International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (2): 863-878
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Anticoagulant, Antiplatelet and Fibrinogenolytic Activity of Serine Protease from *Cicer arietinum* Seed Extract

Chethana Ramachandraiah[a], Sharathkumar MNandish[a], Jayanna Kengaiah[a], Chandramma Srinivas[a], Ashwini Shiviah[a], Sujatha MHanume gowda[b] and Devaraja Sannaningaiah[*a]

[a]Department of Studies and Research in Biochemistry and Centre for Bioscience and Innovation, Tumkur University, Tumkur, Karnataka.

[b]Department of Biochemistry Jnanasahyadri, Kuvempu university, Shankarghatta, Shimoga, Karnataka.

Received: 21 Jan 2019 / Accepted: 22 Mar 2019 / Published online: 1 Apr 2019
***Corresponding Author Email:** sdevbiochem@gmail.com

Abstract

The present investigation aims to examine the role of anticoagulant, antiplatelet and fibrino(geno)lytic effect of *Cicer arietinum Seed Extract (CASE).* CASE showed similar protein banding pattern from 200kDa to 14kDa in both reduced and non-reduced conditions suggesting the presence of monomeric proteins. CASE showed proteolytic activity with the specific activity of 0.1units/mg/min. CASE delayed the clot formation process of both Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP), from the control 210 sec to 410 sec and 300 sec to 600 sec respectively. CASE found to alter the clotting time of Activated Partial Thromboplastin Time but not Prothrombin Time suggesting, triggered anticoagulant effect by CASE could be due to its inference in intrinsic pathway of coagulation cascade. CASE hydrolyzed Aα & Bβ chains without disrupting γ chain of human fibrinogen. However, it hydrolyzed all the chains of human fibrin clot suggesting its limited proteolysis. The identified said activities were completely abolished by PMSF, a serine protease but not IAA, EDTA and 1, 10, Phenanthroline. Revealed the role of serine proteolytic enzyme. Furthermore, CASE inhibited platelet aggregation induced by agonists ADP and epinephrine in PRP. While, thrombin, collagen, arachidonic acid, ADP and epinephrine in washed platelets. The identified platelet aggregation inhibition was found to be 79%and 82% for PRP and 80%,71%,70%,67% & 58% respectively for washed platelets. CASE was non-hemorrhagic, non-edematic and non-hemolytic in nature.

Keywords

Cicer arietinum Seed Extract, Anticoagulant, Antiplatelet, Fibrino(geno)lytic.

*****

INTRODUCTION

Plant extract/products have been using from ancient period for the betterment of human life, as they store large array of therapeutic principles [1]. Nowadays there is a great revolution in the pharmacology due to their diseases curing ability [2]. Hence, seeds, leaves, bark, roots and latex from plants have been extensively used in the treatment regime [3]. For instance, these can be beneficial in the management of diseases like diabetes, cancer, hyperlipidemia, hypertension and stroke. *Cicer arietinum* (Chick pea) belongs to the phylum *Leguminosae* and the family *Fabaceae.* Chickpea is one of the earliest cultivated legumes remains as old as 7,500 years were found in the Middle East. Chickpeas are grown in the Mediterranean, western Asia, Indian subcontinent and Australia. Chick pea stores diversified group of macro and micro nutrients. The major macro nutrients include carbohydrates, proteins and lipids. While, micro nutrients include minerals and vitamins. In addition, it also richest source of alkaloids, tannins, phenols, flavonoids, coumarins, saponins, terpenoid and phytosterols [4,5]. The said cocktails of Chick pea are ascribed to provide immense therapeutic applications such as anti-cancer, anti-inflammatory and anti-fungal effects [6,7,8]. Recent investigation suggests that the participation of Chick pea in gene activation and DNA repair. Folate present in chickpeas cut down the risk of neural tube defects in new born babies and avoid the early abortion in pregnant women [9]. Butyrate helps to suppress the cell proliferation, induces apoptosis and lowers the risk of colorectal cancer [10,11]. Vit C acts as an antioxidant by quenching the free radicals those damage the tissue [12]. The choline present in chickpea provides better sleep, improve muscle movement and increase the memory. In addition, it also maintains the structure of cellular membranes, aids in the transmission of nerve impulses, assists in the absorption of fat and reduces chronic inflammation [13]. Lycopene present in the Chick pea decreases the risk of prostate cancer [14]. Isoflavone biochanin A and formononetin inhibits the growth of colon cancer cells [15]. Despite, said immense therapeutic applications, the role of *Cicer arietinum Seed* on coagulation and platelet function was least explored. Thus, the current study reports on the anticoagulant, anti-platelet and fibrino(geno)lytic activities of *Cicer arietinum* Seed Extract.

MATERIALS

Folin-Ciocalteau reagent, Sodium Dodecyl Sulphate, Acrylamide, Tris-Hcl, Ammonium per sulphate, TEMED, Fat Free Casein, Phenyl Methyl Sulphonyl Fluoride (PMSF), Ethylene Di-Amine Tetra Acetic acid (EDTA), Iodo-Acetic Acid (IAA), 1,10, Phenanthroline were purchased from Sigma Chemicals Company (St. Louis, USA). Molecular weight markers were from Bangalore Genie Private limited, India. APTT and PT Reagents were purchased from AGAPPE diagnostic Pvt. Ernakulum, 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).

