Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(1): 1303-1310
Received: 21-11-2018
Accepted: 26-12-2018

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Anti-hemostatic protease from *Jatropha curcas* latex with fibrinogen lytic activity

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Abstract
The *Jatropha curcas* plant has been used since ancient time to treat open wounds, haemorrhage, to stop blood loss and to enhance wound healing process in folklore/tribal medicine by the people of tropical, subtropical and semi-arid countries. Discovery of new therapeutic molecule(s) is necessary to treat the thrombotic and other disorders in coagulation. There is a need to evaluate and isolate active molecules with medicinal property. This study is conducted to evaluate and characterize the *Jatropha curcas* plant latex for its fibrinogen lytic, collagenolytic and its action on blood coagulation cascade to substantiate the use of this plant latex as antithrombotic medicine.

Keywords: *Jatropha curcas*, fibrinogen, anti-coagulant, cytotoxicity, inflammation, wound healing

Introduction
Thrombosis is becoming a leading cause of mortality throughout the world. Mainly medicines with antithrombotic-anticoagulant and anti-platelet activity such as heparin and coumarin are clinically used. They inhibit either coagulation pathway in the blood circulation system or inhibit activation and aggregation of platelets. However, the nonspecific mode of action such as heparin induced thrombocytopenia, poor availability and risk of animal pathogen contamination limits their therapeutic applications [1]. Therefore, it is necessary to search for new anticoagulants that target specific coagulant enzymes or particular step in the clotting factors. Some medicinal plants/plant extract contains anti-thrombotic agents, including platelet aggregation inhibitors, vasodilators, thrombin inhibitors, fibrino(geno)lytic enzymes and so on, which may lead to identification of new molecule of therapeutic application [2].
Anticoagulants are the predominantly used drugs for the primary and secondary thromboembolic complications of vascular diseases. Anticoagulants act by inhibiting any of the coagulation factors/enzymes of the blood coagulation cascade [4]. Anticoagulants are used to prevent/treat thromboembolic disorders and acute stroke with atrial fibrillation [5].
Plants belonging to family Apocyanaceae, Moraceae and Euphorbiaceae are known to be a rich source of latex derived from laticifer cells. Plant latex is a complex emulsion or colloidal usually white, yellow or pinkish in colour that oozes out from various parts of plants upon injury. This plays a vital role in plant morphogenesis, defense system against various pathogens and senescence [6, 7]. Latex contains various components such as resins, gums, proteins, lipids, hydrolytic enzymes and secondary metabolites derived from alkaloids, tannins and terpenes [8].
Jatropha curcas is widely distributed plant in countries of tropical, subtropical and semi-arid regions. This is a multi-purpose plant with potential medicinal value. The plant/plant latex extracts have a long history of being used to treat open wounds, haemorrhage and to enhance wound healing process in folklore/tribal medicine in many countries [9].
In this study, for the first time we are reporting a protease with anticoagulant property (both *in vitro* and *in vivo* studies) from *J. curcas* latex extract and evaluated to explore its mechanism as antithrombotic medicine.

Materials and Methods
Plant Material
The *Jatropha curcas,* fam. Euphorbiaceae, common name: physic nut was collected near Gubbi, about 20 Km from Tumkur district, Karnataka, India (GPS 13°18'22.1"N, 76°57'00.3"E). The plant was authenticated by Dr. P. Sharanappa, Professor, Department of Studies & Research in Bioscience, Hemagangotri, University of Mysore, Hassan, India. Specific voucher specimens (TU15DOSRBC001) of this plant were deposited in the

Herbarium of Department of Studies and Research in Botany, Tumkur University, Tumkur, India for future reference.

Chemicals and Reagents
Fibrinogen (from human plasma), collagen type-I (from rat tail), collagen type IV (from human placenta), Gelatin (from porcine skin), PMSF (phenyl methyl sulphonyl flouride), EDTA (ethylene diamine tetra acetic acid) and E-64 were purchased from Sigma Aldrich (St Louis, MO, USA). Prothrombin time (PT) and activated partial thromboplastic time (APTT) kits purchased from Tulip group (Goa, India). All other reagents used were of analytical grade.

Instruments
- Spectrophotometer
- Electrophoresis unit
- Coagulometer
- Centrifuge
- Magnetic stirrer

Animals
Adult Swiss Albino mice (20-25 g) were obtained from the Liveon Biolabs Pvt. Ltd., #46 and 47, Water Tank Road, KIADB – Phase II, Antharasanahalli, Tumkur – 572 106, Karnataka, India. The animal care and experimental procedures performed were in compliance with the Institutional Animal Ethics Committee Constituted under CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) rules (Ref: 1610/ROBiBt/S/2012/CPCSEA), India.

Collection of plant latex
Latex was collected early morning from *Jatropha curcas* plant by excising leaves and branches of the plant, allowing the latex to drain into a sterile clean test tube. The sample was kept at -20 ºC overnight. Then the sample was centrifuged at 6000 rpm at 5 ºC for 10 mins. The supernatant was used for further processing.

Ammonium salt precipitation
The latex supernatant obtained after centrifugation was precipitated using ammonium sulphate. Briefly, the sample was precipitated by using saturated ammonium sulphate (40% w/v) and the solution was kept at 4 °C for 24 h and centrifuged at 5000 rpm for 15 min. Supernatant was discarded and the precipitate was dialysed in distilled water overnight. The dialysed fraction was dissolved in a minimum amount of sodium phosphate buffer (0.01 M, pH 7.0) and stored at -20 ºC until further use. Ammonium sulphate precipitated latex sample used for further analysis is named as 'latex extract'.

