CHAPTER-III

ROLE OF CUCUMBER (*CUCUMIS SATIVUS*) SAP EXTRACT PROTEOLYTIC ACTIVITY ON CLOT FORMATION, CLOT DISSOLUTION AND PLATELET FUNCTIONS
INTRODUCTION

Blood clotting and dissolution are two important steps in hemostasis process, it is necessary for maintenance of human normal physiological function. The clotting of blood or fibrin formation was facilitated by many blood clotting factors via extrinsic, intrinsic and common pathway and fibrin dissolution was initiated by plasmin a serine protease by a process known as fibrinolysis [Fleming and Melzig, 2012]. There is a balance between coagulation activation and inhibition if this equilibrium is altered by any methods it leads to profuse bleeding and thrombosis complications may occur.

Blood coagulation disorders are a health disorders worldwide. To treat these disorders researches is progressing for better and inexpensive treatment without or minimize side effects. Cardiovascular diseases are the leading reason for the rate of death in worldwide and are considered as a global epidemic. According to the world health organization (WHO) in 2015, due to this disease the death rate may be about 20 millions. It is substantial and rapidly growing problem around the world, affecting people from all socioeconomic backgrounds. Currently, several anticoagulant drugs (warfarin, heparin and there derivatives) the fibrinolytic drugs (streptokinase, urokinase, alteplase, reteplase and tenecteplase) and the anti-platelet drugs (aspirin, diiypyramidole, ticloidine, clopidrogel and prasugrel) are available for the treatment, while each of these drugs has its own profile of side effects [Mackman, 2008].

In spite of a long history of hemostasis research, an ideal thrombolytic and anti-platelet agent has yet to be developed. Therefore, there is a need to search for effective and safe fibrinolytic agents which dissolve the thrombus effectively with mechanism of action. Nowadays the research is going on to find safe and effective fibrinolytic proteases as drug
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from food source to treat hemostasis disorders. Therefore, in the present study an effort has been made to disclose the role of cucumber (Cucumis sativus L.) fruit, sap proteolytic activity on hemostasis including clot formation, clot dissolution and platelet activation and inhibition processes and the results are presented.
MATERIALS AND METHODS

Materials:

Cucumbers were purchased from Krishna Raja Market, Mysore, Thrombin, Urokinase, ADP, Collagen type I and Epinephrine were purchased from Sigma Chemicals Company (St. Louis, USA). From Tulip Diagnostics Pvt. Limited UNIPLASTIN, LIQUICELIN-E and factor-X and VII deficient plasma were purchased. From healthy donors the blood was collected (Human ethical Sanction order- IHEC-UOM No.71). All other chemicals used were of analytical grade.

Methods:

Plasma re-calcification time

The plasma re-calcification time assay was carried out using citrated human plasma according to the method of Quick (1935). The human citrated plasma (0.2ml) was pre-incubated with different concentration of CSE (2.5-100 µg) respectively in presence of Tris–HCl (20 µl, 10 mM, pH 7.4) buffer at 37 °C for 5 min and for the inhibition of proteolytic activity of CSE, the CSE (25 µg) was pre-incubated with known protease inhibitor like EDTA, EGTA, 1,10, phenanthroline, PMSF and IAA (10mM) for 30 min at 37 °C and for the pre-incubated mixture, add 0.25 M CaCl2 (20 µl) and clotting time was recorded in seconds against light source.
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Fibrinogenolytic activity

According to the methods of Ouyang and Teng (1976) the fibrinolytic activity of CSE was determined. Human plasma fibrinogen (50 µg) was incubated with different concentrations of CSE (1 to 30 µg) and made up to the total mixture of 40 µl with Tris-HCl buffer (10 mM, pH 7.5) independently for 12 h at 37°C. For time dependent activity CSE (10 µg) was incubated with human plasma fibrinogen (50 µg) for different time intervals (30 min to 24 h). For inhibition assay the different known protease inhibitors (10mM) were pre-incubated with CSE (10 µg) for 30 min at 37°C independently and again the mixture of CSE and different protease inhibitors were incubated with human plasma fibrinogen (50 µg) respectively. Finally the reaction of fibrinogenolytic activity was terminated by adding denaturing buffer (20 µl) which contain 4% SDS, 4% β- mercaptoethanol and 1 M urea. The activity was analyzed by 10% SDS-PAGE with 0.1% coomassie brilliant blue (R-250) and destined with ethanol: acetic acid: water (5:1:4).