METHODS

Preparation of *Cicer arietinum* (Chick pea) Seed Extract

Cicer arietinum seeds were purchased from local market, Tumkur. The seeds were washed with distilled water then the seed coat was removed. The edible parts of the seeds were homogenized using double distilled water and centrifuged at 8000*g*for 10min at 4°C. The supernatant was collected and proteins were precipitated using 30% of ammonium sulphate. The precipitated protein sample was again centrifuged at 6,000*g* for 20 min; supernatant was collected dialyzed overnight. The protein sample obtained was stored at -20°C until use. This extracted protein sample was used throughout the study and referred as *Cicer arietinum* Seed Extract (CASE). Protein concentration was determined as described by Lowry et, al., 1951[16], using Bovine serum albumin (BSA) as standards.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% was carried out according to the method of Laemmli et al.,1970 [17]. The crude CASE (100µg) prepared under reducing and non-reducing conditions was used for SDS-PAGE. The electrophoresis was carried out using Tris (25mM), glycine (192mM) and SDS (0.1%) for 2hr 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 (40:10:50v/v). Molecular weight standards from 200kDa to 14.3kDa were used.

Periodic Acid-Schiff (PAS) staining

PAS staining was carried out according to the method of Leach et al.,1980 [18], after electrophoresis; the gel was fixed in 7.5% acetic acid solution and stored at room temperature for 1hr. 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

45min. After that, the gel was placed in Schiff's reagent at 4°C for 24hr and was de-stained using 10% acetic acid to visualize a pink colour band.

Colorimetric estimation of Caseinolytic Activity

Caseinolytic activity was assayed according to the method of Murata et al.,1963 [19], using denatured casein as substrate. Briefly, 0.4 ml casein (2%) in 0.2 M Tris–HCl buffer pH8.5 was incubated separately with CASE in a final volume of 1 ml for 2 .30h at 37^0C. The reaction was stopped by adding 1.5 ml of 0.44 M trichloroacetic acid and allowed to stand for 30 min. The reaction mixture was centrifuged at 1,500 g for 15 min. An aliquot (1 ml) of the supernatant was mixed with 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of Folin reagent (1:2, v/v). The color developed was read at 660 nm. Activity was expressed as units/h. One unit of enzyme activity was defined as the amount of enzyme required to increase an absorbance of 0.01 at 660 nm/h at 37^0C.

Preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP)

PRP and PPP were prepared as described by Ardlie and Han et al.,1974 [20]. The platelet concentration of PRP was adjusted to 3.1×10 8 platelets/ml with PPP. The PRP was maintained at 37°C with prior to use within 120min and 180min for plasma recalcification time and platelet aggregation process respectively.

Coagulant activity

Plasma recalcification time

The plasma re-calcification time was determined according to the method of Quick et al., [21]. Briefly, the CASE (0-100µg) was pre-incubated with 0.2ml of citrated human plasma in the presence of 10mM Tris HCl (20µl) buffer pH 7.4 for 1min at 37°C, 20µl of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded. For inhibition studies, CASE (40µg) was preincubated for about 15 min with 5 mmol/l each of PMSF, IAA, EDTA, EGTA and 1,10-phenanthroline.

Bleeding time

The bleeding time was assayed as described previously. Briefly, CASE(0-100µg) in 30µl of PBS was injected intravenously through the tail vein of a group of five mice. After 10min, mice were anaesthetized using diethyl ether and a sharp cut of 3mm length at the tail tip of a mouse was made. Immediately, the tail was vertically immersed into PBS which is pre-warmed to 37°C. Bleeding time was recorded from the time bleeding started till it completely stopped and it was followed for 10min. For inhibition studies, CASE (40µg) was preincubated for about 15 min with 5 mmol/l each of PMSF, IAA, EDTA, EGTA and 1,10-phenanthroline.

Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT)

Briefly, 100 µL of normal citrated human plasma and CASE (0-100µg) were pre-incubated for 1 min. APTT and PT were carried out according to the manufacturer protocol using the coagulation analyzer (Labitec, Germany). For APTT, 100 µL of LIQUICELIN-E phospholipids preparation derived from Rabbit brain with ellagic acid was added. The clotting was initiated by adding 100 µL of 0.02 M $CaCl_2$ 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 (INR) for PT at each point were calculated from the values of control plasma incubated with the buffer for an identical period of time.

Human fibrinogenolytic activity

Fibrin(ogeno)lytic activity

Fibrinogenolytic activity was determined as described previously by Ouyang and Teng et al.,1976 [22]. CASE (0–50µg) was incubated with the human plasma fibrinogen (50µg) in a total volume 40µl of 10mM Tris–HCl buffer pH7.4 for 4h at 37°C. After the incubation period, reaction was terminated by adding 20µl denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol. It was then analysed by 10% SDS-PAGE. For inhibition studies, CASE (20µg) was preincubated for about 20min with 5mM each of PMSF, IAA, EDTA and 1,10-Phenanathroline.