SDS-polyacrylamide gel electrophoresis
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of (Laemmli *et al.,* 1970) [10]. Briefly, latex extract (160 µg) was loaded onto 12% polyacrylamide gel containing 0.1% SDS. Electrophoresis was carried under non-reducing conditions at a constant voltage of 90V using a Tris-glycine buffer (pH 8.3). After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 to visualize the bands.

Native page zymogram
Native PAGE (12%) polymerized at a final concentration of 0.2% with gelatin/casein was carried out according to the method of (Laemmli *et al.,* 1970) [10]. Briefly, latex extract (80 µg) was loaded onto 12% Native PAGE under non-reducing conditions. Electrophoresis was carried out at 90 V at room temperature. After electrophoresis, the gel was incubated in Tris-HCl buffer (50 mM, pH 7.0) containing 10 mM $CaCl_2$ and 150 mM NaCl at 37 ºC for 16 h. The gel was then stained with Coomassie brilliant blue R-250. The activity was analysed by visualizing translucent band.

Periodic acid schiff's base (PAS) staining
SDS-PAGE was carried out as mentioned above. PAS staining was done according to the method of (Leach *et al.,* 1980) [11]. Briefly, SDS-PAGE was performed as mentioned earlier. The gel was fixed in 7.5% acetic acid at room temperature for 1 h. The gel was washed with nitric acid and stored in 0.2% aqueous periodic acid at 4 ºC for 45 mins. Later, the gel was soaked in Schiff's reagent and stored overnight at 4 ºC. The gel was destained with 10% acetic acid to view the reddish pink bands.

Fibrinogenolytic activity assay
The fibrinogenolytic activity assay was performed as described by (Ouyang and Teng, 1976) [12]. Latex extract (0, 4, 8, 20, 40, 60 and 80 µg) was incubated with human fibrinogen (50 µg) in 30 µL of 50 mM Tris-Hcl buffer pH 7.0 containing 150 mM NaCl. After 3 h, the reaction was terminated by adding 10 µL of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The sample was analysed on 12% SDS-PAGE by staining with Coomassie brilliant blue R-250.

Effects of pH and temperature on fibrinogenolytic activity
The pH and temperature kinetic studies were conducted using fibrinogen as substrate. For determination of the optimum pH for enzyme activity, 50 mM buffers (sodium acetate [pH 4.0 – 5.0], sodium phosphate [pH 6.0 –7.0] and Tris–HCl [pH 8.0 – 10.0]) containing 150 mM NaCl was used. For the optimum temperature study, the enzyme activity of latex extract was measured under standard assay conditions at various temperatures (4ºC to 60 ºC). The activity was monitored as a percent of fibrinogen subunit degradation. Analyses were done using Image J 1.49v software.

Fibrinolytic activity assay
Fibrin degradation was analysed by SDS-PAGE as described by (Rajesh *et al.,* 2005) [13]. Briefly, 100 µL of platelet poor plasma was mixed with equal volume of 25 mM $CaCl_2$ solution at 37 ºC to get the soft fibrin clot. The fibrin clot formed is transferred into a separate eppendorf tube and washed thoroughly for 5-6 times with 10 mM sodium phosphate buffer. The fibrin clot is incubated with varying concentrations of latex extract (0, 0.4, 0.8, 4, 8, 40 and 80 µg) in 40 µL sodium phosphate buffer (10 mM, pH 7.0) at 37 ºC for 3 h. After incubation, the reaction was stopped by adding SDS-PAGE denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol (20 µL) and kept in boiling water bath for 3 min. An amount of 20 µL of this sample was loaded onto 10% SDS gel and electrophoresis was performed to analyse the fibrin degradation.

The collagenolytic activity assay
The collagenolytic activity assay was performed according to the method of (Kim *et al.,* 2007) [14]. Briefly, collagen type I (10 µg) and collagen type IV (25 µg) were incubated with

varying concentrations of latex extract in 40 µL sodium phosphate buffer (10 mM, pH 7.0) at 37 ºC for 3 h. The reaction was terminated by adding denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min and loaded onto 7% SDS-PAGE and electrophoresis was performed at 90 V. The degradation pattern was analysed by staining the gel with Coomassie brilliant blue R-250.

Prothrombin time

The prothrombin time (PT) was performed to analyse the effect of latex extract on the extrinsic blood coagulation pathway, according to the method described by Tulip Diagnostics (P) Ltd. India. Briefly, different concentrations of latex extract (0, 80, 160, 240 and 320 µg) in 100 µL sodium phosphate buffer (10 mM, pH 7.0) was pre-incubated with 100 µL of platelet poor plasma (PPP) in a borosilicate tube at 37 °C for 5 min. Then 200 µL of pre-warmed (37 °C) Uniplastin reagent (tissue factor) was added and the clotting time was recorded.

Activated partial thromboplastic time

The activated partial thromboplastic time (APTT) was performed to analyse the effect of latex extracts on the intrinsic blood coagulation pathway, according to the method described by Tulip Diagnostics (P) Ltd. India. Briefly, different concentrations of latex extract (0, 80, 160 and 240 µg) in 100 µL sodium phosphate buffer (10 mM, pH 7.0) were pre-incubated with 100 µL of PPP at 37 °C for 5 min and mixed with 100 µL of liquicellin E reagent (platelet substitute) in a borosilicate tube. Tubes were incubated at 37 °C for 5 min. Then 100 µL $CaCl_2$ (25 mM) was added and the clotting time was recorded.

Plasma recalcification time

Plasma recalcification time was determined according to the method described by (Condrea *et al.,* 1983) [15]. Briefly, PPP (100 µL) was incubated with different concentration of latex extract (0, 80, 120, 160, 200 and 240 µg) for 5 min at 37 ºC. $CaCl_2$ (100 µL) was added and time taken for clot formation was recorded.