Activated partial thromboplastin time (APTT) assay

This experiment was carried out according to the kit provided by Tulip Diagnostics Pvt. Limited. Different concentration of CSE (2.5 to 100µg) was pre-incubated with of citrated human plasma (100 µl) independently for 1 min and for the inhibition of CSE proteolytic activity the CSE (25 µg) was pre-incubated with known protease inhibitors (10Mm) and the mixture of CSE and protease inhibitors were again incubated for 1 min with citrate human plasma (100 µl) respectively. Add 100 µl of APTT reagent (LIQUICELIN-E Phospholipids preparation derived from rabbit brain with ellagic acid) which was activated for 3 min at 37°C and clotting time was recorded in seconds against the light source after adding 100µl of 0.25M CaCl₂.
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Prothrombin time (PT)

This experiment was carried out according to the kit provided by Tulip Diagnostics Pvt. Limited. Different concentration of CSE (2.5 to 100µg) was pre-incubated with of citrated human plasma (100 µl) independently for 1 min and for the inhibition of CSE proteolytic activity the CSE (25 µg) was pre-incubated with known protease inhibitors (10Mm) and the mixture of CSE and protease inhibitors were again incubated for 1 min with citrate human plasma (100 µl) respectively. Add 200µl of PT reagent (UNIPLASTIN/LIQUIPLASTIN-rabbit brain thromboplastin) and the time taken for the visible clot formation was recorded in seconds against the light source.

Thrombin-like activity

Thrombin-like activity assay was carried out according to the method of Denson (1969). The total volume mixture (0.4ml) containing human plasma fibrinogen (0.5%) and Tris–HCl (10 mm, pH 7.4) buffer was treated with different concentration of CSE (2-20 µg) independently. For control, 100 ml of diluted thrombin (2.5 NIH units/ml) is used. For inhibition studies, the CSE (100 µg) was pre-incubated with known protease inhibitors (10 mM) for 30min at 37°C independently and 0.5% of human plasma fibrinogen was added. Finally the mixture was agitated gently against a light source to record the clotting time in seconds at room temperature.
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**Factor VIIa-like activity**

Using the congenital factor VII deficient plasma the factor VII-like activity was determined. Before initiation of experiment dissolve the factor VII deficient plasma in the 37°C. For 100 µl of factor VII deficient plasma add different concentration of CSE (10-100µg) independently and incubated for 2 min at 37°C. For the inhibition studies, known protease inhibitors (10 mm) were pre-incubated with CSE (50 µg) for 30 min at 37°C independently and this was added to 100 µl of factor VII deficient plasma and incubated for 2 min at 37°C. Finally, add 30 µl of 0.25 M CaCl₂ and record the time in seconds against a light source. Normal plasma, 200 µl treated with 30 µl of 0.25 M CaCl₂ served as control experiments.

**Plasma clot hydrolyzing activity (Fibrinolytic activity)**

- **Colorimetric estimation:** Fibrin soft clot was prepared by keeping the mixture of citrated human plasma (100µl) and 0.25 M CaCl₂ (30 µl) in 37°C for 3h. The soft fibrin clot was washed thoroughly for 5-6 times using phosphate buffered saline (PBS). The washed fibrin clot was incubated with different concentration of CSE (5-150 µg) and for inhibition studies known protease inhibitors (10 mM) were pre-incubated with CSE (100 µg) at 37°C for 30 min independently and made up to final volume of 500 µl with Tris-HCl buffer (0.2 M, pH 8.5) for 2 h at 37°C. The reaction was terminated with 750 µl of 0.44 M of TCA and stand for 30 min at room temperature. Further, it was centrifugation at 1500 g for 15 min and add 1.25 ml of Na₂CO₃ (0.44 M), 0.25 ml of diluted Folin ciocalteus reagent (1:3) to the 0.5 ml of the supernatant which was taken in a clean glass tube. The optical density was read at 660 nm.
**Plate method:** The platelet poor plasma (PPP) was obtained from blood, which was treated with EDTA (2 mg/ml) and centrifuged at 500g for 15 min. Agarose plate was prepared a mixture of Platelet Poor Plasma (2 ml), 3 ml agarose (1.2%) in 10 mM Tris–HCl, NaCl (0.15 M), sodium azide (0.05%) and CaCl$_2$ (0.25 M) and poured into a flat Petri plate (10 mm 99 cm) and allow to solidify at 25 °C left for 2 h. In dose dependent activity the different concentration of CSE (25-100 µg) and positive control of Urokinase (2.5 units) independently placed on the solidified agarose upper surface. For inhibition studies different known protease inhibitors (10 mM) were pre-incubated with CSE (25 µg) for 30 min at 37°C independently and placed on the solidified agarose surface. Finally the plates were incubated overnight at room temperature. To terminate the reaction 0.01% TCA was added over the solidified agarose surface and the translucent clear zone diameter due to fibrinolytic activity was measured in millimeter (mm) using measuring scale.

