ORIGINAL ARTICLE

Anticoagulant, Antiplatelet and Fibrin Clot Hydrolyzing Activities of Flax Seed Buffer Extract

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ABSTRACT

Background: Flax seeds possess long array of medicinal qualities as it found to show beneficial effects on cardiovascular, hypertension, diabetes, cancer, inflammatory and autoimmune disorders. While, the therapeutic role of flax seed proteins on thrombotic disorder is least explored. Objective: The current study aims to identify the fibrin clot-dissolving, anticoagulant and antiplatelet properties of flaxseed buffer extract (FSBE) using Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP). **Materials and Methods:** Flax Seed Buffer Extract (FSBE) proteins were characterized upon subjected to SDS-PAGE. The presence of cysteine protease in the extract was identified using colorimeter and zymography experiments. Anticoagulant activity was established using invitro recalcification time. While, the fibrinogenolytic, fibrinolytic and human plasma proteins degradation activities were proved using SDS-PAGE. Furthermore, antiplatelet activity was confirmed using Chronology dual channel whole blood/optical lumi aggregation system (Model-700). Toxicity studies were assayed using RBC cells and albino mice. **Results:** FSBE showed divergent protein banding pattern from the molecular mass ranging 200 kDa-14 kDa under both reduced and non-reduced conditions. The FSBE showed proteolytic activity as it hydrolyzed casein and gelatin with the specific activity 0.180 and 0.201 units/mg/min respectively. The proteolytic activity of FSBE was completely abolished only by IAA but PMSF, 1, 10 Phenanthroline and EDTA did not, revealing the presence of cysteine protease in the extract. FSBE showed anticoagulant activity as it enhanced the clotting time from control 220s to 320s. Furthermore, FSBE showed antiplatelet activity by inhibiting ADP and Epinephrine induced platelet aggregation and the observed aggregation inhibition was found to be 63.6% and 16.3% respectively. In addition, it hydrolyzed specifically Aα and Bβ chains of human fibrinogen and all the chains of human fibrin clot. The fibrinogenolytic activity was inhibited by IAA but PMSF, EDTA, 1, 10 Phenanthroline did not, suggesting the role of cysteine proteases. Interestingly, FSBE did not cause edema and hemorrhage in the experimental mice and did not hydrolyze RBC cells suggesting its nontoxic properties.

Key words: Anticoagulant, antiplatelet, cysteine proteases, fibrinogenolysis, flaxseed buffer extract

SUMMARY

- Flax Seed Buffer Extract (FSBE) found to contain oligomeric proteins and exhibits cysteine proteolytic activity
- FSBE exhibited anticoagulant activity by specifically interfering in intrinsic pathway of blood coagulation
- FSBE inhibited agonist ADP and epinephrine induced platelet aggregation
- Furthermore, it dissolved fibrin clot.
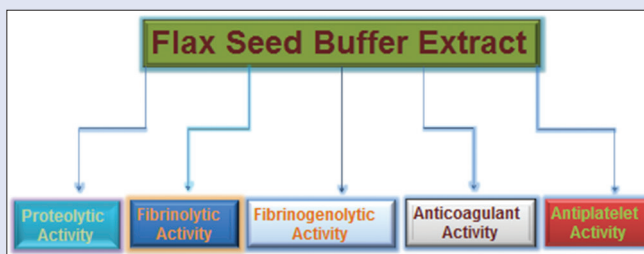
Abbreviations used: FSBE: Flaxseed buffer extract; PMSF: Phenylmethylsulfonyl fluoride; IAA: Iodoacetic acid; EDTA: Ethylenediaminetetraacetic acid; PRP: Platelet-rich plasma; PPP: Platelet-poor plasma; INR: International normalized ratio; MED: Minimum edema dose; MHD: Minimum hemorrhagic dose; APTT: Activated partial thromboplastin time; PT: Prothrombin time; HMWK: High-molecular-weight kininogen.

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INTRODUCTION

In recent time, the traditional medicine/drugs from plant origin have been gaining much importance. The plant-based drugs are continuously using as part of the primary medical emergencies to cure various ailments. Flaxseed is one such medicinally important seeds and belongs to the family of *Linaceae* grown in cooler regions of the world.[1] Flaxseed (*Linum usitatissimum*), traditionally known as Agasebija/Jawas/Alsi in Indian vernaculars, has been cultivated since 5000 BC.[2] The circular fruit encompasses two seeds in each of five compartments. The seeds are flat and oval with a keen tip. The seed is the storehouse for several varieties of phytoconstituents that includes majorly linolenic acid, linoleic acid, omega-3 fatty acids, lignans, cyclic peptides, polysaccharides, alkaloids, cyanogenic glycosides, and cadmium.[3] Flaxseed has the nutritional qualities; thus, the various

products of the flaxseed, namely, whole seed, ground whole seed, flaxseed oil, defatted flaxseed meal, flaxseed mucilage extracts, flaxseed hulls, flaxseed oleosomes, and flaxseed alcohol extracts are being used.[4] The mentioned seed extract found to exhibit long array of beneficial health

effects. Therefore, the flaxseed is widely used as medicine, especially in curing cardiovascular, hypertension, diabetes, cancer, inflammatory, and autoimmune disorders.[5] Flaxseeds helps to lower the blood cholesterol and reduce obesity. In addition, flaxseeds also used in food industry as curative agent. So far, fatty acids such as omega-3 fatty acids, linolenic acid, and cyanogenic glycosides have been characterized and their therapeutic potential has been established.[6] Although flaxseed shares about 35% of the protein content, their therapeutic efficacy is not yet explored. However, Nakai *et al.*, 1981, established only the functional properties of flaxseed proteins. So far, only high-molecular 12S globulin (linin) and the low-molecular-weight 2S conlinin were isolated.[7] Despite immense nutritional and therapeutic potential of various phytoconstituents of flaxseed extract, the beneficial role of proteins/enzymes, especially proteolytic enzymes, is least studied. Thus, the current study mainly focuses on the characterization of anticoagulant, antiplatelet, and fibrinolytic activities of cysteine proteolytic enzyme from the flaxseed extract and the results are presented.

MATERIALS AND METHODS

Reagents

Fat-free casein, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid (IAA), and 1,10-phenanthroline were purchased from Sigma Chemicals Company (St. Louis, USA). Molecular-weight markers were from Bangalore Genei Private Limited, India. Activated partial thromboplastin time (APTT) and prothrombin time (PT) reagents were purchased from AGAPPE Diagnostic Pvt., Ernakulam, Kerala, India. Human plasma fibrinogen was purchased from Sigma Chemicals Co., St. Louis, USA. All other chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the platelet-rich plasma (PRP).

