 1996). The stem sap stops bleeding and itching of cuts and scratches (Morton, 1980; Labadie *et al.,* 1989). It also possesses significant anticancer, hepatoprotective and pesticidal activity (Panda, *et al.,* 2009; Hartwell, 1969). The leaf decoction of *Jatropha gossypiifolia* is used for bathing wounds (Panda, *et al.,* 2009). The stem latex has been shown to possess coagulant activity and therapeutic effects and the mechanism of action of the latex of *J. gossypiifolia* as a haemostatic agent has been documented (Oduola *et al.,* 2005) and the leaf extract has been used as an anticoagulant for biochemical and hematological analyses (Oduola *et al*., 2005).

Despite the grand variety of popular uses, no study has been done regarding the blood clotting activity of the products of *J. gossypiifolia* as the literature suggest and their biological activities has been scarcely studied (Juliana *et al.,* 2014). Therefore, the present study was carried to partially purify and partially characterize the protease enzyme from the latex of *Jatropha gossypiifolia* and to evaluate the claimed anticoagulant effect of Jatropha gossypiifolia latex using human blood.

Materials and methods

Plant materials and collection of latex

The plant materials used in the study was *Jatropha gossypiifolia*. Triplicate samples of plant latex were collected in the morning by nipping mature leaves near the stem or incision of the bark of the trunk and branches of the plant from Ayanavaram, Chennai-TN, India in the month of May. The fluid coming out was collected into a clean sterile test tube, brought to the laboratory and kept at 4°C till the experiment commenced.

Preparation of crude extract and enzyme purification process

Twenty five ml of crude latex were centrifuged at 8000 rpm for 15 min. Supernatant were discarded and the pellet was collected. The collected pellet was dissolved in 4ml of distilled water and was stored in the refrigerator for purification and analysis. The crude enzyme solution was precipitated by ammonium sulphate precipitation methods (50- 70% w/v). The precipitate was dialysed in 0.02M phosphate buffer (pH 7.0). Following dialysis, the fraction obtained (purified latex) was collected in tubes and used for enzyme assay and further analysis (Fig. 2).

Preparation of skim milk agar medium to determine the presence of protease enzymes

Casein (0.25 g) and powdered skimmed milk (0.05 g) were weighed into a clean test tube and mixtures were dissolved in

Fig. 2. Purified protein from the latex.

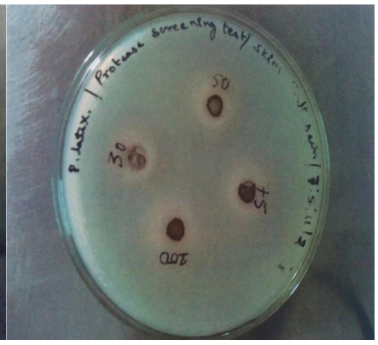

Fig. 3. Skimmed milk agar medium showing zone of inhibition.

20 ml of distilled water. In another test tube, 0.125 g of yeast extract, 0.125 g of glucose and 1.3 g of agar were weighed and mixtures were suspended in 30 ml of distilled water. Both the test tubes were maintained at pH 7 and sterilized by autoclaving at 121°C for 15 minutes. After sterilization both the tubes were mixed in another test tube, was shaken until the milk dissolved and finely mixed which is treated as media. The media is poured into sterilized petri plates and allowed to solidify (Fig. 3). The four well is made in the solidified media plate with the help of gel puncture. After which, 30µl, 50µl, 75µl and 100µl of the purified protein latex (dialysis) was added respectively and the time taken for the milk to clot was taken as a measure of enzyme activity.

Determination of milk clotting activity (MCA)

The milk clotting activities (MCA) of the enzyme extract were determined according to standard method. It is based on the visual evaluation of the appearance of the first clotting of skimmed milk, and is expressed in terms of Soxhlet units (SU). The clotting activity was calculated using the following formula:

$$SU = \frac{2400 \times 5 \times D}{T \times 0.5}$$

Where, T=Clotting time (s)

D = Dilution of the test material

The time taken for the milk to clot was taken as a measure of enzyme activity. The unit of milk clotting activity was defined as the amount of enzyme which clotted 1.0 ml of milk solution in 0.01 M Calcium Chloride ($CaCl_2$) buffer pH 6.5, in one minute at operating temperature of 37°C.

Assay of blood clotting activity

The assay of anticoagulation activity was performed using human blood as substrate. 1ml each of freshly collected whole blood was taken in three glass slides each by clean venipuncture. The analysis was categories into two. In first category, only bloods were kept in first slide (control).

In Second Category, 0.50ml and 1.00 ml of purified latex were added in second and third glass slide respectively (test) and the time taken for clot formation in each glass slide was recorded. The average of the clotting time of the two tubes with purified latex (test) and the two tubes without purified latex (control) were taken as the clotting time respectively. The reference range for whole blood clotting time is 6 to 9 minutes at 37°C (Lee and White. 1913).