**Banding pattern of fibrinopeptides on SDS-PAGE:** Fibrin soft clot was prepared by keeping the mixture of citrated human plasma (100µl) and 0.25 M CaCl$_2$ (30 µl) in 37 °C for 3h. The soft fibrin clot was washed thoroughly for 5-6 times using phosphate buffered saline (PBS). The washed fibrin clot was incubated with different concentration of CSE (5 to 40 µg) for 12 h and time intervals (1 h 30 min to 24 h) in a final volume of 40 µl 10 mM Tris HCl buffer pH 7.4 at 37 °C for 12 h. Inhibition studies were done using different known protease inhibitors (10 mM) which were pre-incubated with CSE (10 µg) at 37 °C for 30 min independently and incubated with washed fibrin clot for 12 h at 37 °C. The reaction was abolished by adding 20 µl sample buffer containing urea (1M), SDS (4%) and β-mercaptoethanol (4%). Finally the samples were boiled approximately for 10 min and centrifuged for 10 min at 6000 rpm. An aliquot of 20 µl supernatant was analyzed in 10% SDS-PAGE.
Plasminogen activation assay

According to method of Chakrabarty [2000], the plasminogen activation assay was performed. Citrated human plasma (20 µl) (a), (a) with 1mg of Urokinase (b), (a) with 50 µg of CSE (c), 50 µg CSE only (d) and 1 mg of Urokinase only (e) was independently incubated for 1 h at 37 °C in 100 µl of 100 mM potassium phosphate buffer and the reaction was initiated by adding azocasein (500 µl- 0.25% of azocasein in 100 mM potassium phosphate buffer pH 7.4). For the inhibition of proteolytic activity of CSE (50 µg) was pre-incubated with different known protease inhibitors (10Mm) independently for 30 min at 37 °C. This inhibitor and CSE mixture were added to 20 µl of citrated human plasma and independently incubated for 1 h at 37 °C in 100 µl of 100 mM potassium phosphate buffer and the reaction was initiated by adding azocasein substrate. For all samples add 400 µl of 25 % trichloroacetic acid abolished the activity and it was centrifuged at 1000g for 15 min. The supernatant (600 µl) was diluted with an equal volume of NaOH (0.5 N) and the optical density was read at 440 nm.

Preparation of platelet-rich plasma and platelet-poor plasma

To obtain PRP (platelet-rich plasma) and PPP (platelet poor plasma), we collected fresh blood from healthy donors, who were non-smokers and non-medicating at least for the previous 15 days, was mixed with anticoagulant trisodium citrate (0.11 M) in the ratio of 9:1 ratio further it was centrifuged for 15 min at 90g to acquire PRP. The remaining blood was again centrifuged for 15 min at 500g to acquire the PPP using plastic wares or siliconized glass wares. The number of platelets in the PRP was attuned to 3.8×108 platelets/ml by diluting with PPP and used within 2 h.
Platelet aggregation

Platelet aggregation was done by a turbidometric method (Born, 1962) using dual channel aggregometer (Chronolog). Different concentrations of CSE (5-50 µg) were pre-incubated with platelet poor plasma (0.45 ml) and make up the total volume to 500µl by PBS (phosphate buffer saline) and kept for constant stirring in cylindrical glass cuvette for 3 min. By adding agonist such as collagen (2µg/ml), ADP (5M/ml) and epinephrine (5mM/ml) independently and aggregation was followed for 6 min. Further, CSE was also checked for its effect on aggregation of platelet in the absence of agonists. As platelets aggregate, the transmission of light increases gradually and an aggregation trace was produced on the recorder. For inhibition of CSE proteolytic activity different known protease inhibitors (10Mm) were pre-incubated with CSE (50 µg) for 30 min at 37 °C independently and aggregation of platelet was done using PRP with respective agonists.

Protein estimation

The protein concentration was determined according to the method of Harrison (1937) using bovine serum albumin (BSA) as a standard solution.