Preparation of flaxseed buffer extract and protein estimation

Flaxseeds were purchased from local market Tumkur. The seeds were washed with distilled water and treated with 0.5M $NaHCO_3$ (1:8 W/V) with constant stirring for 1 h. Then, the mucilage part was removed by filtration and the seeds were washed thoroughly dried at room temperature for 24 h. Then, the dried seeds were powdered, dissolved in 50 mM Tris-HCl buffer, and centrifuged at 8000 g for 15 min, supernatant was collected, and proteins were precipitated using 30% of ammonium sulfate. The precipitated protein sample was again centrifuged at 3500 g for 20 min; pellet was dissolved and dialyzed overnight. The protein sample obtained was stored at −20°C until further use. Protein concentration was determined as described by Bradford *et al.*[8] using bovine serum albumin as standards.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and periodic acid–Schiff staining

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 10% was carried out according to the method of Laemmli.[9] The flaxseed buffer extract (FSBE) (100 μg) prepared under reducing and nonreducing conditions was used for SDS-PAGE. The electrophoresis was carried out using Tris (25 mM), glycine (192 mM), and SDS (0.1%) for 2 h at room temperature. After electrophoresis, the SDS-PAGE gels were stained with 0.1% Coomassie Brilliant Blue R-250 for detection of the protein bands and de-stained with 40% ethanol in 10% acetic acid and water (40:10:50 v/v). Molecular-weight standards from 200 kDa to 14.3 kDa were used.

Periodic acid–Schiff (PAS) staining was carried out according to the method of Leach *et al.*;[10] after electrophoresis, the gel was fixed in 7.5%

acetic acid solution and stored at room temperature for 1 h. Then, the gel was washed with 1% nitric acid solution and kept in 0.2% aqueous periodic acid solution and stored at 4°C for 45 min. After that, the gel was placed in Schiff's reagent at 4°C for 24 h and was de-stained using 10% acetic acid to visualize a pink color band.

Proteolytic activity by colorimetric method

Proteolytic activity was assayed as described by Satake *et al.*[11] Fat-free casein (0.4 ml, 2% in 0.2M Tris-HCl buffer, pH 7.6) was incubated with 50 μg of crude FSBE in a total volume of 1 ml for 2 h and 30 min at 37°C. Undigested casein was precipitated by adding 1.5 ml of 0.44M trichloroacetic acid (TCA) and left to stand for 30 min. The reaction mixture was then centrifuged at 2000 g for 10 min. Sodium carbonate (2.5 ml, 0.4M) and Folin–Ciocalteu's reagent (1:2) were added sequentially to 1 ml of the supernatant, and the color developed was read at 660 nm. One unit of the enzyme activity was defined as the amount of the enzyme required to cause an increase in optical density of 0.01 at 660 nm/min at 37°C. The specific activity was expressed as units/min/mg of protein. For inhibition studies, a similar reaction was performed independently after preincubating the crude FSBE (50 μg) for 30 min with 5 mM each of EDTA, 1,10-phenanthroline, PMSF, and IAA. In all the cases, appropriate controls were kept.

Zymogram

Zymogram was carried as described previously;[12] briefly, FSBE (50 μg and 100 μg) prepared under nonreduced condition was loaded on to polymerized 2% casein/gelatin in resolving gel. After electrophoresis, gels were washed with 10 mM sodium phosphate buffer containing 2.5% of Triton X-100 with constant agitation for 1 h to remove SDS. The gel was incubated overnight at 37°C in Tris-HCl buffer (50 mM) pH 7.6 containing 50 mM $CaCl_2$ and 40 mM NaCl. Gel was then stained to observe the translucent activity bands.

Anticoagulant activity
Plasma recalcification time

The plasma recalcification time was determined according to the method of Quick *et al.*[13] Briefly, the crude FSBE (15–75 μg) was preincubated with 0.2 ml of citrated human plasma in the presence of 10 mM Tris-HCl (20 μl) buffer pH 7.4 for 1 min at 37°C, 20 μl of 0.25M $CaCl_2$ was added to the preincubated mixture, and clotting time was recorded.

Activated partial thromboplastin time and prothrombin time

Briefly, 100 μl of normal citrated human plasma and FSBE (0–100 μg) were preincubated for 1 min. For APTT, 100 μl reagent (LIQUICELIN-E Phospholipids preparation derived from rabbit brain with ellagic acid), which was activated for 3 min at 37°C, was added. The clotting was initiated by adding 100 μl of 0.02M $CaCl_2$ and the clotting time was measured. For PT, the clotting was initiated by adding 200 μl of PT reagent (UNIPLASTIN–rabbit brain thromboplastin). The time taken for the visible clot was recorded in seconds. The APTT ratio and the international normalized ratio for PT at each point were calculated from the values of control plasma incubated with the buffer for identical period of time.

Fibrinogenolytic activity

Fibrinogenolytic activity was determined as described previously by Ouyang and Teng *et al.*[14] FSBE (0–100 μg) was incubated with the human plasma fibrinogen (50 μg) in a total volume of 40 μl of 10 mM Tris-HCl buffer pH 7.4 for 4 h at 37°C. After the incubation period, reaction was terminated by adding 20 μl denaturing buffer containing 1M urea, 4% SDS, and 4% β-mercaptoethanol. It was then analyzed by 10% SDS-PAGE. For inhibition studies, FSBE (100 μg) was

preincubated for about 20 min with 5 mM each of PMSF, IAA, EDTA, and 1,10-phenanthroline.

Fibrin clot-hydrolyzing activity by colorimetric method

Fibrin clot-hydrolyzing activity was determined as described by Rajesh *et al*.[15] Briefly, 100 µl of citrated human plasma was mixed with 20 µl of 0.2M CaCl$_2$ and incubated for 2 h at 37°C. The clot obtained was washed thoroughly for 5–6 times with phosphate-buffered saline (PBS) and suspended in 400 µl of 0.2M Tris-HCl buffer (pH 8.5). The reaction was initiated by adding varied amounts of FSBE (0–100 µg) in 100 µl of saline and incubated for 2 h and 30 min at 37°C. The undigested clot was precipitated by adding 750 µl of 0.44M TCA and allowed to stand for 30 min and centrifuged for 15 min at 1500 g. The aliquots of 0.5 ml supernatant were transferred to clean glass tubes and it was followed by the addition of 1.25 ml of 0.4M sodium carbonate and 0.25 ml of 1:2 diluted Folin–Ciocalteu's phenol reagent. The color developed was read at 660 nm after being allowed to stand for 30 min. One unit of activity is defined as the amount of enzyme required to increase in absorbance of 0.01 at 660 nm/h at 37°C.