Fibrinolytic activity

Colorimetric estimation

Fibrin clot-hydrolyzing activity was determined as described by Rajesh et al., [23]. Briefly, 100µl of citrated human plasma was mixed with 20µl of 0.2M $CaCl_2$ and incubated for 2hr at 37°C. The clot obtained was washed thoroughly for 5–6 times with PBS and suspended in 400µl of 0.2M Tris–HCl buffer (pH 8.5). The reaction was initiated by adding varied amounts of CASE(0–100µg) in 100µl of saline and incubated for 2hr and 30min at 37°C. The undigested clot was precipitated by adding 750µl of 0.44M TCA and allowed to stand for 30min and centrifuged for 15min at 1500g. The aliquots of 0.5ml supernatant was transferred to clean glass tubes and it was followed by the addition of 1.25ml of 0.4M sodium carbonate and 0.25ml of 1:2 diluted Folin–Ciocalteu's phenol reagent. The color developed was read at 660nm after being allowed to stand for 30min. One unit of activity is defined as the amount of enzyme required to increase in absorbance of 0.01 at 660nm/hr. at 37°C.

SDS-PAGE pattern of human plasma clot hydrolyzing Activity

The washed clot was incubated with different doses (0-100µg) of CASE source in a 40µl reaction mixture at 37^0C for 1 h in the presence of 10 mM Tris–HCl buffer (pH 7.6). The reaction was stopped by adding 20 ml sample buffer containing 4% SDS, 4% β-mercapto ethanol and 1 M urea, boiled for 3 min and centrifuged to settle the debris of plasma clot. An aliquot (20 ml) of the supernatant was used to analyze the hydrolyzing pattern of plasma clot in 7.5% SDS-PAGE according to the method of Laemmli et al. For inhibition studies, CASE (60µg) was preincubated for about 20min with 5mM each of PMSF, IAA, EDTA and 1, 10-Phenanathroline.

Human Blood clot lysis Assay

The blood clot lysis assay was determined according to the method of Prasad S. et al.,2006 [24]. Venous blood drawn from healthy volunteers were transferred to different pre-weighed sterile micro centrifuge tubes (500 µl/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube with clot was again weighed to determine the clot weight (clot weight was equal to weight of clot containing tube minus weight of tube alone). Each micro centrifuge tube, containing clot was properly labelled and 50-200µg of CASE was added to the clots. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the obtained fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. the obtained difference in weight taken before and after clot lysis was expressed as percentage of clot lysis.

Human plasma protein hydrolyzing activity

Degradation of human plasma protein was assayed according to the method of Kumar et al.,2010 [25]. The CASE (0–50µg) was incubated with the 100µg of plasma proteins for 12hr at 37°C in a reaction volume of 40µl 10mM Tris–HCl buffer (pH 7.4) containing 10mM NaCl, 0.05% sodium azide. The reaction was terminated by the addition of 20µl denaturing buffer containing 4% SDS and boiled for 5min. It was then analyzed on a 7.5% SDS-PAGE under non-reduced condition.

Preparation of washed platelets

Preparation of washed platelets Washed platelets were prepared as described by the method of Born et al.,1962 [26]. For 9ml of blood sample add 1.5ml of acid citrate dextrose buffer and centrifuged at 300g for 15min. The obtained PRP was collected and centrifuged for 20min at 350g. The obtained pellet was re-suspended in tyrode albumin buffer (pH 6.5) and mixed well then centrifuged for 20min at 350g. The pellet was again re-suspended in tyrode albumin buffer (pH 6.5) and centrifuged again for 20min at 350g and the pellet obtained was re-suspended in tyrode albumin buffer (pH 7.35) containing 2mM $CaCl_2$ $6H_2O$ and this suspension was taken for platelet 2 aggregation study

Platelet aggregation

The turbid metric method of Born et al.,1962 was followed using a Chronology dual channel whole blood/optical lumi aggregation system (Model-700). Aliquots of PRP were pre-incubated with various concentrations of CASE (0–60µg) in 0.25ml reaction volume. The aggregation was initiated independently by the addition of agonists, such as ADP, Epinephrine followed for 6min.

Direct hemolytic activity

Direct hemolytic activity was determined by using washed human erythrocytes. Briefly, packed human erythrocytes and phosphate buffered saline (PBS) (1:9v/v) were mixed; 1ml of this suspension was incubated independently with the various concentration of CASE (0-30µg) for 1hr at 37°C. The reaction was stopped by adding 9ml of ice-cold PBS and centrifuged at 1000g for 10min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540nm. Activity was expressed as percent of hemolysis against 100% lysis of cells due to addition of water that served as positive control and phosphate buffered saline served as negative control.

Animal Studies

Edema inducing activity

The procedure of vishwanath et al.,1987 [27] was followed. Groups of five mice were injected separately into the right foot pads with different doses (0-200µg) of CASE in 20µl saline. The left foot pads received 20µl saline alone served as control. After 1hr mice were anaesthetized 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 (MED) 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., 1969 [28]. Groups of five mice were injected (s.c) separately with various concentrations of CASE (0-200µg) was injected (intradermal) independently into the groups of five mice in 30µl saline. Group receiving saline alone serves as negative control and group receiving venom (2MHD) as positive control. After 3hr, mice were

anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for haemorrhage against saline injected control mice. The diameter of haemorrhagic spot on the inner surface of the skin was measured. The minimum haemorrhagic dose (MHD) was defined as the amount of the protein producing 10mm of haemorrhage in diameter.

Statistical Analysis

The data are presented as mean s± S.E.M of at least five animals in each group. Difference among the data were determined by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Data were considered different at $P < 0.01$.

