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ORIGINAL ARTICLE

Potent Procoagulant and Platelet Aggregation Inducing Serine Protease from *Tridax procumbens* Extract

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ABSTRACT

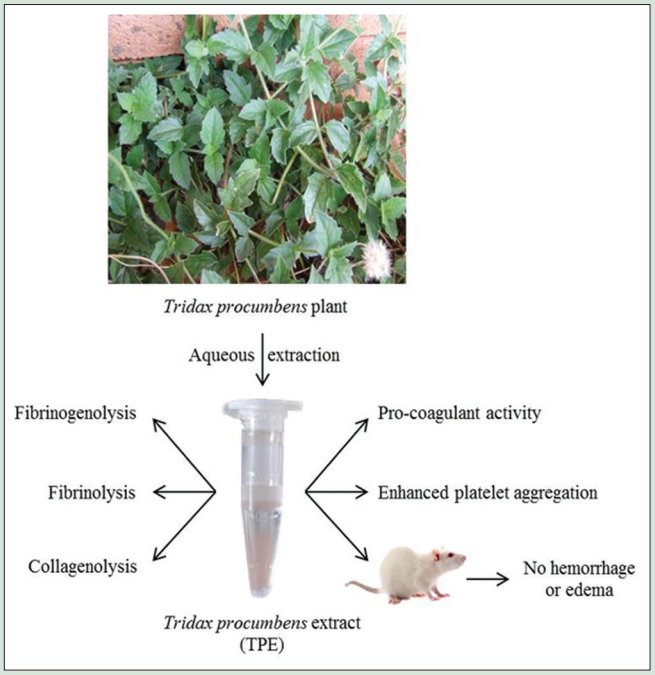
Background: *Tridax procumbens* extract (TPE) has been widely used in tribal/folk medicine to stop bleeding and to enhance wound healing process. Based on its traditional medicinal importance, the TPE is evaluated for its possible role in blood coagulation cascade. **Objective:** This study has been conducted to evaluate the TPE for the presence of protease associated with fibrino (geno) lytic, collagenolytic properties, and its action on blood coagulation cascade and platelet aggregation to substantiate its procoagulant nature. **Materials and Methods:** The TPE was analyzed *in vitro* for proteolytic, fibrinogenolytic, and collagenolytic activity. It was also analyzed for its effect on blood coagulation and platelet aggregation. *In vivo* studies have been conducted for hemorrhagic and edema inducing activity. **Results:** A non-toxic serine protease having procoagulant property associated with fibrino (geno) lytic, collagenolytic (both type I and type IV), and platelet aggregation inducing was identified and evaluated from TPE. The TPE decreased the clotting time of human plasma as evaluated by recalcification time and partial thromboplastin time by 19.8 and 1.53 folds, respectively. This suggests its procoagulant nature. TPE also enhanced the adenosine diphosphate-/epinephrine-induced platelet aggregation by 1.35 and 1.38 folds, respectively. **Conclusion:** The TPE serine protease is a non-toxic procoagulant with fibrino (geno) lytic and collagenolytic activities and induces platelet aggregation. Further, isolation and characterization of active molecule in TPE will allow us to exploit the pharmacological potential of TPE on coagulation cascade.

Key words: Fibrinogenolytic, fibrinolytic, platelet aggregation, procoagulant, *Tridax procumbens*

SUMMARY

- The present study identifies and evaluates the presence of serine protease in TPE. The protease exhibits procoagulant activity associated with fibrino (geno) lytic and collagenolytic properties. It also enhances platelet aggregation induced by adenosine diphosphate and epinephrine.

Abbreviations Used: TPE: *Tridax procumbens* extract; ECM: Extracellular matrix; ADP: Adenosine diphosphate; PMSF: Phenylmethylsulfonyl fluoride; EDTA: Ethylenediaminetetraacetic acid; APTT: Activated partial thromboplastin time; RT: Recalcification time; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; PAS: Periodic acid-Schiff; TCA: Trichloroacetic acid; PPP: Platelet-poor plasma; PRP: Platelet-rich plasma; PBS: Phosphate buffered saline; MHD: Minimum hemorrhagic dose; MED: Minimum edema dose; BSA: Bovine serum albumin; SEM: Standard error of mean.



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INTRODUCTION

Hemostasis is a process of blood coagulation to stop bleeding from damaged blood capillaries. This includes cascade of steps involving different clotting factors. The hemostatic system demands a perfect balance between the coagulation (fibrin formation) and fibrinolysis (fibrin dissolution) to prevent excessive blood loss during tissue injury. Any disruption in this balance may cause thrombosis or hemorrhage.[1] Wound healing is a complex coordinated physiological process of restoring tissue integrity, which comprises several overlapping phases such as hemostasis, inflammation, proliferation, and remodeling of extracellular matrix (ECM).[2,3]

Hemostasis also plays a key role in wound healing by stopping excess bleeding and initiating the wound healing process by activating blood coagulation cascade by fibrinogenolysis/activation of coagulation factor such as Factor X.[4] Blood coagulation is a sequential chain of events involving many inactive precursors of enzymes synthesized from liver