Fibrinolytic activity by sodium dodecyl sulfate polyacrylamide gel electrophoresis

Fibrin clot was prepared as describe above, and the clot obtained was incubated with the various concentrations of FSBE (0–100 µg) in a final volume of 40 µl of 10 mM Tris-HCl buffer (pH 7.4) at 37°C for 6 h. The reaction was stopped by adding 20 µl of sample buffer containing 4% SDS, 1M urea, and 4% β-mercaptoethanol. The samples were kept on boiling water bath for 30 min and centrifuged to settle the debris of the plasma clot. An aliquot of 30 µl supernatant was analyzed in 7.5% SDS-PAGE for fibrin degradation study. For inhibition studies, FSBE (40 µg) was preincubated for about 15 min with 5 mM each of PMSF, EDTA, IAA, and 1,10-phenanthroline. The reaction was stopped by adding 20 µl of sample buffer containing 4% SDS, 1M urea, and 4% β-mercaptoethanol. The samples were kept on boiling water bath for 5 min and centrifuged to settle the debris of plasma clot. An aliquot of 30 µl supernatant was analyzed in 7.5% SDS-PAGE for fibrin degradation.

Degradation of human plasma proteins

Degradation of human plasma protein was assayed according to the method of Kumar *et al*.[16] The FSBE (0–100 µg) was incubated with the 100 µg of plasma proteins for 24 h at 37°C in a reaction volume of 40 µl 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM NaCl, 0.05% sodium azide. The reaction was terminated by the addition of 20 µl denaturing buffer containing 4% SDS and boiled for 5 min. It was then analyzed on a 7.5% SDS-PAGE under nonreduced condition.

Preparation of platelet-rich plasma and platelet-poor plasma

The method of Ardlie and Han[17] was employed for the preparation of human PRP and platelet-poor plasma (PPP). The platelet concentration of PRP was adjusted to 3.1×10^8 platelets/ml with PPP. The PRP maintained at 37°C was used within 2 h for the aggregation process. All the above preparations were carried out using plastic wares or siliconized glasswares.

Platelet aggregation

The turbid metric method of Born[18] was followed using a chronology dual channel whole blood/optical lumi aggregation system (Model-700). Aliquots of PRP were preincubated with various concentrations of FSBE (0–150 µg) in 0.25 ml reaction volume. The aggregation was initiated independently by the addition of agonists such as adenosine diphosphate (ADP) and epinephrine and followed for 6 min.

Direct hemolytic activity by colorimetric method

Direct hemolytic activity was determined using washed human erythrocytes. Briefly, packed human erythrocytes and PBS (1:9 v/v) were mixed; 1 ml of this suspension was incubated independently with the various concentration of FSBE (15–150 µg) for 1 h at 37°C. The reaction was stopped by adding 9 ml of ice-cold PBS and centrifuged at 1000 g for 10 min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540 nm. Activity was expressed as percentage of hemolysis against 100% lysis of cells due to addition of water that served as positive control and PBS served as negative control.

Edema-inducing activity

The procedure of Vishwanath *et al*.[19] was followed. Groups of five mice were injected separately into the right foot pads with different doses (10–200 µg) of FSBE in 20 µl saline. The left foot pads received 20 µl saline alone served as control. After 1 h, mice were anesthetized by diethyl ether inhalation. Hind-limbs were removed at the ankle joint and weighed. Weight increased was calculated as the edema ratio, which equals the weight of edematous leg × 100/weight of normal leg. Minimum edema dose was defined as the amount of protein required to cause an edema ratio of 120%.

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo *et al*.[20] A different concentration of FSBE (0–200 µg) was injected (intradermal) independently into the groups of five mice in 30 µl saline. Group receiving saline alone serves as negative control and group receiving venom (minimum hemorrhagic dose [2MHD]) as positive control. After 3 h, mice were anesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for hemorrhage against saline-injected control mice. The diameter of hemorrhagic spot on the inner surface of the skin was measured. The MHD was defined as the amount of the protein producing 10 mm of hemorrhage in diameter.

Statistical analysis

The data are presented as mean ± standard deviation; statistical analysis was performed by Student's *t*-test. A significant difference between the groups was considered if $P < 0.01$.