Degradation of human plasma protein

The degradation of human plasma protein was performed according to the method of (Kumar *et al.,* 2010) [16]. Briefly, blood was drawn from the healthy individual into heparin containing vial and the plasma was collected by centrifugation (3000 rpm). Plasma (100 µg) was incubated with latex extract (0, 0.08, 0.4, 0.8, 4, 8, 40 and 80 µg) in 40 µL of sodium phosphate buffer (10 mM, pH 7.0) at 37 ºC for 3h. The reaction was terminated by the addition of 20 µL of non-reducing sample buffer and kept in boiling water bath for 3 min. Degradation pattern of plasma protein was analysed on a 7% SDS-PAGE.

Effect of latex extract on clotting time *in vivo*

The effect of the latex extract on the whole blood clotting time *in vivo* was performed according to the method of (Hao Chen *et al.,* 2014)[17]. The mice were injected intravenously with different concentrations (0, 3, 6 and 7.5 mg/kg body weight) of latex extract in 20 µL of sodium phosphate buffer (10 mM, pH 7.0). After 20 minutes, the blood was collected from the retro-orbital sinus region and time taken for blood clot was recorded.

Hemolytic assay

Hemolytic activity was done according to the method of (Shin *et al.,* 1997) [18]. Briefly, isolated RBC were incubated with different concentration of latex extract (400, 800, 1200, 1600 and 2000 µg) for 30 min at 37 ºC. Hemolysis was measured as the amount of haemoglobin released as read at 540 nm.

Haemorrhagic activity

Haemorrhagic activity was assayed as described by (Kondo *et al.,* 1969) [19]. Briefly, latex extract (1600 and 3200 µg) was injected (intradermal) into groups of six mice. Mice were anaesthetized using diethyl ether and sacrificed after 2 h. Dorsal patch of skin surface was removed and observed for haemorrhage against saline injected control mice.

Edema inducing activity

Edema inducing activity was done according to the method of (Vishwanath *et al.,* 1987)[20]. A group of mice was separately injected with latex extract (400 and 800 µg) in a total volume of 20 µL into right foot pads. Left foot pads received only saline served as controls. After 1 h mice were anaesthetized using diethyl ether and sacrificed. Hind limbs were cut at the ankle joint and weighed. Weight increase was calculated as the edema ratio, which equals the weight of the edematous leg×100/weight of a normal leg. Minimum edema dose (MED) was defined as the amount of protein required to cause an edema ratio of 120%.

Myotoxicity

Myotoxicity was determined according to the method of (Gutierrez *et al.,* 1990) [21]. Cytoplasmic marker enzymes, lactate dehydrogenase and creatine kinase levels were determined in the serum. The latex extract (400 µg and 800 µg) in 50 µL saline was injected intramuscularly into the right thigh of a group of four mice. The group receiving 50 µL of saline alone was served as control. After 3 h, the animals were anaesthetized with diethyl ether, abdominal cavities are opened and the blood is drawn from the abdominal vena cava. After clotting, the 1:25 (serum: saline) diluted serum is assayed for LDH and CK enzyme activities using AGAPEE Diagnostic kits. In both the case activity is expressed as IU/L.

LD50

The LD50 was performed according to the method of (Muhammad Ahmed, 2015) [22]. Briefly, for each mouse, the observation was made for 24 hr and symptoms of toxicity and rate of mortality in each group were noted. At the end of study period, expired animals were counted for the calculation of LD50. The arithmetic method of (Karber, 1931) [23] was used for the determination of LD50.

$LD50 = LD100 - \sum (a \times b) / n$

n = total number of animal in a group.

a = the difference between two successive doses of administered extract/substance.

b = the average number of dead animals in two successive doses. LD100 = Lethal dose causing the 100% death of all test animals

Protein estimation

Protein concentration was determined according to the method of (Lowry *et al.,* 1951) [24] using bovine serum albumin (BSA) as standard.

Statistical analysis

All the data were expressed as mean ± standard deviation (SD)

Results

SDS-PAGE, zymogram assay and kinetic studies

Jatropha curcas latex was freshly collected into sterile tube and kept at -20 ºC overnight. The latex was centrifuged and the supernatant was processed by ammonium sulphate precipitation and dialysed as described in the methods section. The dialysed sample is designated as 'latex extract' and used for further studies. SDS-PAGE pattern of latex extract showed protein bands at high molecular weight, few at medium molecular weight and largely concentrated bands at low molecular weight region. Latex extract showed caseinolytic and gelatinolytic activity in zymogram assay with translucent activity bands in the high molecular weight region (Fig. 1.A). The latex extract was devoid of any glycoprotein as it did not show any bands in PAS staining (data not shown). Latex extract did not show any specific pattern of inhibition with protease inhibitors. So, it is difficult to specify the class of protease present in the latex extract. Purification and characterization of individual molecule may provide insights into the class of the protease.

The optimum pH and temperature for latex extract activity was evaluated by fibrinogenolytic activity. The optimum pH was found to between 6 and 7 (Fig. 1.B), and is active in all temperature range of 4 ºC to 40 ºC as it degrades fibrinogen completely. The percentage of degradation of γ chain of fibrinogen was considered for evaluation purpose. This was analysed using Image J 1.49v software (Fig. 1.C)