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and blood cells.[5] These enzymes are termed as coagulation factors which on activation further activates the next factor in the cascade by proteolysis. Blood coagulation takes place in either intrinsic or extrinsic pathways which follows to a common pathway with activated factor V complex or prothrombin activator.[6] The activated factor V complex converts the prothrombin (zymogen form) to thrombin (active form), which is a serine type of protease. Thrombin converts the soluble plasma fibrinogen to soft insoluble fibrin clot.[7,8] The fibrin monomers in fusion with platelet contractile protein undergo polymerization and form a strong hemostatic plug and arrest the bleeding. Later, plasmin (a serine protease) cleaves fibrin clot facilitating the wound healing process and restoring normal blood flow.[9] Thus, proteases play an important role in checking the excessive bleeding from the fresh cut and open wounds.[10]

Tridax procumbens is a common plant which is a native of tropical America but distributed in tropical Africa, Australia, and Asia. It is extensively used in the Indian Ayurvedic system and tribal medicine as procoagulant to stop bleeding and to enhance wound healing process. Different extracts of the plant showed antioxidant, anti-inflammatory, antihypertensive, anticancer, and antimicrobial activities.[11] However, there are no data available for the presence of protease and its possible role in blood coagulation cascade. Thus, the present study is the first report on the identification and evaluation of a serine protease from *T. procumbens* aqueous extract.

MATERIALS AND METHODS

Plant material

The *T. procumbens* plant was collected near Gubbi, about 20 Km from Tumkur district, Karnataka, India (GPS 13.30°51'27"N, 76.95°25'26"E). The plant was authenticated by Dr. P. Sharanappa, Professor, Department of Studies and 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), casein, gelatin (from porcine skin), epinephrine, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), Pepstatin A, and E-64 were purchased from Sigma Aldrich (St Louis, MO, USA). Activated partial thromboplastin time (APTT) kit was purchased from Tulip group (Goa, India). All other reagents used were of analytical grade.

Animals

Adult male Swiss Albino mice (20–25 g) were obtained from the Central Animal House Facility, Shree Siddaganga College of Pharmacy, BH Road, Tumkur, Karnataka State, India. The animal care and experimental procedures performed were in compliance with the Institutional Animal Ethics Committee constituted under Committee for the Purpose of Control and Supervision of Experiments on Animals rules, India with reference number SSCPT/IAEC. Clear/152/2016-17.

Tridax procumbens extract (TPE) preparation

The collected *T. procumbens* plants were shade-dried. The leaves and stem were collected, weighed, and homogenized in a blender, and extraction was carried out stirring the crushed plant in distilled water on a magnetic stirrer at 500 rpm at room temperature for 24 h. The extract was filtered through muslin cloth, and the filtrate was centrifuged at 8000 rpm for 20 min at 4°C. The supernatant was collected, lyophilized, weighed, and stored at −20°C until further use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli.[12] Briefly, the TPE (60 μg) was prepared by keeping in boiling water bath for 3 min, containing non-reducing sample buffer. Standard molecular weight markers ranging from 7 to 175 kDa were used. These were loaded onto 12% SDS-PAGE, and electrophoresis was carried out using Tris (25 mM), glycine (192 mM), and SDS (0.1%) for 2 h at 90 V at room temperature. After electrophoresis, the gel was stained with 0.25% coomassie brilliant blue R-250 to visualize the protein bands.

Gelatin zymogram

Zymogram was carried out according to the method of Laemmli.[12] Briefly, the 12% gel was incorporated with 0.2% gelatin as substrate for the detection of proteolytic activity. The TPE (25 μg) was incubated with SDS-PAGE non-reducing sample buffer for 30 min at 37°C, and electrophoresis was carried out at 90 V at room temperature. After electrophoresis, the gel was washed with 2.5% of Triton X-100 for 1 h to remove SDS. The gel was incubated in incubation buffer containing Tris–HCl (50 mM, pH 8), $CaCl_2$ (10 mM), and NaCl (150 mM) for 18 h at 37°C. The gel was then stained with 0.25% Coomassie brilliant blue R-250 to observe the translucent activity bands.

Periodic acid Schiff's base (PAS) staining

PAS staining was done according to the method of Leach *et al*.[13] Briefly, SDS-PAGE was performed as mentioned earlier. After electrophoresis, 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 for 45 min at 4°C. Later, the gel was soaked in Schiff's reagent and stored overnight at 4°C. The gel was destained with 10% acetic acid to view the reddish pink bands.

Proteolytic activity

Proteolytic activity was assayed according to the method of Satake *et al*.[14] using casein (2% in 200 mM Tris – HCl buffer, pH 8.0) as substrate. Briefly, 0.4 ml of casein was incubated with different concentration of TPE (0, 25, 50, 75, 100, 150, and 200 μg) for 2.5 h at 37°C. The reaction was terminated by the addition of 1.5 ml of 0.44 M trichloroacetic acid and allowed to stand for 30 min. The mixture was centrifuged at 3000 rpm for 5 min. The supernatant (1 ml) was used to determine the release of free amino acids (tyrosine) at 660 nm by incubating with 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of 1:2 diluted Folin–Ciocalteu's reagent for 20 min. The color developed was read at 660 nm. One unit (U) of proteolytic activity was defined as the amount of enzyme required to increase in absorbance of 0.01 at 660 nm in 1 min. Proteolytic activity was expressed as units/mg/min.[15]

pH and temperature kinetics study

The pH and temperature kinetics study was conducted to determine optimum condition for proteolytic activity of TPE. For pH kinetics, study substrate casein (2%) was prepared in different pH buffers (sodium acetate [pH 4.0–5.0], sodium phosphate [pH 6.0–7.0], and Tris–HCl [pH 8.0–10.0]), and proteolytic activity was assayed as mentioned earlier. For temperature kinetics study, the proteolytic activity of TPE was measured by assaying at different temperatures (4°C–60°C). Residual activity was analyzed for optimum pH and temperature.