Statistical Analysis

The experiments were repeated for three independent observations. Results were expressed as mean values mean ± SEM. Data were compared by analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons; significance was accepted at P < 0.05 (*), P < 0.01 (**), and P < 0.001 (**). Data were analyzed using by GraphPad Prism software.
RESULTS

The role of CSE in hemostasis was established by testing on human citrated plasma. CSE decreased the clotting time dose dependently (2.5-150 µg); the clotting time was declined since 266 ± 3.5 Sec to 141 ± 0.7 Sec (Fig 3.1), EDTA and EGTA abolished, while PMSF and IAA did not affect the plasma recalcification time of CSE (Table 3.1).

CSE hydrolyzed human fibrinogen dose dependently (Fig. 3.2A) and time dependently (Fig 3.2B). All, such as Aα, Bβ and γ chains are hydrolyzed. The intensity of the respective bands gradually decreases with increasing doses of CSE and with the materialization of low molecular mass degradation peptide end products. Preferentially the Bβ chain is hydrolyzed over Aα chain while the γ chain is hydrolyzed with a less preference. The protease inhibitors EDTA, EGTA and 1,10 phenanthroline affected the human fibrinogen hydrolytic property of CSE where as PMSF and IAA didn’t affect the fibrinogenolytic activity of CSE (Fig 3.2C).

CSE also reduced the prothrombin time (PT) of human citrated plasma dose dependently from 16 ± 2 Sec to 5 ± 2 Sec (Fig 3.3). Further, pre-incubation of CSE with EDTA inhibited the PT activity while it was insensible to PMSF and IAA pre-treatment (Table 3.1). CSE also showed the thrombin time (TT) dose dependently, but the effect observed was very feeble as compared to the control where thrombin showed the clotting time of 26 ± Sec. Further, the thrombin time was also abolished by EDTA while PMSF and IAA did not affect (data not shown).

When tested on the factor VII deficient congenital human plasma, CSE induced the clotting in a dose dependent manner, and at 100 µg, the clotting time observed was 254 ± 13 Sec (Fig 3.4), and this effect was abolished by EDTA but not by PMSF and IAA (Table 3.1).
The factor VII deficient plasma was insensitive to the added CaCl$_2$, while the normal human citrated plasma showed the recalcification time of 266 ± 4 Sec which served as control.

CSE hydrolyzed the washed fibrin clot dose dependently with the specific activity of 0.475 ± 0.026 units/mg/min. PMSF and IAA inhibited the clot hydrolyzing activity and the inhibition achieved was (95 ± 2%) and (11 ± 4%) respectively while, EDTA didn’t inhibit (Fig. 3.5A and B). The fibrinolytic activity was further confirmed by another semi-quantitative fibrinolytic assay, which was done using agarose plate in which the CSE (100 µg) revealed the clear zone of fibrin hydrolysis of 7.5 ± 0.04 mm diameter. PMSF, benzydamine hydrochloride and IAA inhibited, while EDTA and EGTA did not inhibit the fibrinolytic activity of CSE (Fig. 3.5C and D). Urokinase (2.5 units) revealed the zone of hydrolysis of 12 ± 0.02 mm which served as positive control. All chains of fibrin clot are degraded; however $\alpha$ polymer and $\alpha$ chain are hydrolyzed in preference nearly to a similar extent over $\beta$ chain and $\gamma\gamma$ dimmers are hydrolyzed much slowly (Fig 3.6 A and B), whereas the PMSF and IAA inhibited the fibrinolytic activity while EDTA, 1,10 phenanthroline and EGTA did not inhibit the fibrin degradation activity of CSE (Fig 3.6C).

In plasminogen activation assay, the urokinase didn’t hydrolyze azocasein while CSE hydrolyzed the azocasein dose dependently when incubated independently (Table 3.2). The azocasein hydrolyzing activity of CSE was inhibited to the extent of 86% by IAA and 12% by PMSF while EDTA did not inhibit (Fig 3.7).