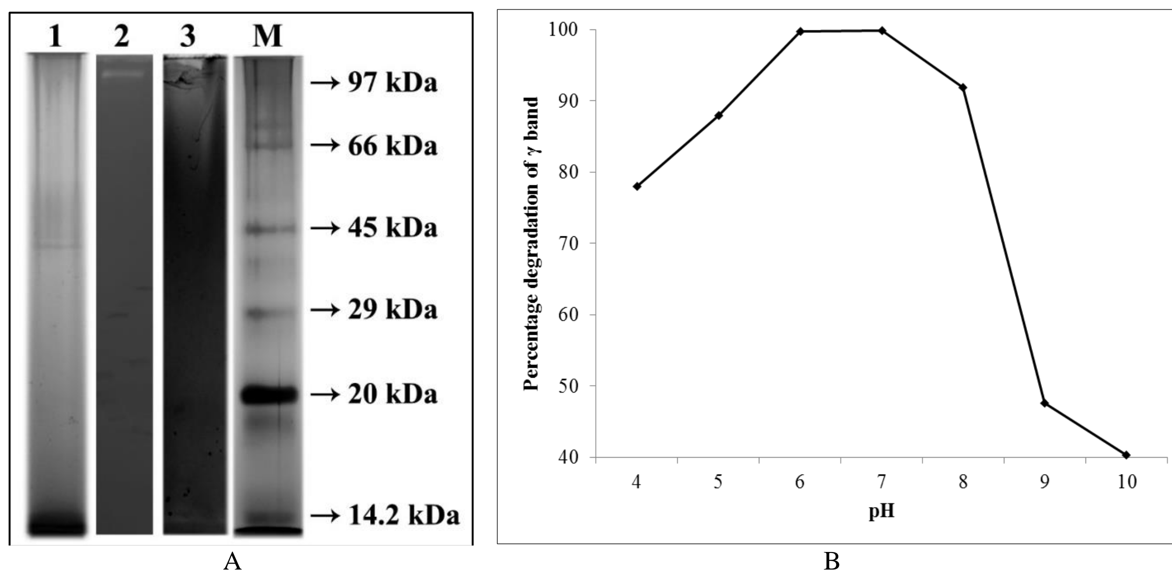

Fig 1: SDS-PAGE, zymogram assay and kinetic studies. (A) *J. curcas* latex extract (160 µg) was loaded onto 12% SDS-PAGE (1), 80 µg of latex extract was loaded onto 12% native PAGE incorporated with 0.2% casein (2) and gelatin (3) and electrophoresis was carried out under non-reducing conditions. After electrophoresis, the gel was kept in incubation buffer for 16 h at 37 ºC. Later the gel was washed and stained with Coomassie Blue R-250 to visualize the gelatinolytic activity. Lanes: 1 – *J. curcas* latex extract (160 µg); 2 – caseinolytic zymogram (80 µg); 3 – gelatinolytic zymogram (80 µg); M – molecular weight markers. (B) Effect of pH on fibrinogenolytic activity. Latex extract (40 µg) was pre-incubated with different buffer systems of varying pH for 40 min. Fibrinogen (50 µg) was incubated with latex extract and digested for 3 h at 37 ºC. The reaction was terminated by adding SDS-PAGE denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The samples were loaded onto 12% SDS-PAGE and the percentage of γ subunit of fibrinogen degradation was analysed.

Degradation of extracellular matrix and plasma proteins

Latex extract showed fibrinogenolytic activity in a concentration dependent manner (Fig. 2.A). The result was analysed using Image J 1.49v software (Fig. 2.B). Latex extract cleaved all the three chains of fibrinogen (Aα, Bβ and γ) with preferential cleavage in the order of Aα>Bβ>γ. Aα and Bβ chains were readily cleaved at the concentration of 8 µg followed by γ-chain at the concentration of 80 µg. The major degradation products were observed at a molecular weight range of 26 and 37 kDa.

Latex extract showed fibrinolytic activity by degrading partially cross-linked human fibrin clot prepared from human plasma. Latex extracts completely degraded α – polymer, β – chains and γ – γ dimer in a concentration dependent manner as observed in 10% SDS-PAGE under reduced condition (Fig. 2.C) and was analysed using the Image J 1.49v software (Fig. 2.D).

Latex extract completely degrades all the bands of both type I (Fig. 2.E) and type IV (Fig. 2.F) collagen in a concentration dependent manner. Latex extract at the concentration of 14 µg and 20 µg completely degrades the chains of collagen type I (α1, α2 and γ) and collagen type IV (α1, α1 and α2) respectively.

Latex extract completely hydrolyses the human plasma proteins in a concentration dependent manner as observed in SDS-PAGE under non-reduced condition. Plasma proteins are completely hydrolysed at 80 µg of latex extract (Fig. 2.G).