RESULTS

The present work designates on the anticoagulant, antiplatelet and fibrino(geno)lytic activities of *Cicer arietinum* seed extract (CASE). The CASE showed similar protein banding pattern on SDS-PAGE (Fig.1a). CASE was positive for PAS-staining as maximum proteins were taken the stain at the region around 200kDa to 14.3kDa those were compared with the fibrinogen the positive control (Fig.1b). CASE digested casein suggesting its proteolytic ability and the specific activity was found to be 0.1 units/mg/min. Only PMSF was completely neutralized the caseinolytic activity of CASE, but not IAA, EDTA & 1.10, Phenanthroline (Table1).

CASE increased the clotting time from control 210 sec to 410 sec in platelet rich plasma and from 300 sec to 600 sec in platelet poor plasma at the concentration of 100µg. (Fig.2a). An anticoagulant activity of CASE was also established by invivo mouse tail bleeding assay. CASE injected intravenously to the mice delayed bleeding time significantly in a dose dependent manner and the noted bleeding time was >600sec. ($P< 0.01$) at 100µg against PBS treated control of 180±5s (Fig.2c). Both *in-vitro* and *in-vivo* anticoagulant activity of CASE was entirely neutralized by only PMSF but not IAA, EDTA and1,10. Phenanthroline. (Fig.2b &2d). Furthermore, CASE specifically enhanced the clotting time of only APTT but not PT, reveals the involvement of intrinsic pathway of blood coagulation cascade. (Table 2). CASE degraded human fibrinogen and fibrin clot. CASE specifically hydrolysed Aα & Bβ chain at the concentration of 0-50µg with the 4h incubation time (Fig.3a), when incubation time was extended up to 24h, it did not affect γ chain (Fig.3b). The fibrinogenolytic activity was completely abolished by PMSF but not IAA, EDTA, 1, 10-Phenanthroline (Fig.3c). CASE dissolved both whole blood and fibrin clot signifying its clot dissolving property. When whole blood clot was incubated with CASE (0-200 µg) it showed dose dependent clot dissolving efficiency that was compared with the positive control streptokinase (60 U/ml). Water taken as a negative control showed negligible clot lysis percentage. The percentage of clot lysis was found to be 55 % at the concentration of 200 µg of CASE against the positive control streptokinase 66.8 %. (Fig. 4). Furthermore, CASE hydrolysed washed plasma clot and the specific activity was found to be 2.3units/mg/min (Fig.5a). The plasma clot hydrolysed pattern of CASE was also analysed in the SDS-PAGE. CASE degraded α polymer and γ-γ dimer of human fibrin, whereas α-chain & β-chain remains resilient to proteolysis over the incubation period of 12 h (Fig 5b). Where it completely degraded all the chains of fibrin clot over the incubation time of 48h (Fig5c). The human fibrin clot hydrolysing activity was inhibited by PMSF (Fig.5d). Furthermore, plasma protein degradation assay was carried out to identify the substrate specificity of CASE on plasma proteins. CASE preferably degraded only fibrinogen bands without affecting the other plasma proteins when incubated at 37°C for 12h at the concentration of 50µg (Fig.6). CASE was analysed for platelet aggregation using platelet rich plasma and washed platelets with agonists such as ADP and Epinephrine. Remarkably, CASE inhibited ADP and epinephrine induced platelet aggregation of about 79% and 82% respectively at the concentration of 60 µg in PRP. (Fig.7 & 8). CASE was also inhibited the thrombin, collagen, arachidonic acid, ADP and Epinephrine induced platelet aggregation of about 70%, 58%, 67%, 71% & 80% respectively at the concentration of 60µg. (Fig.9-13). The order of inhibition observed among the agonists was Epinephrine >ADP >Thrombin> Arachidonic Acid & Collagen. Moreover, CASE did not hydrolyse RBC up to the concentration of 200 µg when compare to the water positive control and PBS taken as a negative control, that concludes CASE is non-toxic to RBC cells (Fig. 14). In addition, it did not cause haemorrhage and edema in experimental mice up to the concentration of 200 µg (Fig.15). While positive control daboiarusselli venom induced haemorrhage and edema in experimental mice.

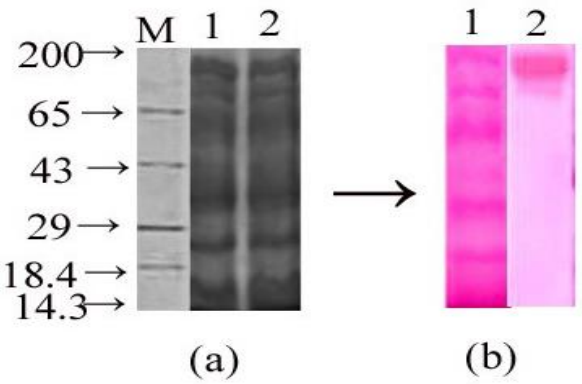

Fig. 1 (a) SDS-PAGE 10 % (b) Glycoprotein staining. (a) CASE as shown in SDS-PAGE (10 %): CASE (100 µg) under non-reduced (a1) and reduced conditions (a2), (b) PAS staining of CASE: positive control fibrinogen (c1) and CASE (c2). M represents the molecular weight marker in kDa from top to bottom: Myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), lactalbumin (18.4) and lysozyme (14.3) BSA: bovine serum albumin, CASE: Cicer arietinum Seed Extract.