CSE inhibited the epinephrine, collagen and ADP induced platelet aggregation dose dependently and the order of inhibition was found to be epinephrine > collagen > ADP with the respective IC$_{50}$ values of 22 ± 2.5, 20 ± 3 and 11 ± 2 µg/ml (Fig. 3.8, 3.9, 3.10, 3.14A, B).
and C). The platelet aggregation property of CSE was abolished by PMSF while it was insensitive to EDTA and IAA (Fig. 3.11, 3.12, 3.13, 3.14D, E and F).
DISCUSSION

Hemostasis is a balanced process between clot formation and dissolution. It involves a series of enzymatic reactions, where the inactive proteins are converted into active form by proteases in each step of the pathway. The blood clotting cascade was initiated by the damaged collagen beneath the blood vessel endothelial or by the release of thromboplastin/tissue factor in site of injury. Thrombin acts on the fibrinogen and forms fibrin clot. To maintain the balance in the system the clot dissolution was imperative, this was achieved by the endogenous serine protease called as plasmin, which was activated by tissue plasminogen activators.

Any imbalance in hemostasis pathway leads to hemorrhage or the development of a thrombus that adheres to the wall of the blood vessels. Accumulation of excess fibrin in the blood vessels, alter the blood flow and leads to heart attack and other serious cardiovascular diseases. If the blockage was not removed, the blood vessel will severely damaged or die due to insufficient supply of oxygen. If the damaged region is large, the normal conduction of electrical signals through the ventricle will be disrupted, leading to irregular heartbeat, cardiac arrest or death.

Worldwide, the death rate due to cardiovascular diseases (CVDs) and stroke are increasing every year and in recent years, even below 25 year old young people’s are also highly vulnerable and suffering from deadly CVDs. In 2003 the national institute of health (USA) was spending 2286 and 330 million dollars to find a novel treatment/drug for CVDs and stroke respectively. So, many synthetic and natural derived compounds and enzymes were used to treat CVDs and stroke, but these drugs have shown to have their own limitations and side effects. Now scientists are extensively working to discover a novel drug from a food
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source, which may reduce the percentage of side effects and increase the efficiency of drug action.

CSE readily reduced the plasma recalcification time and thus it showed strong procoagulant activity and the procoagulant activity was abolished by both EDTA and EGTA. EDTA is a non specific divalent metal ion chelator while EGTA is specific for calcium ions. Therefore, it appears that the procoagulant activity was due to the metalloprotease/s which is most likely calcium ion dependent. The metalloproteases known, are predominantly the zinc ion dependent enzymes, however, there are metalloprotease which are dependent both on calcium and zinc ions for activity have been reported, for example, NN-PF3, the metalloprotease which was isolated from the Indian cobra (Naja naja) venom contain both zinc and calcium ions and the activity was abolished both by EDTA and EGTA [Jagadeesha et al., 2002]. Normally the metal ions in metalloprotease are known to play both structural and catalytic role, and effecting any of the metal ion will affect the activity, thus CSE protease/s needs thorough investigation of its metal ion composition and biological activity. CSE did not alter the APTT while it reduced PT suggests that it is affecting the extrinsic/tissue factor pathway of coagulation. Both EDTA and EGTA abolished the PT of CSE. Further, CSE readily caused the clotting of factor VII deficient plasma; therefore, it is highly likely that the CSE interfered in the plasma coagulation process and work in place of factor VII of the extrinsic pathway. This activity was also abolished by both EDTA and EGTA. In addition, CSE also hydrolyzed the fibrinogen and caused the formation of a fibrin clot, but at a very slow rate as clot formation seen only after 30 min of incubation and it was also abolished by EDTA and EGTA. Thus, during tissue damage, CSE appear to promote the clot formation through metalloprotease/s as both EDTA and EGTA abolished the plasma recalcification time, PT and coagulation of factor VII deficient plasma. Although not studied
extensively, only few studies reported the procoagulant activity of plant products that are working through the extrinsic pathway of coagulation, for example, the methanolic leaf extract of *Ageratum conyzoides* and the cysteine protease from *Ficus carica* were found to show procoagulant activity through the extrinsic pathway of coagulation [Bamidele et al., 2010; Richter et al., 2002]. However, the procoagulant activity has been reported in different extracts of plant latex, fruits like kiwi, pineapple and raspberries [Zhao 2007; Torres-Urrutia et al., 2011], while the activity has been extensively studied in animal venoms [Markland 1998; Meier et al., 1991; Kini 2005]. As the injury enters into the healing phase, this will further trigger a complex event, including clot dissolution, scavenging of the debris and tissue regeneration through controlled proliferation of cells including angiogenesis. Plasmin hydrolyses the fibrin network and the fragments or breakdown products, which will eventually be engulfed and removed by phagocytic system. This step appears crucial, as little alteration of this step would result in prominent scar and keloids formation. Further, excess accumulation of collagen and other connective tissue materials would lead to fibrosis and has been commonly seen in many surgical wounds.