Effect of protease inhibitors

The nature of protease activity was determined by using various standard protease inhibitors such as EDTA, E-64, PMSF, and pepstatin A at 5 mM

concentration. The TPE (3 μg) was preincubated with inhibitors for 30 min at 37°C. Proteolytic activity was performed by fibrinogenolytic activity assay, and the percentage of inhibition by the standard protease inhibitors was calculated by measuring the density of the bands using the ImageJ 1.49 v data analysis software (National Institutes of Health, Bethesda, Maryland, United States).

Fibrinogenolytic activity assay

The fibrinogenolytic activity assay was performed as described by Gubbiveeranna *et al.*[16] Briefly, human fibrinogen (50 μg) was incubated with different concentration of TPE (0, 0.05, 0.1, 0.25, 0.5, 1, 2, and 3 μg) in 30 μL of Tris-HCl buffer (50 mM, pH 8.0) for 3 h at 37°C. The reaction was terminated by adding 10 μL of reducing sample buffer containing 1 M urea, 4% SDS, and 4% β-mercaptoethanol and kept in boiling water bath for 3 min. The hydrolyzed products were analyzed on 12% SDS-PAGE by staining with Coomassie brilliant blue R-250.

Fibrinolytic activity assay

Fibrin degradation was analyzed according to the method of Shivaiah and Kempaiah.[17] Briefly, 100 μL of platelet poor plasma (PPP) was mixed with equal volume of 25 mM $CaCl_2$ solution at 37°C to get the soft fibrin clot. The fibrin clot formed was transferred into a separate Eppendorf tube and washed thoroughly for 5–6 times with 10 mM sodium phosphate buffer, pH 8.0. The washed fibrin clot was incubated with varying concentration of TPE (0, 0.4, 0.8, 1.2, and 1.6 μg) in 40 μL Tris-HCl buffer (50 mM, pH 8.0) for 3 h at 37°C. After incubation, the reaction was terminated by adding 20 μL of reducing sample buffer containing 1 M urea, 4% SDS, and 4% β-mercaptoethanol 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 analyze the fibrin degradation.

Degradation of extracellular matrix proteins

Collagen type-I and type-IV was incubated with different concentration of TPE (0, 2, 4, 6, and 8 μg for type-I and 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 μg for type-IV, respectively) in a total volume of 30 μL of Tris-HCl buffer (50 mM, pH 8.0), for 3 h at 37°C . The reaction was stopped by adding 5 μL denaturing sample buffer containing SDS and β-mercaptoethanol and kept in boiling water bath for 3 min. The degradation products were analyzed on 7% SDS-PAGE and stained with 0.25% Coomassie Brilliant Blue R-250.

Coagulation studies

Recalcification time

Recalcification time (RT) was determined according to the method described by Condrea *et al.*[18] To study the RT, human blood was obtained from healthy volunteers who were not under medication within the previous 10 days. The blood was collected in 3.2% tri-sodium citrate in the ratio 9:1. The citrated human blood was centrifuged at 3000 rpm for 20 min at 37°C to obtain PPP. PPP (100 μL) was incubated with different concentration of TPE (0, 10, 20, 30, and 40 μg in a volume of 20 μL of sodium phosphate buffer) in borosilicate tubes for 3 min at 37°C. Later, 100 μL of 25 mM $CaCl_2$ was added to initiate clotting, and the clotting time was recorded. The sodium phosphate buffer (10 mM, pH 8.0) alone without the TPE was taken as the control.

Activated partial thromboplastin time

The effect of TPE on APTT of PPP was measured using commercial kits. Briefly, PPP (100 μL) was incubated with different concentration of TPE (0, 10, 20, 30, and 40 μg in a total volume of 20 μL of sodium phosphate buffer) in borosilicate tubes at for 3 min 37°C. After incubation, 100 μL of liquicellin E reagent (platelet substitute) was added, and tubes were incubated for 5 min at 37°C. Later, 100 μL of 25 mM $CaCl_2$ was added to initiate clotting, and the clotting time was recorded. The sodium phosphate buffer (10 mM, pH 8.0) alone without the TPE was taken as the control.

Effect of TPE against adenosine diphosphate/epinephrine-induced platelet aggregation

Platelet aggregation was monitored by light transmission in Chronolog model 700-2D optical lumi-aggregometer. Human blood was obtained from healthy volunteers who were not under medication within the previous 10 days. The blood was collected in 3.2% tri-sodium citrate in the ratio 9:1. Platelet-rich plasma (PRP) was prepared by centrifuging citrated human blood for 15 min at 900 rpm. The supernatant was called PRP. The remaining blood was centrifuged at 3000 rpm for 20 min, and the supernatant was called PPP. The PRP (0.25 ml) was transferred to siliconized glass cuvette containing a Teflon-coated stir bar. The sample was maintained at 37°C and was allowed to reach equilibrium with a constant stirring speed of 1200 rpm. The instrument was calibrated, and aggregation was initiated by adenosine diphosphate (ADP)/epinephrine. To study the effect of TPE, PRP was preincubated with different concentration of TPE (0, 10, 20, and 30 μg) for 5 min before the addition of ADP/epinephrine. The extent of aggregation was continually monitored by optical mode for the change in turbidity for 6 min in which PRP and PPP independently represented 0 and 100% transmittance, respectively.