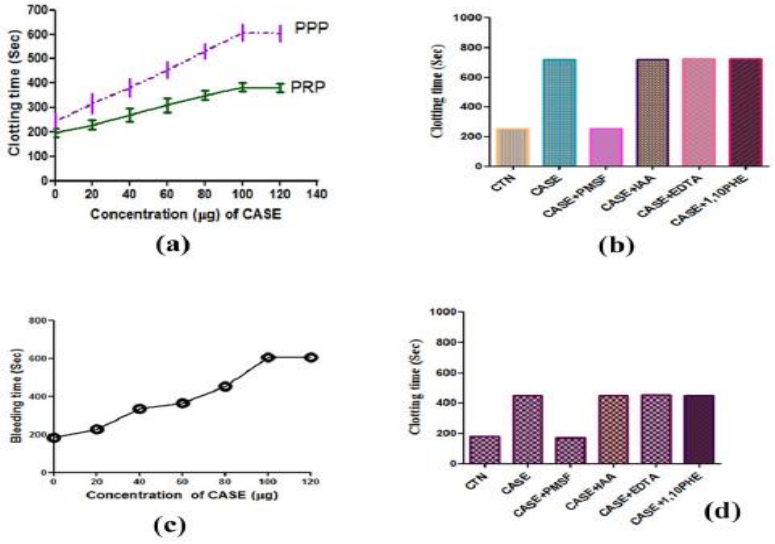

Fig. 2 Plasma re-calcification time. (a)CASE (0–100µg) was pre-incubated with 0.2 ml of citrated human plasma PRP/PPP in the presence of 20 µl 10 mM Tris–HCl buffer (pH 7.4) for 1 min at 37° C. 20 µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded. (b) Effect of protease inhibitors on the anticoagulant activity of CASE. CASE (40µg) was preincubated with 5 mmol/l PMSF, IAA, EDTA, 1,10-phenanthroline, respectively, for 15 min at 378C. IAA, iodoacetic acid; PMSF, phenyl methyl sulphonyl fluoride. CASE: Cicer arietinum Seed Extract. (c)Tail bleeding time was measured 10 min after intravenous administration of PBS or various doses of CASE. Each point represents the mean SD of three independent experiments ($P < 0.01$). (d) CASE (60µg) was preincubated with 5 mmol/l PMSF, EDTA, IAA and 1,10-phenanthroline, respectively, for 15 min at 37^0C.; PBS, phosphate-buffered saline. CASE: Cicer arietinum Seed Extract.

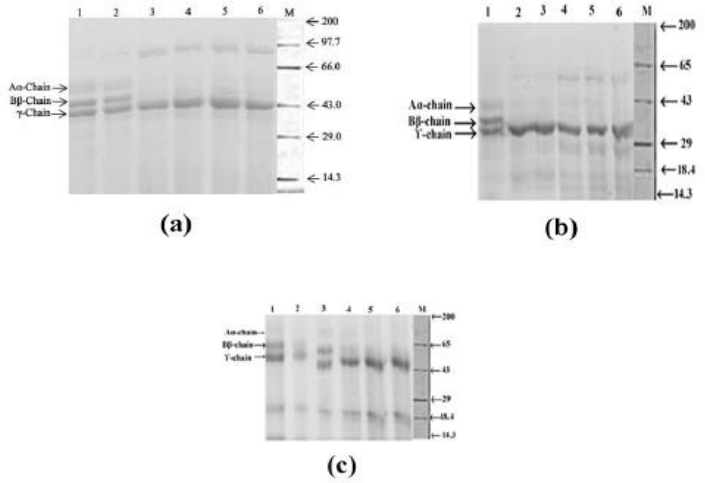

Fig.3 Effect of CASE on Fibrinogenolytic activity. (a) Dose-dependent effect: fibrinogen alone 50µg (a1), fibrinogen treated with 10µg (a2), 20µg (a3), 30µg (a4), 40µg (a5), 50µg (a6) of CASE respectively, incubated for 4hr at 37°C and then separated on 10% SDS-PAGE under reduced condition.: CASE20µg was incubated with fibrinogen 50µg for 0hr (b1), 4hr (b2), 8hr (b3), 12hr (b4), 16hr (b5) and (b) Time-dependent effect 24hr (b6) respectively at 37°C. CASE20µg was pre-incubated with protease inhibitors for 30min at 37°C. Further reaction (c) Inhibition study: was initiated by adding 50µg of fibrinogen and incubated for 4hr, fibrinogen alone (c1), CASE 10µg (c2), fibrinogen 50µg and CASE 10µg with 5mM PMSF (c3), fibrinogen 50µg and CASE 10µg with 5mM IAA (c4), fibrinogen 50µg and CASE 10µg with 5mM EDTA (c5), fibrinogen 50µg and CASE 10µg with 5mM 1, 10, phenanthroline (c6). CASE: Cicer arietinum Seed Extract.