Treatment and management of cardiovascular disease and stroke is mainly relied on the available anticoagulant drugs and antiplatelet drugs because the basic pathophysiological process in heart attack and stroke is the formation of a thrombus, which consists of platelets and fibrin. Ardeparin, Dalteparin, Enoxaparin, Urokinase and Warfarin were anticoagulant drugs commonly use to treat heart disease, but these drugs have some side effects like breathing and swallowing difficulties, pain in the head, chest, abdomen, joint and muscle, tingling, hypotension, weakness, hypersensitivity and shock. Generally tissue plasminogen activators like alteplase, tenecteplase and streptokinase were used to treat stroke, but the side effects were nausea, vomiting, headache, rash pruritus, intra cerebral hemorrhage and
genitourinary bleeding. To overcome from these side effects now research was going on to find novel drug from food/plant source. Clausena suffruticosa, Leea indicia, Leucas aspera, [Rahman et al., 2013] hydrolyses the fibrin clot. CSE hydrolyzed azocasein suggesting the plasminogen activation property and this was abolished by IAA and thus the role of cysteine protease in the process. This is the first study reporting plasminogen activation by the cysteine protease; however cysteine proteases are abundantly present in lysosomes. Further, CSE readily hydrolyzed all chains of fibrin such as the α-polymer, α-chain, β-chain, and γ-γ dimer dose and time dependently of plasminogen free washed human fibrin clot and suggesting the fibrinolytic or plasmin-like activity and the activity was abolished by PMSF, suggesting the role of serine protease. Several plants serine proteases degrade fibrin clot such as Euphorbia hirta [Patel et al., 2012], Clausena suffruticosa, Leea indicia and Lucas aspera [Eduardo Fuentes et al., 2014]. Interestingly CSE inhibited platelet aggregation induced by ADP, epinephrine and collagen extensively and this activity was abolished by PMSF suggesting the role of serine proteases. Similarly the antiplatelet activity was reported from several fruits and vegetables, including kiwi (Actinidia chinensis Planchon), scallion (Allium schoenoprasum L.), black grapes (Vitis vinifera L.), garlic (Allium sativum L.), strawberry (Fragaria x ananassa L. Dutch.), onion (Allium cepa L.), pineapple (Ananas comosus L. Mar.), tomato (Solanum Lycopersicon Mill.), melon (Cucumis Melo L. Var. Inodorous) and green beans [Torres-Urrutia et al., 2011]. Many serine proteases isolated from plant source such as bromelain from Ananas comosus [Metzig et al., 1999] and Crinumin from Crinum asiaticum [Singh et al., 2011] showed the inhibition of platelet aggregation. CSE play a dual role in hemostasis process, as a procoagulant and anticoagulant like Jatropha curcas latex, which possesses both procoagulant and anticoagulant activities [Osoniyi, 2003].
In summary/conclusion, this study for the first time, reporting the factor VII-like activity of a CSE protease(s) with the two contrasting effects on hemostasis. The properties such as factor VIIa-like activity and activator of prothrombin complex, accounted for pro-coagulation while, fibrinolytic, activation of plasminogen and inhibition of ADP, collagen and epinephrine induced platelet aggregation led to anti-coagulation properties of CSE.
Fig 3.1: Effect of CSE on plasma recalcification time: Dose dependent effect of CSE (2.5-150 µg) on human citrated plasma and the assay was carried out as illustrate in the method section and results were presented as the mean ± SEM.
Fig 3.2: Hydrolysis of fibrinogen by CSE: A represents the dose dependent degradation of human fibrinogen (50 µg), with different concentration of CSE lane 1 (fibrinogen alone), 2 (1 µg), 3 (2.5 µg), 4 (5 µg), 5 (10 µg), 6 (20 µg) and 7 (30 µg) respectively. B represents the time dependent hydrolysis of human fibrinogen (50 µg) with the CSE (10 µg), lane 1 (fibrinogen alone), 2 (30 min), 3 (1 h, 30 min), 4 (3 h), 5 (6 h), 6 (12 h) and 7 (24 h) respectively and C represents the effect of protease inhibitors (10mM) on the fibrinogenolytic activity of CSE. Lane 1 (fibrinogen alone), 2 (EDTA), 3 (EGTA), 4 (1,10, phenonthroline), 5 (PMSF), 6 (IAA) and M represents the following molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).
Fig 3.3: Effect of CSE on Prothrombin time: Dose dependent effect of CSE (5-100 µg) on citrate human plasma with prothrombin time reagent and the assay was carried out as illustrate in the method section and results were presented as the mean ± SEM.
Fig 3.4: Effect of CSE on congenital factor VII deficient human plasma: Dose dependent effect of CSE (5-100 µg) on congenital factor VII deficient human plasma and the assay was carried out as illustrate in the method section and results were presented as the mean ± SEM.
Fig 3.5: Effect of CSE on fibrin clot hydrolysis and inhibited by protease inhibitors: A represents the consequence of CSE on fibrin clot hydrolysis. B represents the effect of protease inhibitors on fibrinolytic activity of CSE. Protease inhibitors (10 mM) were pre-incubated independently with CSE (100µg) for 30 min at 37 °C. C represents the consequence of CSE on fibrin clot hydrolysis by the agarose plate method. 1 (2.5 units urokinase), 2, 3, 4 (25, 50, and 100 µg CSE respectively). D represents the consequence of protease inhibitors (10Mm) on fibrin degradation by CSE. 1 (2.5 units urokinase), 2 (CSE 25 µg), 3 (CSE 25 µg + EDTA), 4 (CSE 25 µg + PMSF), 5 (CSE 25 µg + benzydamine hydrochloride), 6 (CSE 25 µg+ EGTA) and 7 (CSE 25 µg + IAA) respectively and the assay was carried out as illustrated in the method section and results were presented as the mean ± SEM. (***P<0.0001, significant when compared to CSE).
Fig 3.6: Banding pattern of fibrinopeptides on SDS-PAGE: A represents the dose dependent degradation of human fibrin with different concentration of CSE lane 1 (fibrin alone), 2 (5 µg), 3 (10 µg), 4 (20 µg), 5 (30 µg) and 6 (40 µg) respectively. B represents the time dependent hydrolysis of human fibrin with CSE (10 µg), lane 1 (fibrin alone), 2 (30 min) 3(1 h 30 min), 4 (3 h), 5 (6 h), 6 (12 h) and 7 (24 h) respectively. C represents the consequence of protease inhibitors (10mM) on fibrinolytic activity of CSE. Lane 1 (fibrin alone), 2 (CSE 10µg), 3 (EDTA), 4 (EGTA), 5 (1,10, phenonthrole), 6 (PMSF), 7 (IAA) and M represents the following molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5). For all the above mentioned assays 10% SDS-PAGE was used for analysis under reduced condition.
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Fig 3.7: Effects of protease inhibitors on the proteolytic activity of CSE towards activation of plasminogen: Different protease inhibitors (10mM) were pre-incubated independently with CSE (50 µg) at 37 °C for 30min and the assay was carried out as illustrate in the method section and results were presented as the mean ± SEM.
Fig 3.8: Effect of CSE on aggregation of platelets induced by epinephrine: Dose-dependent effect of CSE (5-50 µg) on epinephrine (5mM/ml) induced platelet aggregation using human platelet rich plasma.
Fig 3.9: Effect of CSE on aggregation of platelets induced by collagen: *Dose-dependent* effect of CSE (5-50 µg) on collagen (2µg/ml)-induced aggregation of platelet using human platelet rich plasma.
Fig 3.10: Effect of CSE on aggregation of platelet induced by ADP: Dose-dependent effect of CSE (5-50 µg) on ADP (5mM/ml) induced aggregation of platelet using human platelet rich plasma.
Fig 3.11: Effect of protease inhibitor on inhibition of epinephrine induced platelet aggregation by CSE: Different inhibitors (10mM) are pre-incubated with CSE for 30 min at 37 °C and the assay was carried out as illustrate in the method section.
Fig 3.12: Effect of protease inhibitor of inhibition of collagen induced platelet aggregation by CSE: Different inhibitors (10mM) are pre-incubated with CSE at 37 °C for 30 min and the assay was carried out as illustrate in the method section.
Fig 3.13: Effect of protease inhibitor on inhibition of ADP induced platelet aggregation by CSE: Different inhibitors (10mM) are pre-incubated with CSE at 37 °C for 30 min and the assay was carried out as illustrate in the method section.
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Fig. 3.13: Effect of CSE and protease inhibitors on platelet aggregation by using human platelet rich plasma: A, B and C represent the dose dependent effect of CSE (5-50 µg) on aggregation of platelets which was induced by epinephrine, collagen and ADP respectively. D, E and F represent the effect of EDTA, PMSF and IAA (10 mM) on platelet aggregation activity of CSE (50 µg) induced by ADP, collagen and epinephrine respectively. During inhibition studies the protease inhibitors were pre-incubated separately with CSE at 37 °C for 30 min and platelet aggregation the assay was carried out as illustrate in the method section and the data was analyzed with one-way ANOVA and Bonferroni’s multiple comparison Test (**P<0.0001, significant when compared to aggregation induced by epinephrine, collagen and ADP).
TABLES:

Tables 3.1: Effect of protease inhibitors on recalcification time, prothrombin time and factor VII deficient plasma clotting property of CSE.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Clotting time</th>
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<tbody>
<tr>
<td>Recalcification time (Sec)</td>
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</tr>
<tr>
<td>Control (Normal plasma)</td>
<td>262 ± 04</td>
</tr>
<tr>
<td>CSE (25µg)</td>
<td>182 ± 07 * ****</td>
</tr>
<tr>
<td>CSE (25µg)+ PMSF (10mM)</td>
<td>179 ±14</td>
</tr>
<tr>
<td>CSE (25µg)+ IAA (10mM)</td>
<td>180 ±13</td>
</tr>
<tr>
<td>CSE (25µg)+EDTA (10mM)</td>
<td>No clot formation b ***</td>
</tr>
<tr>
<td>CSE (25µg)+EGTA (10mM)</td>
<td>No clot formation b ***</td>
</tr>
<tr>
<td>Prothrombin time (Sec)</td>
<td></td>
</tr>
<tr>
<td>Control (Normal plasma)</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>CSE (25µg)</td>
<td>13 ± 2 * ***</td>
</tr>
<tr>
<td>CSE (25µg)+ PMSF (10mM)</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>CSE (25µg)+ IAA (10mM)</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>CSE (25µg)+EDTA (10mM)</td>
<td>No clot formation b ***</td>
</tr>
<tr>
<td>CSE (25µg)+EGTA (10mM)</td>
<td>No clot formation b ***</td>
</tr>
<tr>
<td>Factor VII deficient plasma clotting time (Sec)</td>
<td></td>
</tr>
<tr>
<td>Control (Normal plasma)</td>
<td>262 ± 04</td>
</tr>
<tr>
<td>CSE (50µg)</td>
<td>382 ± 20 * ***</td>
</tr>
<tr>
<td>CSE (50µg)+ PMSF (10mM)</td>
<td>379 ± 21</td>
</tr>
<tr>
<td>CSE (50µg)+ IAA (10mM)</td>
<td>381 ± 23</td>
</tr>
<tr>
<td>CSE (50µg)+EDTA (10mM)</td>
<td>No clot formation b ***</td>
</tr>
<tr>
<td>CSE (50µg)+EGTA (10mM)</td>
<td>No clot formation b ***</td>
</tr>
</tbody>
</table>