Hemolytic activity

The hemolytic activity of TPE was determined against mammalian washed erythrocytes, as described by Shin *et al.*[19] Briefly, human blood was obtained from healthy volunteers who were not under medication within the previous 10 days. The blood was collected in 3.2% tri-sodium citrate in the ratio 9:1.The citrated human blood was centrifuged at 3000 rpm for 20 min at 37°C. The supernatant was discarded, and the pellet was washed thrice with cold saline (0.9%). Packed erythrocytes (1 ml) were suspended in 9 volumes of phosphate-buffered saline. The erythrocytes suspension (1 mL) was incubated with different concentration of TPE (0, 10, 20, 30, and 40 μg). The reaction volume was made up to 2 ml with saline and was incubated for 30 min at 37°C. After incubation, 2 ml of saline was added and centrifuged at 1500 rpm for 2–3 min at 4°C. The amount of hemoglobin released into the supernatant was measured at 540 nm. Saline and distilled water were used as minimal and maximal hemolytic controls, respectively.

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo *et al.*[20] Briefly, TPE (250 and 500 μg) in saline was injected intradermally into groups of six mice each. Group receiving saline alone served as negative control and group receiving *Daboia russelii* venom (20 μg, minimum hemorrhagic dose [2MHD]) served as positive control. Mice were anaesthetized using diethyl ether inhalation and sacrificed after 3 h. Dorsal patch of skin surface was carefully removed without stretching, and the inner surface was observed for hemorrhagic spot against saline-injected control mice. The MHD was evaluated as the amount required to produce a hemorrhagic spot of 10 mm.

Edema-inducing activity

Edema-inducing activity was done according to the method of Vishwanath *et al.*[21] Briefly, TPE (250 and 500 μg) in a total volume of 20 μL saline was injected into the right foot pads of a group of six mice each. Left foot pads of the mice received only saline and served

as controls. Mice were anaesthetized using diethyl ether inhalation and sacrificed after 2 h. 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 was defined as the amount required to cause an edema ratio of 120%.

Protein estimation

Protein concentration was determined according to the method of Lowry *et al.*[22] In this method, bovine serum albumin (BSA) was used as standard. The protein concentration of TPE was measured by comparing with known concentration of BSA.

Statistical analysis

The results are expressed as the means ± standard error of mean. Data were analyzed by one-way ANOVA and *post hoc* Dunnett *t*-test for multiple comparisons. $P < 0.05$ was accepted as statistically significant.

RESULTS

Properties of TPE

TPE was subjected to 12% SDS-PAGE under non-reducing condition. SDS-PAGE pattern of the TPE showed protein bands distributed between 200 and 30 kDa molecular weights range as compared with the standard molecular weight markers. PAS staining of the gel did not reveal any pink-colored bands suggesting that no glycoproteins were present (data not shown). *T. procumbens* extract prepared contained 2 mg/mL of the protein concentration as analyzed by Lowry's method. TPE was analyzed for proteolytic activity using casein as substrate, and the specific activity was found to be 3.33 units/mg/min. The proteolytic activity was supported by zymogram assay, where TPE showed translucent activity bands in the high molecular weight region [Figure 1]. The effect of pH [Figure 2a] and temperature [Figure 2b] on the proteolytic activity was analyzed. The enzyme activity of TPE was measured under standard proteolytic activity assay condition with different pH buffers and at different temperature conditions using casein as substrate. Residual activity was calculated to analyze the optimum pH and temperature. The optimum pH and temperature for the proteolytic activity was found to be pH 8.0 and 37°C, respectively.

Effect of protease inhibitors on TPE

TPE was incubated with standard protease inhibitors such as EDTA, E-64, PMSF, and pepstatin A for 30 min and assayed for proteolytic activity. The proteolytic activity of TPE was completely inhibited by PMSF, suggesting the presence of serine type of protease [Table 1].

Fibrino(geno)lytic activity of TPE

The pharmacological study of TPE was studied using human fibrinogen and fibrin clot. The human fibrinogen is a 340 kDa soluble plasma glycoprotein composed of three subunits (Aα, Bβ, and γ). TPE was studied for fibrinogenolytic activity, and the hydrolysis of fibrinogen was examined using SDS-PAGE. The TPE showed fibrinogenolytic activity in a concentration dependent manner [Figure 3]. The TPE cleaved all the subunits of fibrinogen (Aα, Bβ and γ) with preferential cleavage in the order of Aα>Bβ>γ. The Aα and Bβ subunits of fibrinogen were more susceptible for hydrolysis by TPE compared to γ subunit. All the subunits were hydrolyzed at a concentration of 3 μg.

The TPE showed fibrinolytic activity as analyzed by SDS-PAGE. TPE partially degraded cross-linked human fibrin clot prepared from human plasma. The α-polymer and α-chains were completely degraded at a concentration of 1.6 μg, whereas γ-dimer and β-chains were partially degraded [Figure 4]. This confirms that fibrin was more resistant to hydrolysis by TPE compared to the fibrinogen.