RESULTS AND DISCUSSION

The current study presents the anticoagulant, antiplatelet, and fibrinolytic activities of cysteine protease from the FSBE. Initially, protein banding pattern was analyzed on SDS-PAGE and visualized by coomassie brilliant blue and silver staining. Interestingly, FSBE exhibited dissimilar protein banding pattern from the range 200 kDa to 14 kDa on 10% SDS-PAGE under reduced and nonreduced conditions [Figure 1a and c], suggesting the presence of both monomeric and oligomeric proteins with varied molecular mass. In addition, FSBE was subjected to basic PAGE [Figure 1b]; it showed only one protein band and revealed the extract stored maximum acidic proteins. Further, FSBE was analyzed for probable carbohydrate content in the proteins; interestingly, it taken up PAS staining at the low-molecular-weight region around 29 kDa to 18 kDa that was compared with the positive control fibrinogen [Figure 1d], suggesting that the presence of low-molecular-weight proteins in the extract is glycoproteins. Osborne (1892) was the first scientist to shown the evidence on the presence of proteins in the flaxseed extract. Subsequently, several researchers worked on the flaxseed proteins. For the first time, Vassel and Nesbitt (1945) were isolated two proteins, namely, high-molecular-weight protein 12S "linin" and low-molecular-weight 2S "Conlinin" while their therapeutic potential has not revealed yet. Although the proteins linin and conlinin were isolated from the flaxseed,
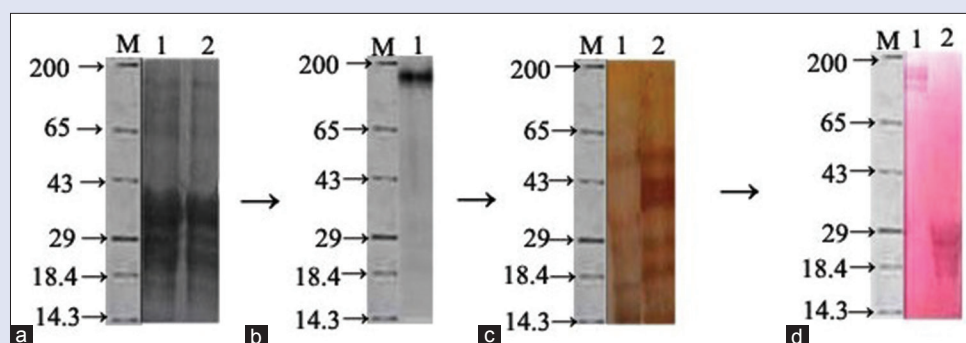
Figure 1: (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis 10%. (b) Native (basic) polyacrylamide gel electrophoresis. (c) Silver staining. (d) Glycoprotein staining. (a) FSBE as shown in sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%): FSBE (100 μg) under nonreduced (a1) and reduced conditions (a2), (b1) Native basic polyacrylamide gel electrophoresis of FSBE: FSBE (100 μg) under non-reduced condition. (c) Silver staining of FSBE: FSBE (30 μg) under nonreduced (c1) and reduced conditions (c2), (d) Periodic acid–Schiff staining of FSBE: positive control fibrinogen (d1) and FSBE (d2). M represents the molecular-weight marker in kDa from top to bottom: Myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), lactalbumin (18.4), and lysozyme (14.3) BSA: Bovine serum albumin, FSBE: Flaxseed buffer extract
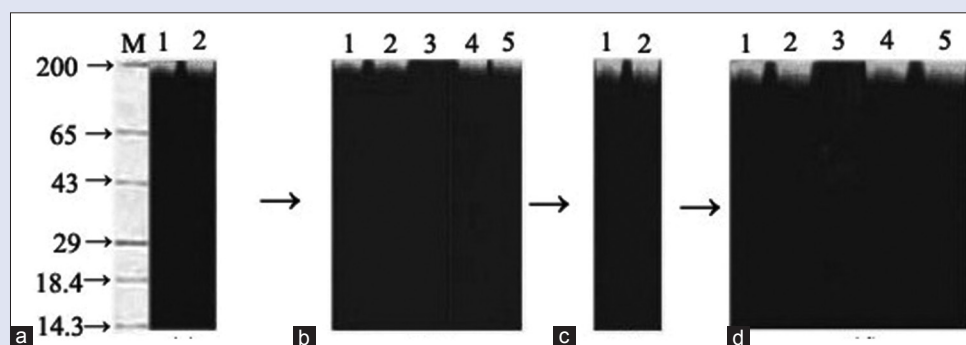

Figure 2: (a) Casein zymogram (b) Casein zymogram with inhibitor (c) Gelatin zymogram (d) Gelatin zymogram with inhibitor.(a) FSBE casein zymogram: FSBE 50 μg (a1) and 100 μg (a2) under nonreduced conditions.(b) FSBE Casein zymogram with inhibitor: FSBE 50 μg alone (b1), FSBE 50 μg was pretreated with 5 mM PMSF (b2), FSBE 50 μg was pretreated with 5 mM iodoacetic acid (b3), FSBE 50 μg was pretreated with 5 mM 1,10-phenanthroline (b4), FSBE 50 μg was pretreated with 5 mM ethylenediaminetetraacetic acid (b5) under nonreduced conditions.(c) FSBE gelatin zymogram: FSBE 50 μg (c1) and 100 μg (c2) under nonreduced conditions.(d) FSBE gelatin zymogram with inhibitor: FSBE 50 μg alone (d1), FSBE 50 μg was pretreated with 5 mM PMSF (d2), FSBE 50 μg was pretreated with 5 mM iodoacetic acid (d3), FSBE 50 μg was pretreated with 5 mM 1,10-phenanthroline (d4), FSBE 50 μg was pretreated with 5 mM ethylenediaminetetraacetic acid (d5) under nonreduced conditions. M represents the molecular-weight marker in kDa from top to bottom: Myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), β-lactalbumin (18.4), and lysozyme (14.3). BSA: Bovine serum albumin, FSBE: Flaxseed buffer extract

there are no reports available on the presence of proteolytic enzyme/s in the extract.

Thus, an attempt was made on proteolytic activity using casein and gelatin as the substrates. Curiously, FSBE exhibited proteolytic activity as it hydrolyzed casein and gelatin with the specific activity 0.180 and 0.201units/mg/min at 37°C, respectively. To strengthen FSBE protease activity, zymography experiments were also carried out using casein and gelatin as the substrate. To the surprise, FSBE showed a translucent activity band around 200-kDa region in both casein [Figure 2a] and gelatin [Figure 2c] zymography experiments that clearly reveal the presence of proteolytic enzyme in the extract. Furthermore, to understand type of protease present in the extract inhibition, studies were carried out using protease inhibitors such as PMSF, IAA, EDTA, and 1,10-phenanthroline. Interestingly, only IAA, a cysteine protease inhibitor completely inhibited the proteolytic activity of the extract, and it was confirmed in colorimetric [Table 1] assay and zymography [Figure 2b and d] experiments as well. It clearly indicates that FSBE is having cysteine protease. Indeed, FSBE is the richest source for proteins (35%), yet proteolytic enzymes have been not identified. On the

other hand, proteolytic enzymes, namely, serine, metallo, and cysteine proteases have been extensively studied in other seeds such as bitter gourd, jackfruit, peanut, *Araucaria angustifolia*, *Citrullus colocynthis*, maize, and sorghum seeds.[21-24] High-molecular-weight serine protease was reported from jackfruit seed extract while the presence of high-molecular-weight serine proteases was also reported in the brown spider venom.[25]