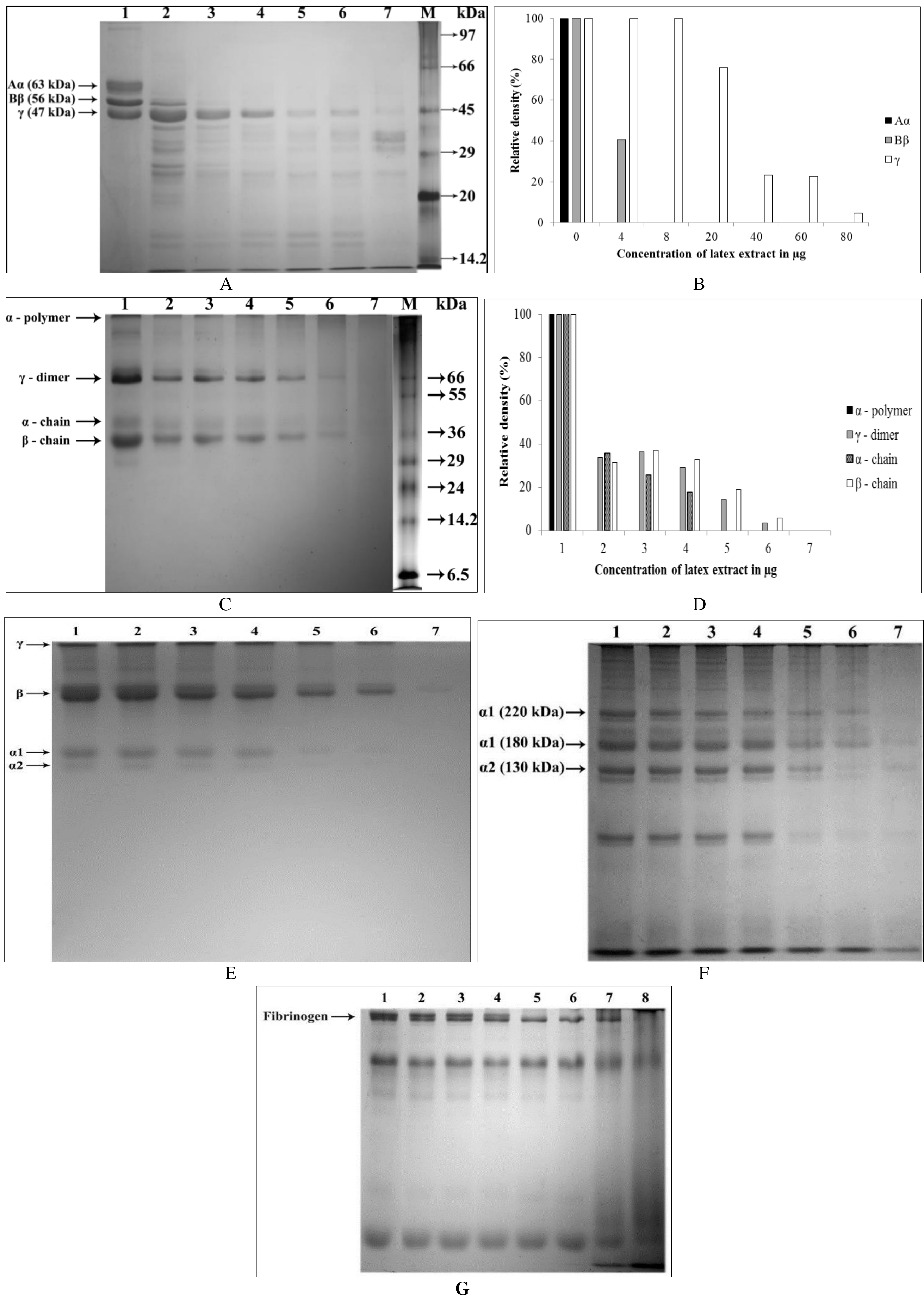

Fig 2: Degradation of extracellular matrix and plasma proteins. (A) Fibrinogenolytic activity of latex extract. Hydrolysis pattern of latex extract on human fibrinogen are shown on SDS-PAGE 12%. Fibrinogen was incubated with 0, 4, 8, 20, 40, 60 and 80 µg of latex extract (lanes 1, 2, 3, 4, 5, 6 and 7 respectively) for 3 h at 37 ºC. The reaction was terminated by adding SDS-PAGE denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The activity was visualized on 12% SDS-PAGE using Coomassie Blue R-250 staining. (B) Densitometric analysis of fibrinogen chains corresponding to fibrinogenolytic activity of latex extract. The densitogram study

of the Fig. 2.A using Image J 1.49v software was done and the fibrinogen subunits band density in control is considered as 100%. (C) Fibrinolytic activity of latex extract. PPP (100 µL) was mixed with 25 mM $CaCl_2$ (100 µL) at 37 ºC to obtain soft fibrin clot. The soft fibrin clot was washed 5 – 6 times with sodium phosphate buffer (10 mM, pH 7) and suspended in 0, 0.4, 0.8, 4, 8, 40 and 80 µg of latex extract (lanes 1 – 7 respectively) at 37 ºC for 3 h. The reaction was stopped by adding SDS-PAGE denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The fibrin products were analysed on 10% SDS-PAGE under reducing conditions. (D) Densitogram of fibrinolytic assay by latex extract. The densitogram study of the Fig. 4.A using Image J software Version 1.49. The fibrin subunits band density in control is considered as 100%. (E) Type I and type IV (F) Collagenolytic activity of latex extract. An amount of 10 µg of type I (Fig. 5. A) and 25 µg of type IV (Fig. 5. B) collagen were incubated separately with 0, 0.8, 4, 8, 10, 12 and 14 µg of latex extract (lanes 1, 2, 3, 4, 5, 6 and 7 respectively in Fig. 2.E) and 0, 0.08, 0.4, 0.8, 4, 8 and 20 µg of latex extract (lanes 1, 2, 3, 4, 5, 6 and 7 respectively in Fig. 2.F) at 37 ºC for 3 h. The reaction was terminated by adding denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The activity was visualized on 7% SDS-PAGE using Coomassie Brilliant Blue R-250 staining. (G) Effect of latex extract on human plasma protein. Varying concentrations such as 0, 0.08, 0.4, 0.8, 4, 8, 40 and 80 µg latex extract (lanes 1 – 8 respectively) were incubated with 100 µg of human plasma protein in a total reaction volume of 40 µL of sodium phosphate buffer (10 mM, pH 7.0) at 37 °C for 3 h. The reaction was terminated by the addition of 20 µL SDS-PAGE non-reducing denaturing buffer and kept in boiling water bath for 5 min. An amount of 20 µL was loaded onto a 7% SDS-PAGE under non-reducing conditions and Coomassie Blue R-250 stained.

In vitro coagulation studies

Latex extract interfered in the blood coagulation cascade as it increased the PT from 21.2 sec to 79.2 sec at a concentration of 300 µg. This indicates the strong anti-coagulant property of the extract (Fig. 3.A) involving in the extrinsic pathway of coagulation cascade.