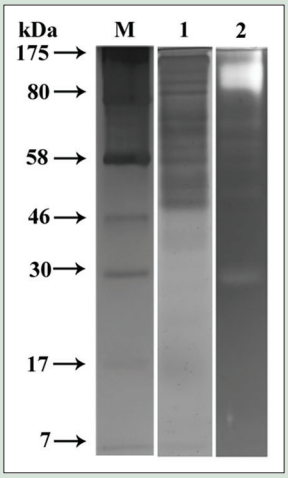

Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis and zymogram assay. The TPE was loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) under non-reducing condition. For gelatinolytic zymogram, TPE was preincubated with non-reducing sample buffer for 30 min at 37°C and loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) incorporated with 0.2% gelatin. Electrophoresis was carried out, and the gel was washed with 2.5% Triton X-100 for 1 h to remove sodium dodecyl sulfate and incubated in incubation buffer for 18 h at 37°C. Gels were stained with Coomassie Blue R-250. Lane M: Molecular weight marker in kDa; Lane 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of TPE (60 μg); Lane 2: Gelatin zymogram of TPE (25 μg)

Table 1: Effect of protease inhibitors on *Tridax procumbens* extract

Inhibitor	Percentage of inhibition
PMSF	100
E-64	22
EDTA	25
Pepstatin A	12

TPE (3 μg) was preincubated separately with 5 mM of standard protease inhibitors EDTA, E-64, PMSF and pepstatin A for 30 min at 37°C. The samples were run on SDS-PAGE under reduced condition. The results are expressed as percentage of inhibition using the ImageJ 1.49v software. TPE: *Tridax procumbens* extract; PMSF: Phenylmethylsulfonyl fluoride; EDTA: Ethylenediaminetetraacetic acid

Effect of TPE on extracellular matrix molecules

TPE was studied for its effects on collagen type I and type IV molecules. TPE degraded the subunits of collagen type I and type IV of ECM molecules in a dose-dependent manner. In collagen type I, both α1 and α2 – chains were most susceptible for hydrolysis compared to the other subunits. The α – chains were hydrolyzed at a concentration of 8 μg while the β and γ chains were more resistant to TPE hydrolysis compared to α1 and α2 subunits [Figure 5a].

TPE cleaved all the subunits of collagen type IV in a preferential manner (α2> α1). The α2 chain was hydrolyzed at 15 μg concentration while the α1 subunit was degraded at 20 μg concentration [Figure 5b]. This suggests that collagen type IV was more resistant to hydrolysis by TPE compared to collagen type I.

Coagulation studies

The effect of TPE on blood coagulation was analyzed by RT and APTT assays. TPE on incubation with plasma reduced the RT in a

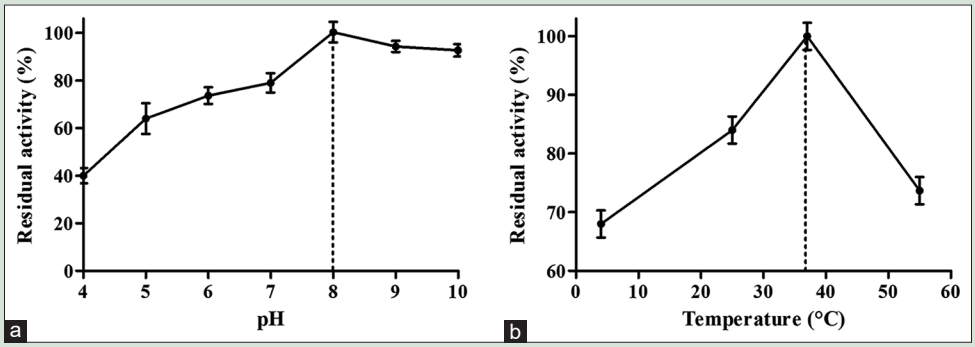

Figure 2: Effect of pH and temperature on proteolytic activity of TPE. (a) Effect of pH was analyzed using casein substrate in different pH buffers (sodium acetate pH 4–5, sodium phosphate pH 6–7, Tris – HCl pH 8–10) containing 0.3 M NaCl. (b) Effect of temperature was analyzed by assaying proteolytic activity at various temperature (4°C–60°C). Residual activity was analyzed for optimum pH and temperature. Each value in the figures represents as mean ± standard error of mean

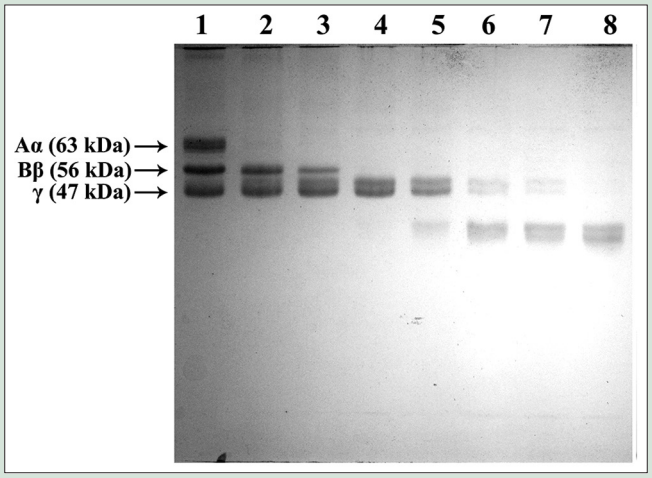

Figure 3: Fibrinogenolytic activity of TPE. Human fibrinogen (50 μg) was incubated with different concentration of TPE for 2.5 h at 37°C. sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) was performed in reducing condition to visualize degradation pattern. Lane 1: Fibrinogen (50 μg); Lanes 2–8: Fibrinogen incubated with 0, 0.05, 0.1, 0.25, 0.5, 1, 2 and 3 μg of TPE, respectively

dose-dependent manner. The normal RT of plasma is 198 s, and with 40 μg of TPE, the clotting time was reduced to 10 s [Figure 6]. This confirms that TPE reduced the clotting time by 19.8 folds.