Furthermore, FSBE was analyzed for its probable interference in coagulation cascade; plasma recalcification time was done using both human platelet-rich and platelet-poor plasma. Interestingly, FSBE showed anticoagulant effect in both PRP and PPP as it enhanced the clotting time from control 220s to 320s and 220s to 346s, respectively, at the concentration of 60 μg and did not altered much upon increased dose of 75 μg [Figure 3]. To make sure that, triggered anticoagulation by the FSBE could be due to which pathway of the coagulation cascade. APTT and PT were carried out. Remarkably, FSBE, in particular, prolonged the clot formation process of only APTT but not PT revealed that the observed anticoagulation by the FSBE is due to the interference in intrinsic pathway of blood coagulation cascade [Table 2]. Blood

coagulation cascade is a physiological phenomenon that could be activated to arrest the bleeding following an injury. It mainly comprises three pathways, namely, intrinsic, extrinsic, and common pathway. Activation of intrinsic pathway takes place due to trauma and contact of high-molecular-weight kininogen, Prekallikrein (PK), and factor XII with underlining collagen on endothelium.[22] The factor XII complex activates PK into kallikrein (K); the activated K, in turn, activates factor XII into factor XIIa. Then activated factor XIIa converts factor XI into

factor XIa, factor XIa converts factor IX into factor IXa, and it, in turn, converts factor X into factor Xa.[23] On the other hand, extrinsic pathway activates due to the release of tissue factor (TF) into the bloodstream upon tissue injury. The TF activates factor VII into factor VIIa; it, in
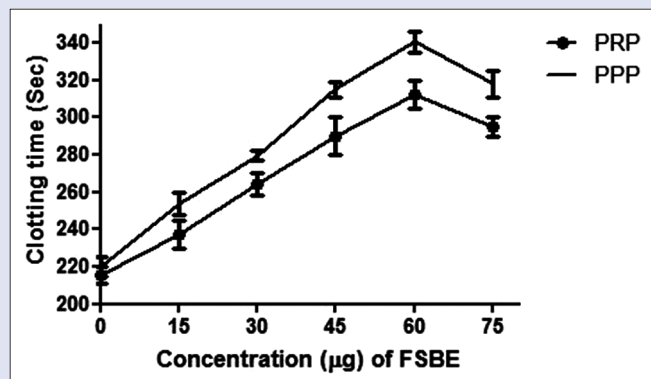

Figure 3: Plasma recalcification time. (a) FSBE (0–60 µg) was preincubated with 0.2 ml of citrated human plasma platelet-rich plasma/platelet-poor plasma in the presence of 20 µl 10 mM Tris-HCl buffer (pH 7.4) for 1 min at 37°C. 20 µl of 0.25M CaCl₂ was added to the preincubated mixture and clotting time was recorded

Table 1: Effect of inhibitors on the proteolytic activity of flaxseed buffer extract

Inhibitor (5mM each)	Activity/Residual Activity (%)
None	100
EDTA	97.55
1,10-Phenanthroline	89.10
IAA	11.10
PMSF	95.80

Table 2: Dose-dependent effect of flaxseed buffer extract on clotting time of normal human plasma

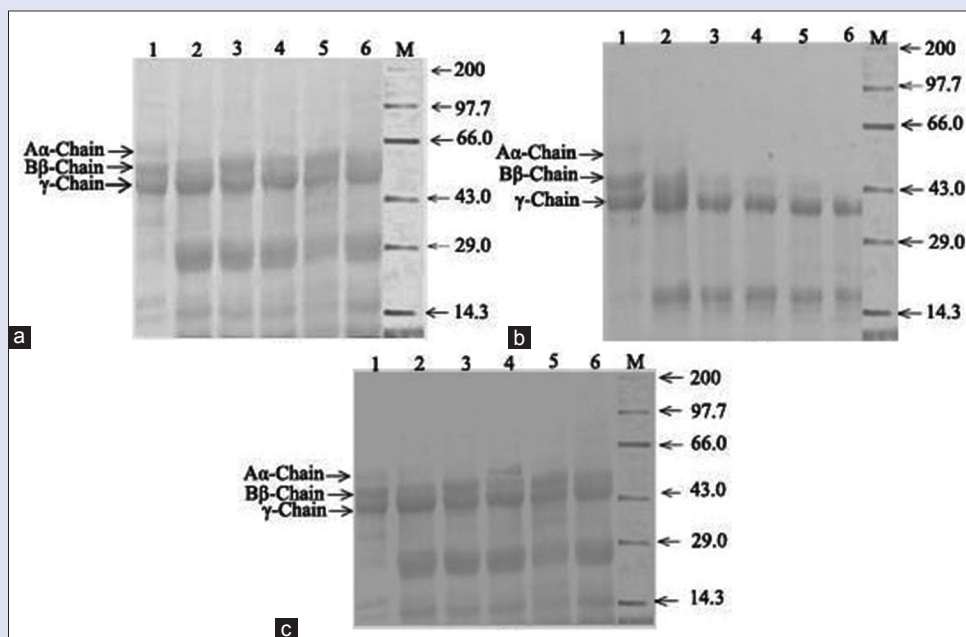| FSBE (µg)	PT clotting time in sec	PT (INR values)	APTT clotting time in sec	APTT ratio
0	11.0±0.05	0.93±0.01	38.3±0.04	1.39±0.02
20	11.2±0.02	0.96±0.08	47.6±0.05	1.73±0.03
40	11.4±0.04	1.01±0.02	51.7±0.03	1.88±0.01
60	11.6±0.03	1.05±0.01	56.2±0.05	2.04±0.07
80	11.8±0.1	1.09±0.06	67.5±0.09	2.45±0.04
100	12.1±0.08	1.13±0.1	70.1±0.02	2.66±0.01