Activated partial thromboplastin time (APTT) is the indicator of the intrinsic pathway of coagulation cascade. Latex extract prolonged the APTT from 42.6 sec to 182.2 sec at a concentration of 200 µg indicating its involvement in the intrinsic pathway of blood coagulation pathway (Fig. 3.B).

Pre-incubation of latex extract with the plasma prolonged the clotting time upon addition of the $CaCl_2$. Latex extract interfered with plasma recalcification time by prolonging the recalcification time from 117 sec to 377 sec at a concentration of 320 µg (Fig. 3.C). Plasma was made completely non-coagulable at a concentration of 400 µg.

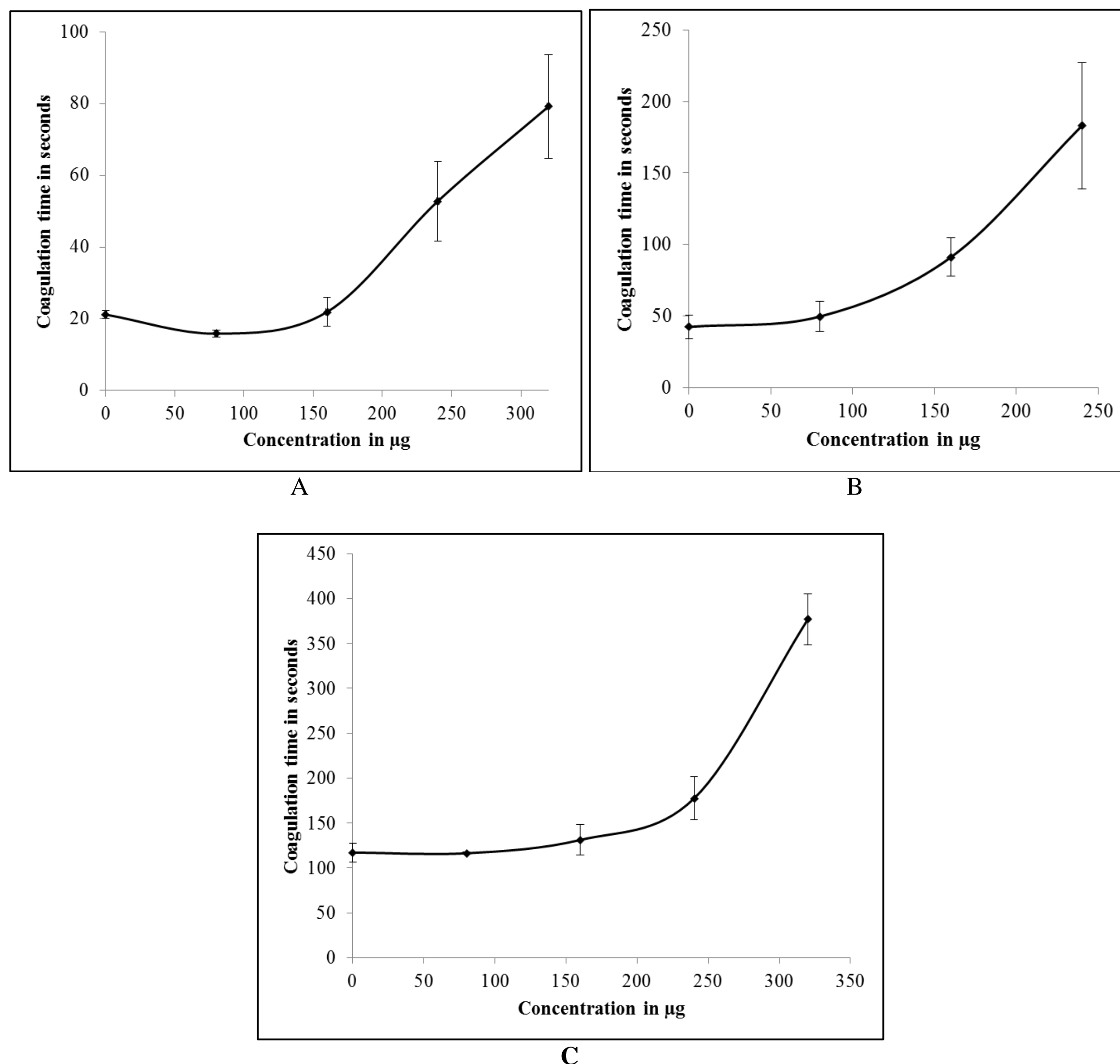

Fig 3: *In vitro* coagulation studies. (A) Effects of latex extract on Prothrobin time (PT). (B) Effect of latex extract on activated partial thromboplastin time (APTT). (C) Effect of latex extract on plasma recalcification time.

Effect of latex extract on clotting time *in vivo*

The latex extract increased the clotting time of the blood in a mouse model in a dose dependent manner. The injection of latex extract increased the blood clotting time from 99.4 sec to 141.5 sec at a concentration of 7.5 mg/kg body weight (Fig. 4).

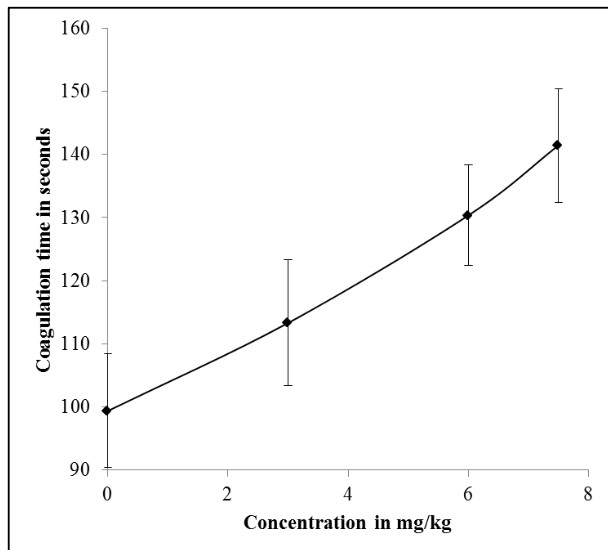

Fig 4: Effect of latex extract on clotting time *in vivo*. Varying concentrations (0, 3, 6 and 7.5 mg/kg body weight) of latex extract in 20 µL of sodium phosphate buffer (10 mM, pH 7.0) was injected intravenously into the mice. After 20 minutes, the blood was collected from the retro-orbital sinus region and time for blood clot was recorded.