Assay for proteolytic activity (PA)

Protease activity (PA) of the purified enzyme was determined by the method of Shimogaki *et al.* (1991) with slight modification, using casein as substrate. The casein solution (0.65%, w/v) was prepared in 10ml of 50mM potassium phosphate buffer pH 7.5 at 37°C (Adjust pH with 1 M HCl and either 1 M NaOH or 1 M HCl) and heat treated at 80-90°C for 10 min in water bath, cooled and used as substrate.

The reaction mixture consists of 1.0 ml of the substrate, 1.0 ml of the buffer and 1.0 ml of the enzyme extract thoroughly mixed together while the control consists of 2.0 ml of distilled water and 2.0 ml of the phosphate buffer. This was incubated for exactly 10 minutes at 37°C and the reaction was terminated by adding 1.0 ml of cold (2°C) 1% Trichloroacetic Acid (TCA). The tubes were allowed to stand for a minimum of 1 h at 2°C in a refrigerator to allow the undigested protein to precipitate. Thereafter, the mixtures were centrifuged at 6000 rpm for 20 minutes under room temperature and the absorbance of the clear supernatant was measured at 310 nm, the blank was used as control. The unit

of activity was defined as the amount of protease which caused an increase of one unit of absorbance per minute of digestion at 310 nm.

Enzyme optimizations

The protease enzyme is subjected to various temperatures and pH to study the activity of enzymes for optimum temperatures and optimum pHs as follows:

a) Effect of pH on the proteolytic enzyme activity

The optimum pH was also determined on casein according to the method of (Kunitz, 1947). The reaction mixture consists of 1.940 ml of the phosphate buffer at various pHs such as pH ranges from 3-12, by adjusting the pH of the substrate (skimmed milk) with 0.1 M HCl or 0.1 M NaOH as appropriate. The maximum milk clotting activity obtained was taken to be 100%. Sixty µl of the enzyme solution were added to each test tube and incubate for 10 minutes at room temperature after which 2 ml of 1% casein were added to each test tube which were thoroughly mixed and incubated for 30 minutes at room temperature (35°C) in a water bath. The reaction was terminated by adding 2.0 mL of cold (2°C) 10% Trichloroacetic Acid (TCA) to each test tube.

The tubes were allowed to stand for a minimum of 1 h at 2°C in a refrigerator to allow the undigested protein to precipitate. Thereafter, the mixtures were centrifuged at 4500 rpm for 30 min under room temperature and the absorbance of the clear supernatant was measured by spectrophotometer at 280 nm, the blank distilled water was used as control. The unit of activity was defined as the amount of protease which caused an increase in one unit of absorbance per minute of digestion at 280 nm.

b) Effect of temperature on the proteolytic enzyme activity

The optimum temperature for the activity of the purified enzyme was determined by assaying the milk-clotting activity at temperatures ranges from 5°C - 85°C, by using the activity at the pH determined as optimum. Sixty µl of purified enzyme solution in 1.940 ml of 0.02M phosphate buffer (pH 3.0) was incubated for 10 minutes in four test tubes at different temperatures such as (5°C-75°C), after which 2 ml of 1% casein were added to each test tube which were thoroughly mixed and incubated for another 30 minutes at room temperature (35°C) in a waterbath. The reaction was terminated by adding 2.0 ml of cold (2°C) 10% Trichloroacetic Acid (TCA) to each test tube. The tubes were allowed to stand for a minimum of 1 h at 2°C in a refrigerator to allow the undigested protein to precipitate.

The mixtures were centrifuged at 4500 rpm for 30 min under room temperature and the absorbance of the clear supernatant was measured at 280 nm, the blank distilled water was used as control. The unit of activity was defined as the amount of protease which caused an increase of one unit of absorbance per minute of digestion.

Statistical analysis

All experiments were performed in triplicate and the results are expressed as mean standard deviation (SD).

Results

1. Purification of protease enzyme

The protease from the latex of *Jatropha gossypiifolia* Linn. has been successfully purified to homogeneity by a simple purification procedure and has also been partially characterized. The protease enzyme was purifies from the crude enzyme extract through 2 steps of purification including ammonium sulphate precipitation also known as salting out and dialysis procedures. The zones of inhibition were formed on the casein milk agar plate (Media) which indicates the presence of protease enzymes in the purified latex of *Jatropha gossypiifolia* Linn.

2. Blood clotting activity

The present study showed that the purified latex of *J. gossypiifolia* has anticoagulant properties. The anticoagulant effect of the purified latex was achieved at 1.00 ml of the purified latex. The clotting time without adding purified latex was 7 minutes 7 seconds while it was almost 1 hour when purified latex was added.

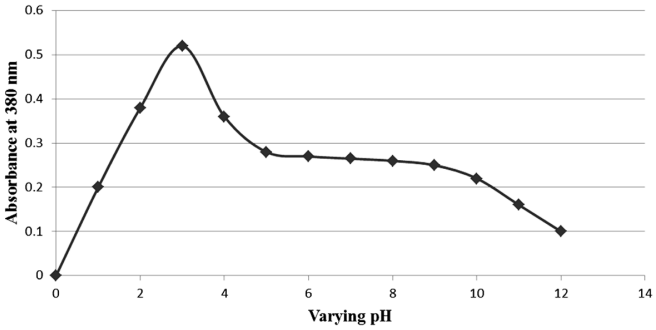

Fig. 4. Effect of pH on activity of purified enzyme.