Figure 4: Fibrinogenolytic activity. (a) Dose-dependent effect (b) Time-dependent effect (c) Inhibition study. (a) FSBE dose-dependent effect: fibrinogen alone 50 µg (a1), fibrinogen treated with 20 µg (a2), 40 µg (a3), 60 µg (a4), 80 µg (a5), and 100 µg (a6) of FSBE, respectively, incubated for 4 h at 37°C and then separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reduced condition. (b) FSBE time-dependent effect: FSBE 40 µg was incubated with fibrinogen 50 µg for 0 h (b1), 4 h (b2), 8 h (b3), 12 h (b4), 16 h (b5), and 24 h (b6), respectively, at 37°C. (c) FSBE inhibition study: FSBE 40 µg was preincubated with protease inhibitors for 30 min at 37°C. Further reaction was initiated by adding 50 µg of fibrinogen and incubated for 4 h, fibrinogen alone (c1), FSBE 40 µg (c2), fibrinogen 50 µg and FSBE 40 µg with 5 mM PMSF (c3), fibrinogen 50 µg and FSBE 40 µg with 5 mM iodoacetic acid (c4), fibrinogen 50 µg and FSBE 40 µg with 5 mM ethylenediaminetetraacetic acid (c5), fibrinogen 50 µg and FSBE 40 µg with 5 mM 1,10-phenanthroline (c6). M represents the molecular-weight marker in kDa from top to bottom: myosin-H-chain (200) phosphorylase b (97.2), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29) and lysozyme (14.3). BSA: Bovine serum albumin, EGTA: Ethylene di-amine tetra-acetic acid, IAA: iodoacetic acid, PMSF: Phenylmethylsulfonyl fluoride, FSBE: Flaxseed buffer extract
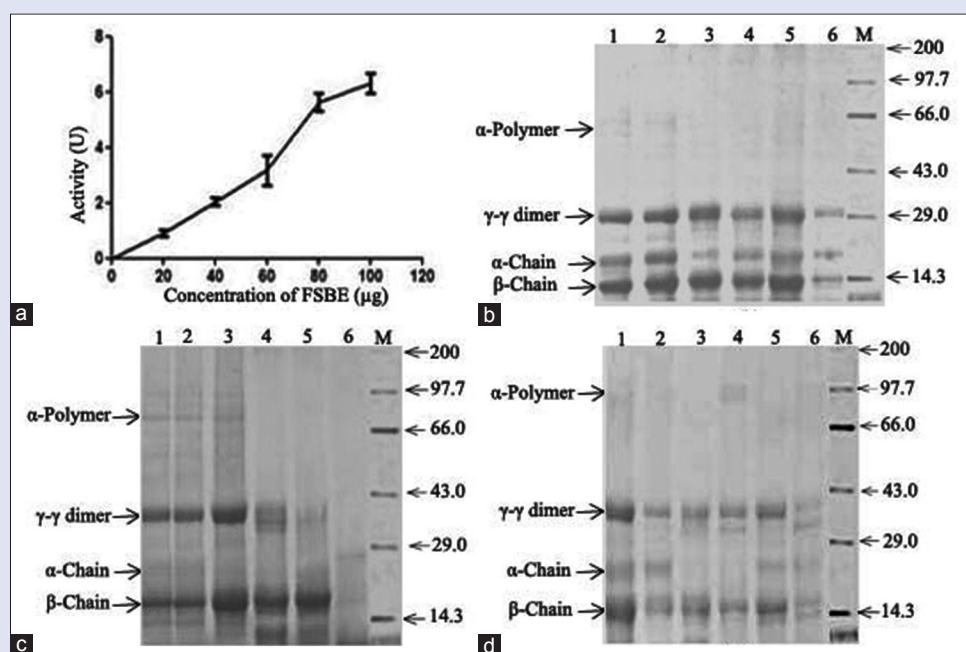
Figure 5: Fibrinolytic activity: (a) Colorimetric assay. (b) Dose-dependent effect. (c) Time-dependent effect. (d) Inhibition study. (a) Washed plasma clot was incubated with 0–100 µg of FSBE for 2.30 h and then the optical density was measured at 660 nm. (b) FSBE dose-dependent effect; washed plasma clot was incubated for 12 h and then separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5%), washed plasma clot alone (b1), plasma clot treated with 20 µg (b2), 40 µg (b3), 60 µg (b4), 80 µg (b5), and 100 µg (b6) of FSBE, respectively.(c) FSBE time-dependent effect; FSBE 40 µg was incubated with fibrin clot at 37°C, fibrin clot alone (c1), 0 h (c2), 6 h (c3), 12 h (c4), 18 h (c5), and 24 h (c6) of FSBE.(d) FSBE inhibition study: FSBE 40 µg was preincubated with protease inhibitors for 30 min at 37°C. Further reaction was initiated by adding fibrin clot and incubated for 12 h, fibrin clot alone (d1), FSBE 40 µg (d2), fibrin clot and FSBE 40 µg with 5 mM PMSF (d3), fibrin clot and FSBE 40 µg with 5 mM iodoacetic acid (d4), fibrin clot and FSBE 40 µg with 5 mM ethylenediaminetetraacetic acid (d5), fibrin clot and FSBE 40 µg with 5 mM 1,10-phenanthroline (d6). M represents the molecular-weight marker in kDa from top to bottom: Myosin-H-chain (200) phosphorylase b (97.2), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), and lysozyme (14.3). BSA: Bovine serum albumin, EGTA: Ethylene di-amine tetra-acetic acid, IAA: Iodoacetic acid, PMSF: Phenylmethylsulfonyl fluoride, FSBE: Flaxseed buffer extract
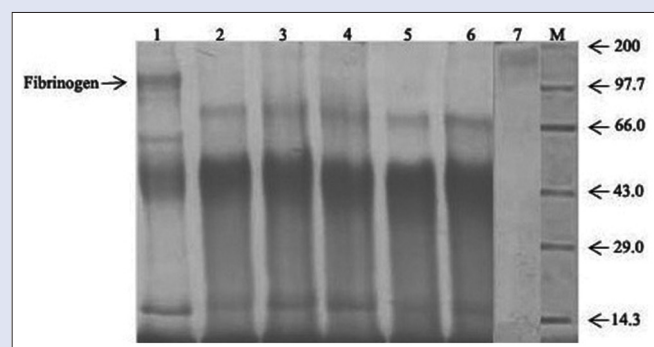

Figure 6: Degradation of plasma proteins. Plasma protein (100 µg) was incubated with FSBE in 40 µl of 10 mM Tris-HCl buffer (pH 7.4) at 37°C and then analyzed on 7.5% Sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreduced condition. Plasma protein (100 µg) alone (1), plasma protein treated with 20 µg (2), 40 µg (3), 60 µg (4), 80 µg (5), 100 µg (6) of FSBE and 20 µg of fibrinogen as control (7). M represents the molecular-weight markers in kDa from top to bottom myosin-H-chain (200) phosphorylase b (97.2), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), and lysozyme (14.3). BSA: Bovine serum albumin; FSBE: Flaxseed buffer extract.