Hemolytic assay

Latex extract is devoid of hemolytic activity with the human RBC cells in correspondence to the control (saline).

Toxicity studies

The latex extract is devoid of toxicity as confirmed by haemorrhagic activity, edema inducing activity and myotoxicity in *in vivo* studies using mouse models.

Discussion

Anticoagulants are very important in short term treatment of arterial and venous thrombotic disorders and for the long-term prevention of recurrences [25]. Although there are several treatments available, these have limitations related to their clinical applications such as inefficiency, potential for development of thrombocytopenia, immunosuppression and osteoporotic effectively with the long-term application as side effects [26, 27]. The present study explored the presence of protease in *J. curcas* latex extract with potent anticoagulant property as evaluated by both *in vitro* and *in vivo* studies.

The *Jatropha curcas* latex extract showed protein bands in SDS-PAGE under non-reduced condition. In casein and gelatin zymograms, the latex extract showed transluscent bands in the high molecular weight region indicating the proteolytic activity. The optimum activity showed between pH 6-7 and was active upto 40 ºC.

Latex extract showed fibrinogenolytic, fibrinolytic and collagenolytic (both type-I and type-IV) activity in a dose dependent manner by hydrolysing all the subunits of the proteins. Degradation of fibrinogen is mainly related to its anti-coagulant property as the sites of degradation leads to fibrinogen subunits which inhibits the formation of fibrin. Fibrinolytic and collagenolytic activity is mainly associated with clearance of thrombotic clots and enhancing wound healing property during physiological events.

Blood coagulation is formed due to the formation of insoluble fibrin from the soluble plasma protein which is catalysed by thrombin. The thrombin is activated from its zymogen form prothrombin either or both by intrinsic and extrinsic pathways. The PT which reflects the extrinsic pathway might be prolonged due to deficiencies of any of the factors VII, X, V, prothrombin or fibrinogen. Similarly, the a PTT for intrinsic pathway might be prolonged with the deficiency in any of the factors such as IX, VIII, X, V, prothrombin or fibrinogen [17]. The interference of the *J. curcas* latex extract in the blood coagulation cascade was analysed by recalcification time (RT), activated partial thromboplastin time (APTT) and prothrombin time (PT) assays using citrated human plasma. Latex extract increased the RT by 3.22 folds, aPTT by 4.28 folds and PT by 3.74 folds. This clearly indicates the involvement of latex extract components, both in *intrinsic* and *extrinsic* or in the common pathway of blood coagulation. These results clearly indicate the anti-coagulant property associated with the latex extract.

The latex extract also increased the clotting time of the blood in *in vivo* study. Upon injection to mice the latex extract increased the blood coagulation time by 1.42 folds. The inhibition of the clot formation may be due to inhibition of any clotting factors in intrinsic or extrinsic or common pathway of the blood coagulation. This clearly substantiates the anti-coagulant effect of the latex extract *in vivo* in a concentration dependent manner and supports the *in vitro* data. The showed activities were exclusively associated with protease present in the latex extract as the observed activities lost upon heat denaturation of the sample.

The latex extract was devoid of toxicity as none of the organs in injected animals were affected. It was supported by toxicity studies such as LD_{50}, haemorrhage, myotoxicity and edema inducing activities.

Proteases interfering in the coagulation cascade are of interesting components to study because of their wide range of applications. Anticoagulants are used extensively in clinical and medical treatments. They are used to treat thromboembolic disorders, atrial fibrillation, left ventricular thrombus and for prevention or treatment of deep venous thrombosis, pulmonary embolism. and prophylaxis of thrombotic events. Anticoagulant drugs help by preventing the adhesion of the thrombus during thromboembolism [28].

Conclusions

In conclusion, the *J. curcas* latex extract has potent anti-coagulant protease which hydrolyses fibrinogen, fibrin and collagen. The latex extract is non-toxic with anti-coagulant property which makes it to be a source for promising antithrombotic agent(s). Isolation and characterization of this protease could be used to decipher blood coagulation cascade. Further, the purified active component can be studied for its clinical applicability for thrombotic disorders.

Acknowledgements

We are grateful to the Dr. B.R. Ambedkar study center, Tumkur University, Tumkur, Karnataka, India.