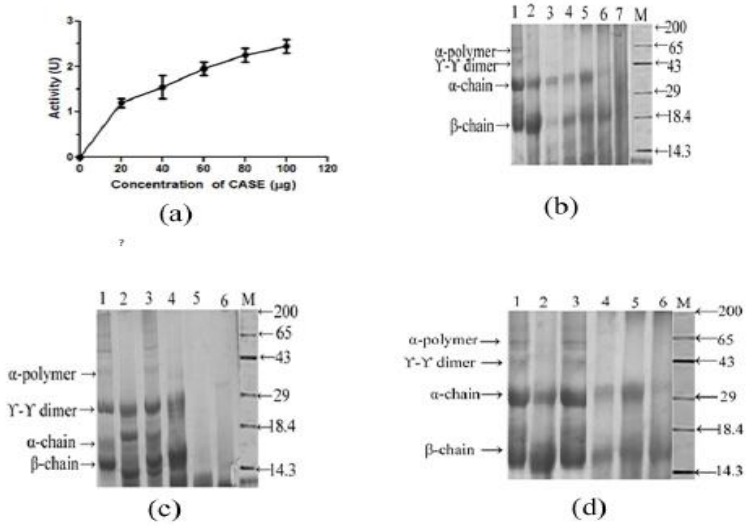

Fig.4 Fibrinolytic activity. (a) Colorimetric assay (b) Dose-dependent effect (c) Time-dependent effect. (d) CASE Inhibition study (a) Washed plasma clot was incubated with 0–100 µg of CASE for 2.30 hr and then the OD was measured at 660 nm. **(**b) CASE Dose-dependent effect**:** Washed plasma clot was incubated for 12 hr and then separated on SDS-PAGE (7.5 %), washed plasma clot alone (b1), plasma clot treated with 10 µg (b2), 20 µg (b3), 30 µg (b4), 40 µg (b5) and 50 µg (b6) of CASE respectively. (c) CASE Time-dependent effect**:** CASE 40 µg was incubated with fibrin clot at 37° C, fibrin clot alone (c1), 0 hr (c2), 6 hr (c3), 12 hr (c4), 18 hr (c5) and 24 hr (c6) of CASE. (d) CASE Inhibition study**:** CASE 60 µg was pre-incubated with protease inhibitors for 30 min at 37° C. Further reaction was initiated by adding fibrin clot and incubated for 12 hr, fibrin clot alone (d1), CASE 60 µg (d2), fibrin clot and CASE 60 µg with 5 mM PMSF (d3), fibrin clot and CASE60 µg with 5 mM IAA (d4), fibrin clot and CASE 60 µg with 5 mM EDTA (d5), fibrin clot and CASE 60 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, CASE: Cicer arietinum Seed Extract.

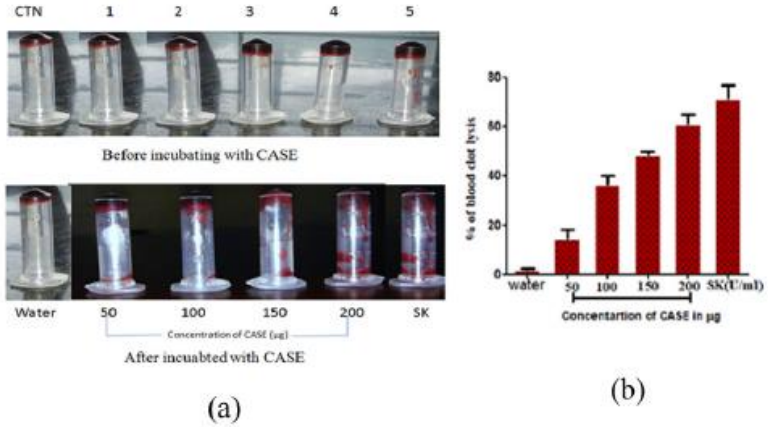

Fig. 5 Blood clot lysis study. (a) Photographic image of blood clot lysis assay (b) Percentage of blood clot lysis. (a) Venous blood was transferred to different pre-weighed sterile micro centrifuge tubes (500 µl/tube) and incubated at 37° C for 45 min. After clot formation, serum was completely removed and each tube with clot was again weighed. The 0-200 µg of CASE was added to the micro centrifuge tube containing clots. All the tubes were then incubated at 37° C for 90 min and observed for clot lysis. (b) After incubation, the obtained fluid was removed and tubes were again weighed and blood clot lysis percentage was calculated.

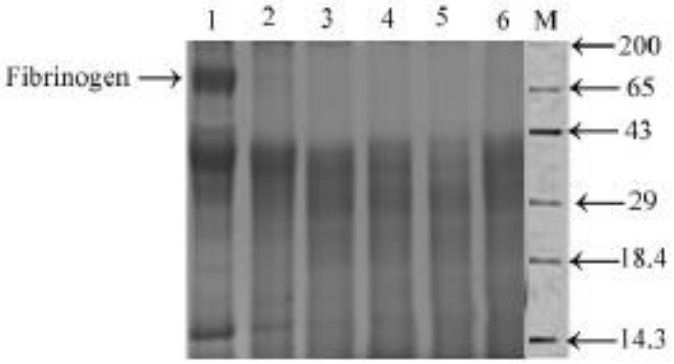

Fig. 6 Degradation of plasma proteins. Plasma protein (100 µg) was incubated with CASE in 40 µl of 10 mM Tris–HCl buffer (pH 7.4) at 37° C and then analyzed on 7.5 % SDS-PAGE under non-reduced condition. 20 µg of fibrinogen as control (1), plasma protein (100 µg) alone (2), plasma protein treated with 10 µg (3), 20 µg (4), 30 µg (5), 40 µg (6), 50 µg (7) of CASE and 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, CASE: Cicer arietinum Seed Extract