TPE upon incubation with plasma reduced the APTT in a dose-dependent manner. The normal APTT of plasma is 43 sec, and with 40 μg of TPE, the clotting time was reduced to 28 s [Figure 7]. Thus, TPE reduced the APTT of the plasma by 1.53 folds. These studies show that the TPE is procoagulant in nature and that it predominantly interferes in the common pathway of blood coagulation when compared to the intrinsic pathway.

Platelet aggregation studies

The effect of TPE on platelet aggregation was studied using PRP. TPE enhanced the platelet aggregation induced by agonists ADP/epinephrine in a concentration dependent manner [Figure 8]. TPE (30 μg) enhanced the platelet aggregation induced by ADP and epinephrine to an extent of 35% and 38%, respectively [Figure 9]. This shows that the TPE increased the ADP- and epinephrine-induced platelet aggregation by 1.35 and 1.38 folds, respectively.

Hemolytic, hemorrhagic, and edema-inducing activities

The TPE did not show hemolysis of erythrocytes indicating it is devoid of hemolytic activity. The TPE did not induce hemorrhage [Figure 10] and edema in the mice animal model study indicating its non-toxic nature.

DISCUSSION

T. procumbens plant has been applied topically on fresh wounds to arrest bleeding and to enhance wound-healing process.[11] The present study has been taken up to validate its traditional usage. This is the first report on identification and evaluation of protease from *T. procumbens* aqueous extract.

TPE was subjected to SDS-PAGE which showed protein bands distributed in high and medium molecular weight range. The protein concentration was found to be 2 mg/mL as analyzed by Lowry's method. TPE exhibited proteolytic activity with specific activity of 3.33 units/mg/min. The optimum activity was observed at pH 8.0 and at 37°C.

In zymogram assay, TPE showed translucent activity band at high molecular weight range. Similar high molecular weight proteases have been reported from medicinal plants such as *Cucumis dipsaceus,*[23] *Oroxylum indicum, Adhatoda vasica, Pongamia pinnata, Acalypha indica, Wrightia tinctoria, Caralluma attenuata, Caralluma umbellata, Morinda citrifolia, Rhinacanthus nasutus, Madhuca longifolia, Datura inoxia, Punica granatum, Lawsonia inermis, Pleurostylia opposita* and *Curcuma longa*.[24]

The proteolytic activity of TPE was inhibited by PMSF suggesting the presence of serine type of protease in TPE. Generally, proteases reported from medicinal plants/latex belong to either serine or cysteine protease class.[25,26]

Proteases are found to be responsible for the observed pharmacological activities in the TPE. TPE hydrolyzed fibrinogen and fibrin in a dose-dependent manner. Fibrinogen, a soluble glycoprotein, is mainly involved in blood coagulation. Fibrinogen, a soluble glycoprotein, is mainly involved in blood coagulation. Upon hydrolysis by thrombin (a serine protease), produces fibrinopeptides A and B. These fibrinopeptides polymerises to form the fibrin soft clot. Fibrin soft clot stabilized by platelet factors forms hard clot. During wound-healing process, plasmin is the key proteolytic enzyme that degrades fibrin clot into soluble fragments, and this is a significant event associated with wound-healing process. TPE hydrolyzed both fibrinogen and fibrin in a dose-dependent manner. Degradation of fibrinogen-forming fibrinopeptides may help in enhancing blood coagulation process. Similarly, degradation of fibrin

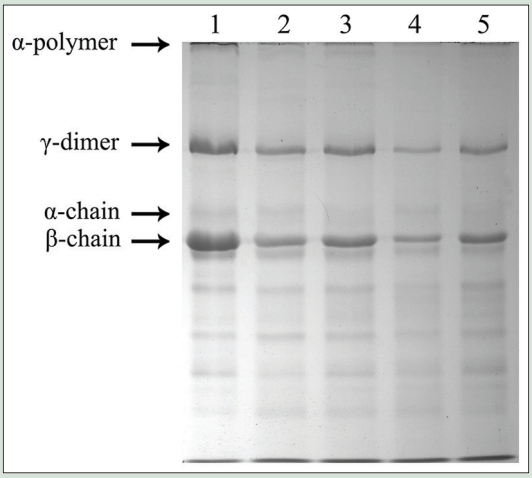

Figure 4: Fibrinolytic activity of TPE. Fibrin clot was incubated with different concentration of TPE for 3 h at 37°C. sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) was performed in reducing condition to visualize the degradation pattern. Lane 1: Fibrin clot (control); Lanes 2–5: Fibrin clot incubated with 0, 0.4, 0.8, 1.2, and 1.6 μg of TPE, respectively