References

1. Izabela Pawlaczyk, Leszek Czerchawski, Wiktor Kuliczkowski, Bożena Karolko, Witold Pilecki, Wojciech Witkiewicz *et al*. Anticoagulant and anti-platelet activity of polyphenolic-polysaccharide preparation isolated from

the medicinal plant *Erigeron canadensis* L. Thromb Res. 2011; 127:328–340.
2. Vinod Gubbiveeranna, Nagaraju S. Ethnomedicinal, phytochemical constituents and pharmacological activities of *Tridax procumbens*: a review. International J. Pharm. Pharmaceutical Sciences. 2016; 8(2):1-7.
3. Ramos MV, Viana CA, Silva AFB, Freitas CDT, Figueiredo IST, Oliveira RSB *et al*. Proteins derived from latex from *C. procera* maintain coagulation homeostasis in septic mice and exhibit thrombin and plasmin-like activities. *Naunyn-Schmiedeberg's* Arch. Pharmacol. 2012; 385(5):455-463.
4. Seiji Kaku, Mami Suzuki, Minori Saitoh, Toshiyuki Funatsu, Toshio Uemura, Ken-ichi Suzuki *et al.* Darexaban: Anticoagulant effects in mice and human plasma *in vitro*, antithrombotic effects in thrombosis and bleeding models in mice and effects of anti-inhibitor coagulant complex and recombinant factor VIIa. Thromb Res. 2013; 131:450-456.
5. Jeremy SP, Doug SW, John WE. Effectiveness and safety of combined antiplatelet and anticoagulant therapy: A critical review of the evidence from randomized controlled trials. Blood Rev. 2011; 25:123-129.
6. Thankamma L. Hevea latex as a wound healer and pain killer. Curr. Sci. 2003; 84(8):971-972.
7. Abdelgadir HA, Staden VJ. Ethnobotany, ethnopharmacology and toxicity of *Jatropha curcas* L. (Euphorbiaceae): A review. S Afr J Bot. 2013; 88:204-218.
8. Jean-Louis Jacob, Jean d'Auzac, Jean-Claude Prevot. The composition of natural latex from hevea brasiliensis. Clinical Reviews in Allergy. 1993; 11:325-337.
9. Shabeena Yousuf Naqash, Nazeer RA. Anticoagulant, antiherpetic and antibacterial activities of sulphated polysaccharide from Indian medicinal plant Tridax procumbens L. Appl Biochem Biotechnol. 2011; 165:902-912.
10. Laemmli VK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259):680-685.
11. Leach BS, Collawn Jr JF, Fish WW. Behavior of glycopolypeptides with empirical molecular weight estimation methods. 1. In Sodium Dodecyl Sulfate. Biochemistry. 1980; 19(25):5734-5741.
12. Ouyang C, Teng CM. Fibrinogenolytic enzymes of *Trimeresurus mucrosquamatus* venom. Biochim. Biophys. Acta. 1976; 420: 298-308.
13. Rajesh R, Gowda CDR, Nataraju A, Dhananjaya BL, Kemparaju K, Vishwanath BS. Procoagulant activity of *Calotropis gigantea* latex associated with fibrinogen lytic activity. Toxicon. 2005; 46:84-92.
14. Kim M, Hamilton SE, Guddat LW, Overall CM. Plant collagenase: Unique collagenolytic activity of cysteine proteases from ginger. Biochimica et Biophysica Acta. 2007; 1770:1627-1635.
15. Condrea E, Yang CC, Rosenberg P. Anticoagulant activity and plasma phosphatidylserine hydrolysis by snake venom phospholipase A2. Thromb. Haemost 1983; 49:151.
16. Kumar MS, Devaraj VR, Vishwanath BS, Kemparaju K. Anticoagulant activity of a metalloprotease: further characterization from the Indian cobra (Naja Naja) venom. J. Thromb. Thrombolysis. 2010; 29:340-348.
17. Hao Chen, Jin M, Wang YF, Wang YQ, Meng L, Li R *et al*. Effect of *Toona microcarpa* harms leaf extract on the coagulation system. Biomed. Res. Int. 2014; Doi: 10.1155/2014/615363.
18. Shin SY, Lee MK, Kim KL, Hahm KS. Structure-antitumor and hemolytic activity relationships of synthetic peptides derived from cecropin A-magainin 2 and cecropin A-melittin hybrid peptides. J Peptidr. Rrs. 1997; 50:279-228.
19. Kondo H, Kondo S, Ikezawa H, Murata R, Ohsaka A. Studies on the quantitative method for determination of haemorrhagic activity of Habu snake venom. Jpn. J Med. Sci. Biol. 1969; 13:43-51.
20. Vishwanath BS, Kini RM, Gowda TV. Characterization of three edema tic inducing phospholipase A2 enzymes from habu (*Trimeresurus flavoviride*) venom and their interaction with the alkaloid aristolochicacid. Toxicon. 1987; 25: 501-515.
21. Gutierrez JM, Arce V, Brenes F, Chaves F. Changes in myofibrillar components after skeletal muscle necrosis induced by a myotoxin isolated from the venom of the snake bothrops asper. Exp Mol Pathol. 1990; 52:25-36.
22. Muhammad Ahmed. Acute toxicity (Lethal Dose 50 Calculation) of herbal drug Somina in rats and mice. Pharmacol Pharm 2015; 6:185-189
23. Karber G. Beitrag zur kollecktiven Behandlung pharmakologischer Reihenversuche. Arch. Exptl. Pathol. Pharmakol. 1931; 162:480-483.
24. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951; 193(1):265-275.
25. Michiels JJ, Stibbe J, Bertina R, Broekmans A. Effectiveness of long term oral anticoagulation treatment in preventing venous thrombosis in hereditary protein S deficiency. Br Med J. 1987; 295:641-643.
26. Bick RL, Frenkel EP. Clinical aspects of heparin-induced thrombocytopenia and thrombosis and other side effects of heparin therapy. Clin Appl Thromb Hemost. 1999; 5(1): S7-S15.
27. Barbour LA, Kick SD, Steiner JF, Loverde ME, Heddleston LN, Lear J *et al.* A prospective study of heparin-induced osteoporosis in pregnancy using bone densitometry. Am J Obst Gynencol. 1994; 170(3):862-869.
28. Sweta RC, Rajpal SK, Jayant YD, Hemant JP, Girdhar MT, Hatim FD. Effect of aqueous extract and fractions of Fagonia Arabica on *in vitro* anticoagulant activity. Clin Appl Thromb Hemost. 2014; 20(8):844-850.