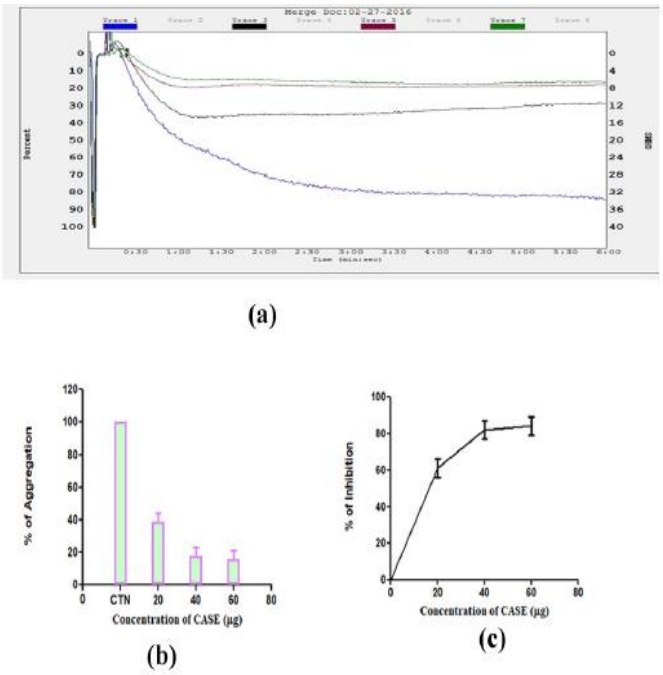

Fig. 7 Platelet aggregation was initiated by adding ADP as an agonist. (a) Traces of platelet aggregation**:** Trace 1 (ADP 10 µM); Trace 2 (ADP 10 µM + 20 µg of CASE); Trace 3 (ADP 10 µM + 40 µg of CASE); Trace 4 (ADP 10 µM + 60µg of CASE). The values represent ±SD of three independent experiments**.** (b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%

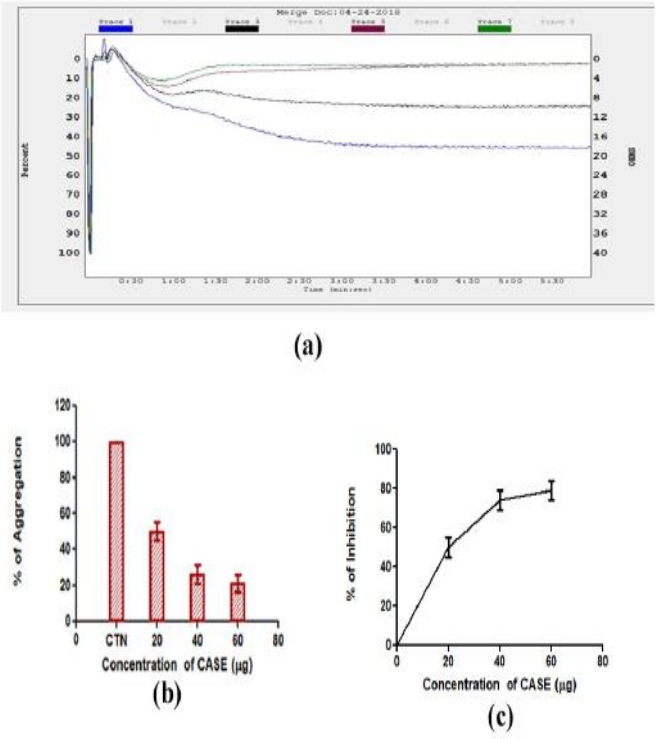

Fig. 8 Platelet aggregation was initiated by adding Epinephrine as an agonist. (a) Traces of platelet aggregation**:** Trace 1 (Epinephrine 5 µM); Trace 2 (Epinephrine 5 µM + 20 µg of CASE); Trace 3 (Epinephrine 5 µM + 40 µg of CASE); Trace 4 (Epinephrine 5 µM + 60 µg of CASE). The values represent of three independent experiments. (b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%.