turn, activates factor X into factor Xa. Either intrinsic or extrinsic pathways after activation, they culminate in common pathway.[26] The common pathway activates when factor Xa generated either from intrinsic pathway or from extrinsic pathway that, in turn, cleaves

prothrombin to thrombin. Thrombin later converts fibrinogen to fibrin the clot/thrombus.[24] Thus, coagulation is highly regulated pathway. However, imbalance due to genetic and environmental factors could alter the normally operating coagulation system that leads to thrombosis, a pathological phenomenon. Thrombosis is the formation of unusual clot in the arteries and veins which subject to the risk of cardio- and cerebro-vascular impediments.[27] FSBE exhibited anticoagulant effect, specifically inhibited APTT. Thus, the triggered anticoagulation by FSBE could be due to the participation in the intrinsic pathway of blood coagulation cascade. Thus, FSBE may be a better candidate in preventing the formation of unusual clot. Anticoagulant from snake venom (ancrod), fungi *Aspergillus oryzae* (brinase), coumarin derivative from sweet clover, and hirudin derivative from saliva of leach are currently being used to treat thrombotic disorders.[28-30] Bitter gourd seed extract and jackfruit seed extract found to exhibit the anticoagulant effect.[12] Since FSBE exhibited anticoagulation, an attempt was made on its ability to hydrolyze fibrinogen. The main reason could be proteases those degrade fibrinogen Aα and Bβ-chain from N terminal end and generates fibrinopeptide A and B are generally called as thrombin like enzymes. They mainly attribute pro-coagulation activity [23]. While, the proteases those degrade fibrinogen from C-terminal end and generates truncated fibrinogen generally causes anticoagulation.[21] Amazingly, FSBE degraded human fibrinogen in dose [Figure 4a] and time-dependent manner [Figure 4b]. FSBE in the dose-dependent assay; it degraded only Aα-chain without affecting the Bβ and γ-chains of fibrinogen at the maximum concentration of 100 µg for the 4 h incubation time at 37°C. However, when FSBE was incubated for 24 h at the concentration
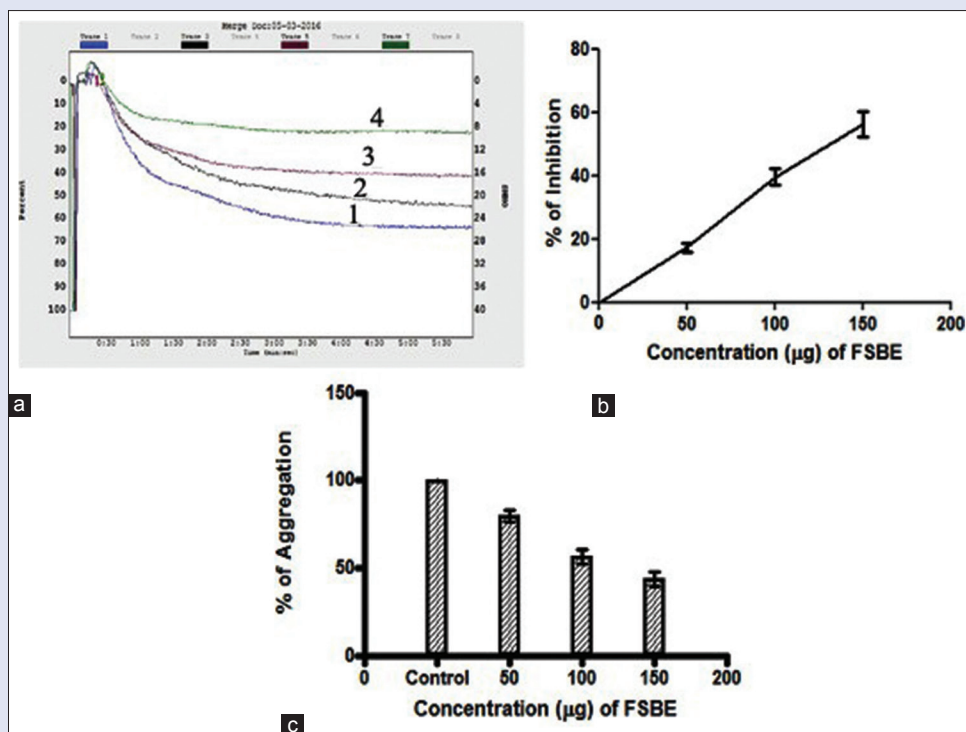
Figure 7: Platelet aggregation was initiated by adding adenosine diphosphate as an agonist. (a) Traces of platelet aggregation: Trace 1 (adenosine diphosphate 10 µM); Trace 2 (adenosine diphosphate 10 µM + 50 µg of FSBE); Trace 3 (adenosine diphosphate 10 µM + 100 µg of FSBE); Trace 4 (adenosine diphosphate 10 µM + 150 µg of FSBE). The values represent ± standard deviation of three independent experiments. (b) Dose-dependent platelet aggregation inhibition%. (c) Dose-dependent platelet aggregation%
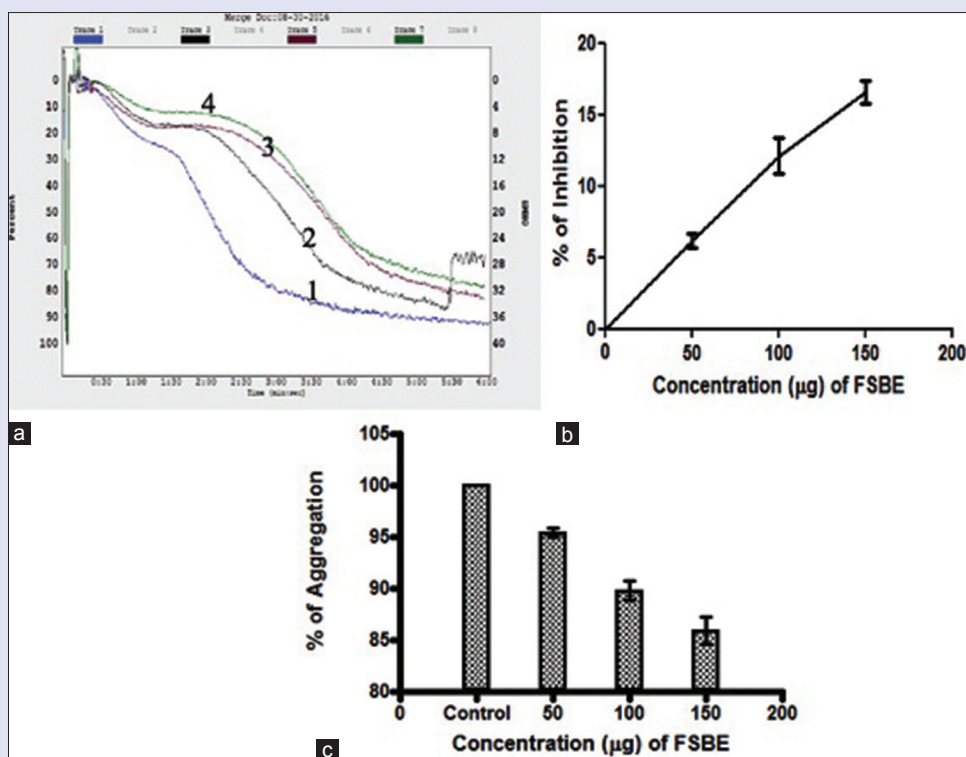