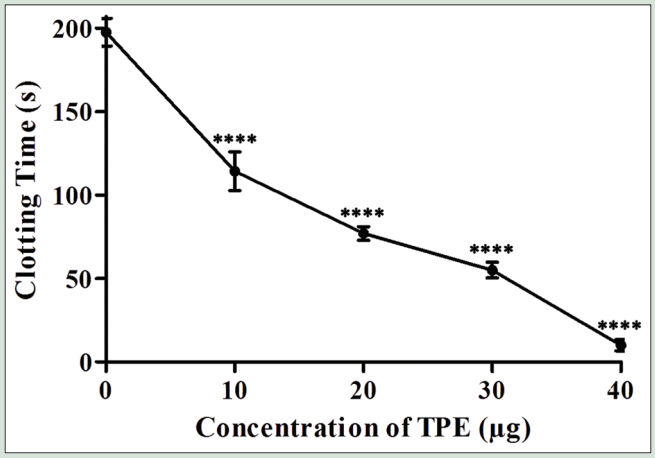

Figure 6: Recalcification time. Platelet-poor plasma (100 μL) was incubated separately with different concentration of TPE (0, 10, 20, 30, and 40 μg) in sodium phosphate buffer (10 mM, pH 8.0) in borosilicate tubes for 3 min at 37°C. Clotting was initiated by adding 100 μL of $CaCl_2$ (25 mM) and clotting time was recorded. The sodium phosphate buffer (10 mM, pH 8.0) alone served as control. Each value represents as mean ± standard error of mean (n = 3). P < 0.0001 was considered as significant

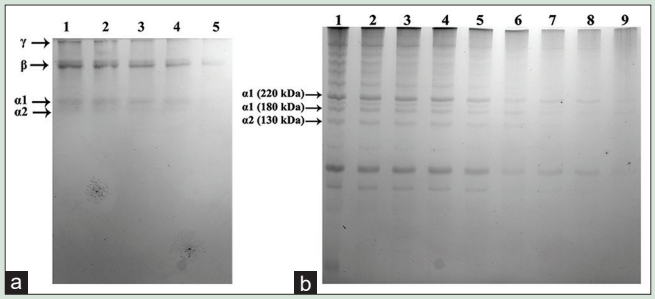

Figure 5: Extracellular matrix degradation by TPE. Collagen type I (10 μg) and type IV (25 μg) were incubated separately with different concentration of TPE for 2.5 h at 37°C. sodium dodecyl sulfate polyacrylamide gel electrophoresis (7%) was performed in reduced condition to visualize the degradation pattern. (a) Lane 1: Collagen type I (10 μg); Lanes 2–5: Collagen type I incubated with 0, 2, 4, 6 and 8 μg of TPE, respectively; (b) Lane 1: Collagen type IV (25 μg); Lanes 2–9: Collagen type IV incubated with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 μg of TPE, respectively

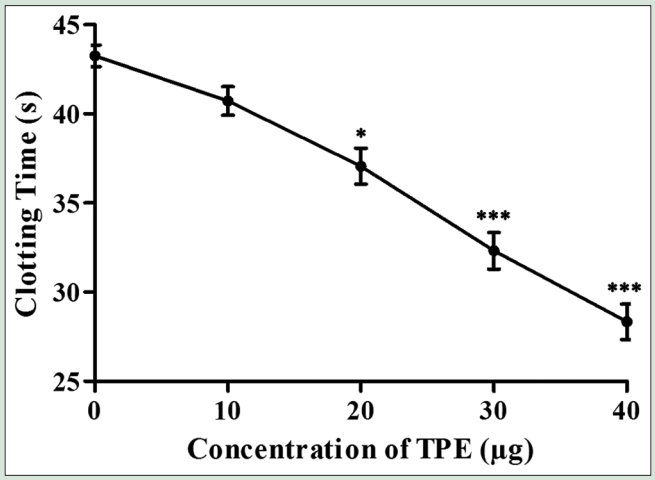

Figure 7: Activated partial thromboplastin time. Platelet-poor plasma (100 μL) was incubated with different concentration of TPE (0, 10, 20, 30, and 40 μg) in sodium phosphate buffer (10 mM, pH 8.0) for 3 min at 37°C. After incubation, liquicellin E reagent (100 μL) was added and incubated for 5 min at 37°C. Clotting was initiated by adding 100 μL of $CaCl_2$ (25 mM) and clotting time was recorded. The sodium phosphate buffer (10 mM, pH 8.0) alone served as control. Each value represents as mean ± standard error of mean (n = 3). P < 0.0001 was considered as significant

helps in enhancing wound-healing process. Several serine proteases from medicinal plants/latex have been reported to arrest bleeding and also enhance wound-healing process.[27,28]

TPE exhibited pronounced procoagulant activity by reducing the RT by 19.8 folds and APTT by 1.53 folds. This suggests the interference of TPE in common pathway and intrinsic pathway of blood coagulation. The observed procoagulant property might be partially due to the fibrinogenolytic activity of TPE.

In platelet aggregation study, TPE enhanced the agonist-induced platelet aggregation. Platelets and its aggregation play a key role in hemostasis. Platelets' aggregation together with fibrin stabilizes the platelet plug formation which stops bleeding from minor injuries. TPE along with ADP and epinephrine enhanced aggregation to 1.35 and 1.38 folds, respectively. This substantiates the *T. procumbens* usage as topical applicant to stop bleeding from injuries.

TPE was devoid of hemolytic activity. Further, TPE is non-edematous and non-hemorrhagic in nature as studied in mice model.

CONCLUSION

Ethnomedicines are regarded as reservoirs of impending therapeutic molecules that may affect the coagulation cascade. The present study is the first report on the identification and evaluation of a serine protease from *T. procumbens* aqueous extract.