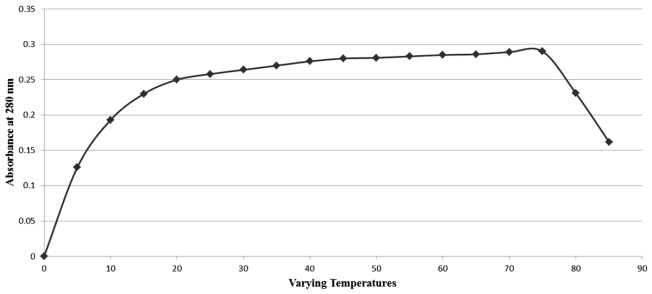

Fig. 5. Effect of temperature on activity of purified enzyme.

3. Milk clotting-activity (MCA) and proteolytic activity (PA)

The enzyme of the *Jatropha gossypiifolia* latex weakly coagulated skimmed milk solution within seven minutes (which corresponds to a specific clotting activity of 8.88 U mg^{-1}), forming a white and unstable curd. The proteolytic activity is found to be 0.06 U mg^{-1}. The ratio of milk-clotting activity to proteolytic activity (148) of *Jatropha gossypiifolia* latex was determined.

4. Enzyme optimisation

a) Effect of pH on the proteolytic enzyme activity

The milk-clotting activity decreases with increased in pH in the pH range 3-12. The highest activity is recorded in pH 3.0. Hence, the protease enzyme purified from latex has optimum at pH 3.0 (Fig. 4).

Although complete purification and characterization has been not carried out, this study serves as basis for further research on *J. gossypiifolia* and extensive studies can be carried out to identify the phytoconstitute with their mechanism of actions responsible for the observed pharmacological activities as it finds frequent use in the Indian traditional medicine.

b) Effect of temperature on the proteolytic enzyme activity

The milk-clotting activity increased with increased in temperature in the temperature range 20-75 °C. The result of this study showed that the protease enzyme purified from latex has maximum activity at 75 °C with casein as substrate. Above the optimum temperature, its activity decreased to approximately 50 % at 85 °C (Fig. 5).

Table 1. Comparision of the ratio of milk clotting activity/proteolytic activity of purified enzyme from the latex of *Jatropha gossypiifolia* and other milk coagulants from plants.

Enzyme	MCA (U mg^{-1})	PA (U mg^{-1})	Ratio (MCA/O.D. PA)
Protease from Ginger	314	0.19	1653
Protease from Quixaba	917	0.16	5731
Protease from *J. gossypiifolia*	88.8	0.06	1480

Discussion

The protease from the latex of *Jatropha gossypiifolia* Linn. 1753 were successfully purified to homogeneity by a simple purification procedure and dialysis and has also been partially characterized. Based on the findings of this study, Anticoagulant activity of the stem latex of Jatropha gossypiifolia was demonstrated, which was also demonstrated by Oduola *et al.* (2005). The increased clotting times recorded in the experiment with purified latex as compared to those purified latex was evidence that the purified latex possess anticoagulation agent thereby providing scientific basis for its use in haematological studies. One of the criteria for selecting a good anticoagulant is that it should not interfere by adding to or subtracting from the components under study. Since the purified extract added to the true value of these components, it is therefore not a suitable anticoagulant when these biochemical parameters are to be estimated. The pure anticoagulant in the extract has to be extracted and characterized. This could be a substitute for K_2EDTA, and may also be useful for assaying biochemical parameters. The findings of this study are in agreement with the traditional uses of the leaves of *Jatropha gossypiifolia* Linn. as a haematological agent in some regions of the worlds. Moreira *et al.* (2013) provided values for coagulant and proteolytic activities of enzyme from latex of Quizaba (*Sideroxylon obtusifolium*) and Ginger, which is presented in Table 1.

The coagulants described by Moreira *et al.* (2013) had a milk coagulation activity higher than that of the protease enzyme from *J. gossypiifolia* latex. These results show that the ratio of coagulant activity to protease activity in the *J. gossypiifolia* latex (1480) may be sufficient for the commercial uses like production of cheese, although, more studies about quality of both milk curds and the cheese formed should be carried out to confirm its usefulness in industry.

There is no doubt that the stem latex of *Jatropha gossypiifolia* has anticoagulant properties and latex extract can be used for haematological investigations, but its active chemical must be isolated and purified before it is used for biochemical analysis and work needs to be done on toxicity studies to eliminate any dangerous side effects before it can be safely recommended for intravascular treatment. Anticoagulants such as heparin and warfarin are used therapeutically to control thrombosis. If the latex is experimented on laboratory animals and the results are satisfactory, it could also be used therapeutically. Although complete purification and characterization has been not carried out, this study serves as basis for further research on *Jatropha gossypiifolia* and extensive studies can be carried out to identify the phytoconstitute with their mechanism of actions responsible for the observed pharmacological activities as it finds frequent use in the Indian traditional medicine.

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