The protease inhibitors were pre-incubated separately with CSE for 30 min at 37 °C. Reclassification time, Prothrombin time and congenital factor VII deficient human plasma clotting properties of CSE were the assay was carried out as illustrate in the method section and analyzed with one-way ANOVA and followed by Bonferroni's multiple comparison test (***<0.0001, a- significant when compare to plasma clotting time of control, b- substantial when compared to CSE treated samples).
Table 3.2: Plasminogen activation assay of CSE

<table>
<thead>
<tr>
<th>S.No</th>
<th>Samples</th>
<th>Activity (Unit*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>20 µl human citrated plasma (I) / 1 mg plasminogen contaminated with fibrinogen (II)</td>
<td>Nil</td>
</tr>
<tr>
<td>b.</td>
<td>20 µl human citrated plasma + 200 IU of Urokinase</td>
<td>2.8 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>1 mg plasminogen contaminated with fibrinogen + 200 IU of Urokinase</td>
<td>6.4 ± 0.68</td>
</tr>
<tr>
<td>c.</td>
<td>20 µl human citrated plasma + 50 µg CSE</td>
<td>4.5 ± 0.003</td>
</tr>
<tr>
<td>d.</td>
<td>50 µg CSE</td>
<td>1.9 ± 0.001</td>
</tr>
<tr>
<td>e.</td>
<td>200 IU of Urokinase</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*The amount of enzyme required for increases the absorbance of 0.01/h at 440 nm is equal to one unit. The results are expressed as mean ± SD.