Figure 8: Platelet aggregation was initiated by adding epinephrine as an agonist. (a) Traces of platelet aggregation: Trace 1 (Epinephrine 5 µM); Trace 2 (Epinephrine 5 µM + 50 µg of FSBE); Trace 3 (Epinephrine 5 µM + 100 µg of FSBE); Trace 4 (Epinephrine 5 µM + 150 µg of FSBE). The values represents of three independent experiments. (b) Dose-dependent platelet aggregation inhibition% (c) Dose-dependent platelet aggregation%
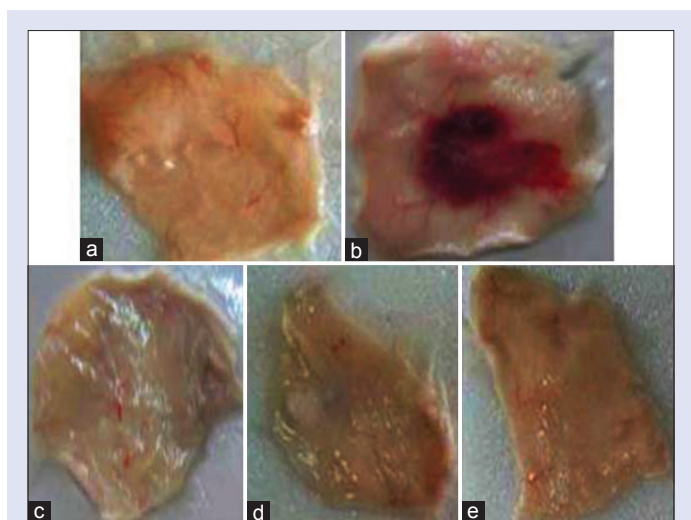
Figure 9: Dose-dependent hemorrhagic activity of flaxseed buffer extract. (a) Saline, (b) Positive control 2 MDH venom, (c) 50 µg, (d) 100 µg, and (e) 200 µg of FSBE was injected independently into mice in a total volume of 50 µl intradermal. FSBE; Flaxseed buffer extract

of 50 µg; it degraded Aα- and Bβ-chains of fibrinogen without affecting γ-chain. Furthermore, the fibrinogenolytic activity of FSBE was inhibited only by IAA but not serine and metalloprotease inhibitors clearly indicate the role of cysteine protease in the extract [Figure 4c]. Several fibrinogenolytic enzymes from venomous organisms from snake, spider, and scorpion were extensively reported.[29-31] The metalloproteolytic activity of bitter gourd and jackfruit seeds found to degrade fibrinogen[12] while serine proteolytic activity of *Artocarpus heterophyllus* latex found to hydrolyze fibrinogen. Cysteine proteases are least studied enzymes in plant seeds while few of the cysteine proteases have been reported from the plant latex.[32]

Furthermore, FSBE was further investigated for its probable clot dissolving ability. Initially, the clot-dissolving ability was analyzed colorimetrically [Figure 5a] and the observed specific activity was found to be 3.88units/mg/min. The fibrinolytic activity of the FSBE was further confirmed by analyzing on SDS-PAGE [Figure 5b]. FSBE degraded only α-polymer; at the higher concentration of 100 µg, when it was incubated for 12 h at 37°C without affecting γ-γ-dimer, α-chain, and β-chain. When the incubation time was increased further for about 24 h, FSBE hydrolyzed all the chains of cross-linked fibrin at the concentration of 100 µg at 37°C [Figure 5c]. Only IAA inhibited the fibrinolytic activity suggesting the role of cysteine protease in the extract [Figure 5d]. Several plasmin-like/fibrinolytic enzymes have been reported from the plant latex, earthworms, caterpillar, and venoms of snake, spider, and honey bees.[29-31] Dissolution of fibrin clot/thrombus plays a key role in the pathophysiology of cerebro/cardiovascular complications.[27]

FSBE was also analyzed for its specificity on protein degradation using plasma proteins. Interestingly, FSBE did not hydrolyze other plasma proteins when it incubated for 12 h at the concentration of 100 µg at 37°C. However, it preferably hydrolyzed fibrinogen present in the plasma as the intensity of fibrinogen band was progressively vanished, which could be compared with positive control fibrinogen alone, suggesting its limited substrate specificity [Figure 6].

To study the intervention of FSBE on platelet function, platelet aggregation was analyzed using agonists such as ADP and epinephrine using platelet-rich plasma. FSBE inhibited the agonists such as ADP [Figure 7] and epinephrine [Figure 8]-induced platelet aggregation of about 63.6% and 16.3%, respectively at the concentration of 150 µg. Among agonists, observed

FSBE inhibited in the order of ADP > epinephrine-induced aggregation. Platelets are anuclear discoid-shaped cells originating from the stem cells of bone marrow megakaryocytes contribute equally along with coagulation factors for arrest of bleeding.[30] Several physiological agonists, namely, ADP, epinephrine, thrombin, thromboxane, arachidonic acid, and collagen activate platelets.[21-24] These activated platelets plug at the site of injury along with fibrin clot and inhibit blood loss.[23] While, in case of hypercoagulation disorders, there will be burst of activation of platelets too contribute for the formation unusual clot.[29-31] Thus, antiplatelet agents do play a key role in inhibiting hyperactivation of platelets. Several antiplatelet agents have been characterized from plants and animal sources.[12] Eptifibatide derivative from rattlesnake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently using in the treatment of coagulation disorders.[26,31,32]

FSBE did not hydrolyze red blood cell; it did not cause hemorrhage and edema in experimental mice up to the concentration of 200 µg while positive control *Daboia russelii* venom-induced hemorrhage and edema in experimental mice, suggesting its nontoxic property [Figure 9].

CONCLUSION

In conclusion, this study for the first time demonstrates the cysteine proteolytic activity of FSBE responsible for anticoagulant, fibrinogenolytic, clot-dissolving, and antiplatelet properties. Thus, it could be a better therapeutic agent for thrombotic disorders.

Conflicts of interest

There are no conflicts of interest.

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