The procoagulant and fibrinogenolytic property with moderate fibrinolytic action of TPE provide the evidence for possible role in forming hemostatic plug to arrest bleeding in fresh cuts and further initiating the dissolution of fibrin clot, thus enhancing the process

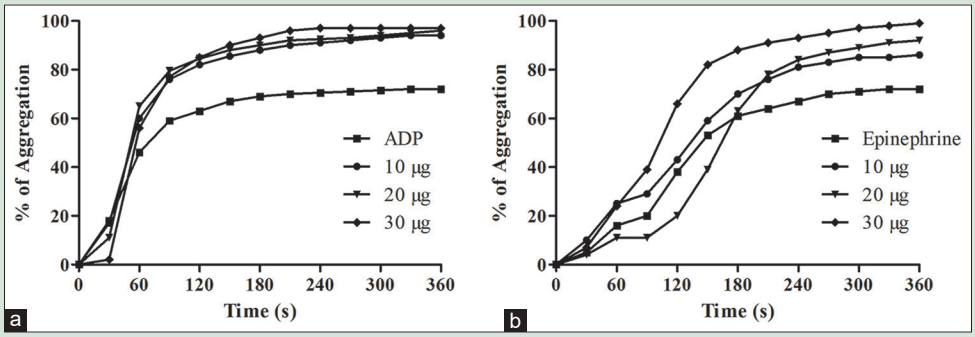

Figure 8: Effect of TPE on platelet aggregation. Platelet aggregation was monitored by light transmission in chrono-log 700 model. Platelet-rich plasma 250 µL was incubated with different concentration of TPE (0, 10, 20, and 30 µg) with continuous stirring for 5 min at 37°C. Platelet aggregation was monitored after addition of agonist adenosine diphosphate/epinephrine to platelet-rich plasma and change in transmittance was monitored for 6 min. (a) Effect of TPE on adenosine diphosphate-induced platelet aggregation and (b) effect of TPE on epinephrine-induced platelet aggregation

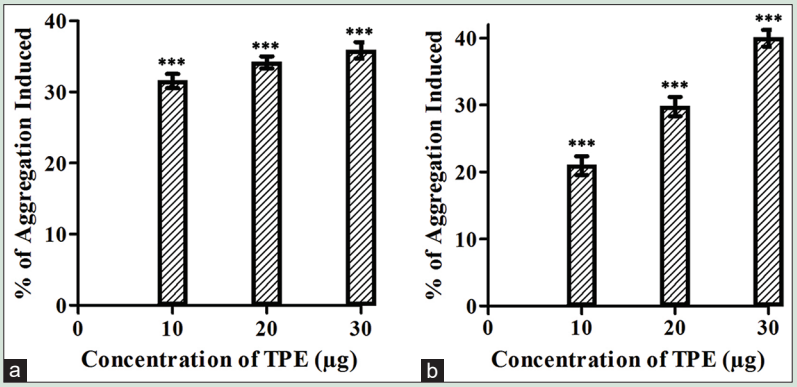

Figure 9: Percentage of platelet aggregation induced. Platelet aggregation without the enzyme was considered as control and the aggregation induced by TPE (0, 10, 20, and 30 µg) when compared to that of control (adenosine diphosphate/epinephrine alone) was calculated. (a) Increase in % of adenosine diphosphate-induced platelet aggregation when treated with TPE; (b) increase in % of epinephrine-induced platelet aggregation when treated with TPE. Each value represents as mean ± standard error of mean ($n = 3$). $P < 0.0001$ was considered as significant

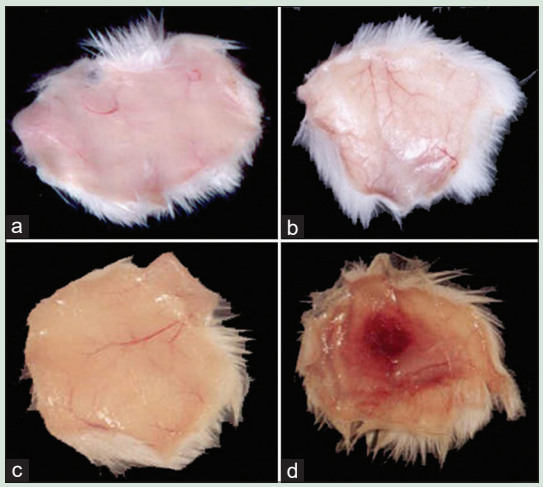

Figure 10: Hemorrhagic activity. Different concentration of TPE (250 and 500 µg) was injected intradermally into groups of six mice each in a total volume of 50 µL saline. Group receiving saline alone served as negative control and group receiving *D. russelii* venom (20 µg, minimum hemorrhagic dose) served as positive control. After 3 h, mice were sacrificed, dorsal patch of skin surface was removed, and the diameter of hemorrhagic spot was measured. (a) Saline, (b) TPE (250 µg), (c) TPE (500 µg), and (d) *D. russelii* venom (20 µg, 20 µg, minimum hemorrhagic dose)

of wound healing. Thus, evaluation of TPE provided insight into its pharmacological property and thereby substantiates its use in arresting bleeding in fresh cuts and initiate wound-healing process.

Further isolation and characterization for active molecule is in progress which may lead to decipher the molecular pathways of blood coagulation.

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Conflicts of interest

There are no conflicts of interest.

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