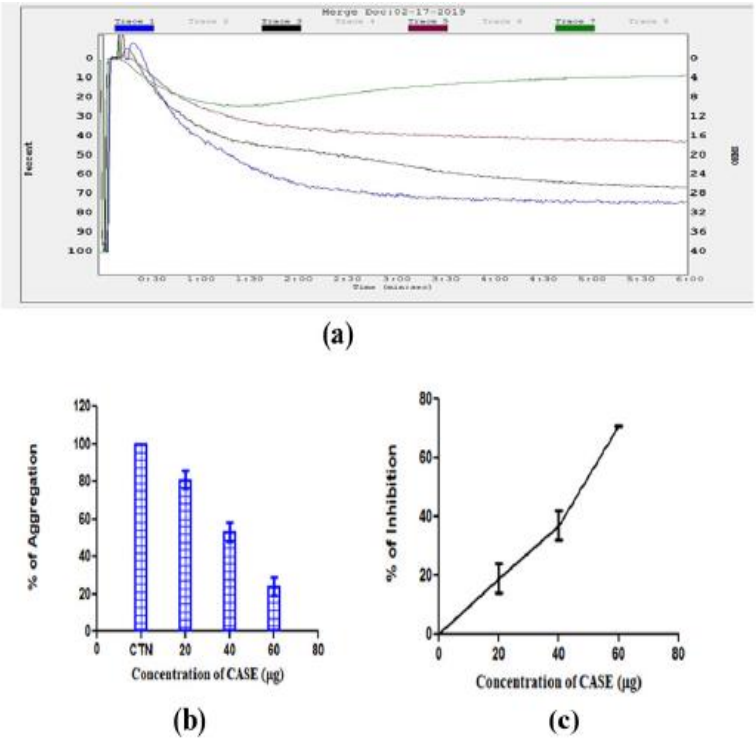

Fig.9 Washed platelet aggregation was initiated by adding ADP as an agonist. (a) Traces of platelet aggregation: Trace 1 (ADP 10µM); Trace 2 (ADP 10µM+20µg of CASE); Trace 3 (ADP 10µM+40µg of CASE); Trace 4 (ADP 10µM+60µg of CASE). The values represent of three independent experiments. (b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%.

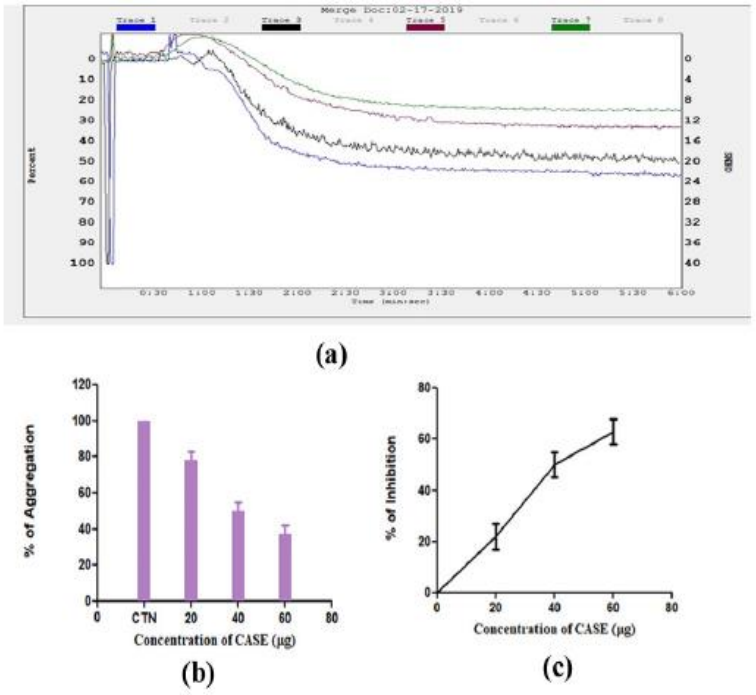

Fig10. Washed Platelet aggregation was initiated by adding Collagen as an agonist. (a) Traces of platelet aggregation: Trace 1 (Collagen 5µM); Trace 2 (Collagen 5µM+20µg of CASE); Trace 3 (Collagen 5µM+40µg of CASE); Trace 4 (Collagen 5µM+60µg of CASE). The values represent of three independent experiments. (b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%.

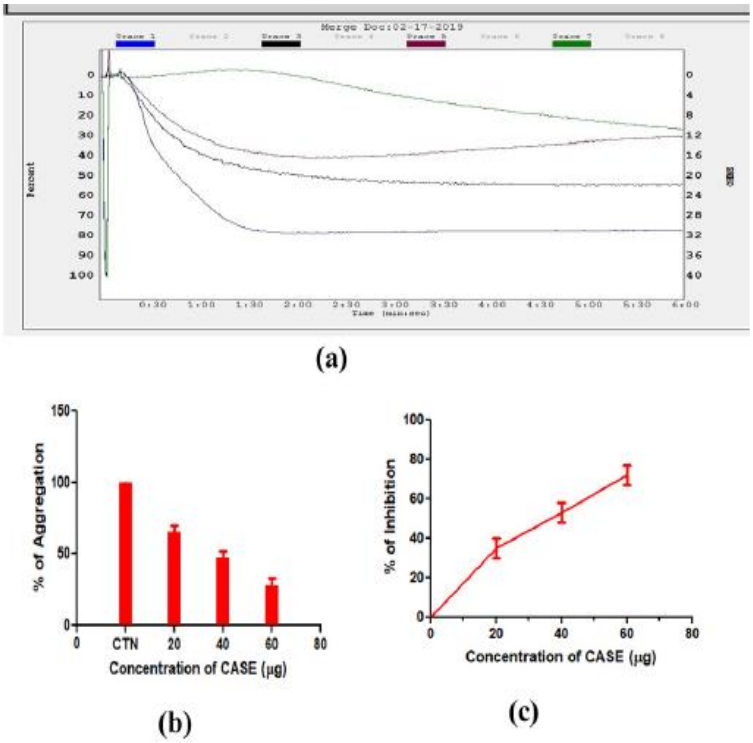

Fig 11. Washed Platelet aggregation was initiated by adding Arachidonic acid as an agonist. (a) Traces of platelet aggregation: Trace 1 (Arachidonic acid 5µM); Trace 2 (Arachidonic acid 5µM+20µg of CASE); Trace 3 (Arachidonic acid 5µM+40µg of CASE); Trace 4 (Arachidonic acid 5µM+60µg of CASE). The values represent of three independent experiments. (b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%.

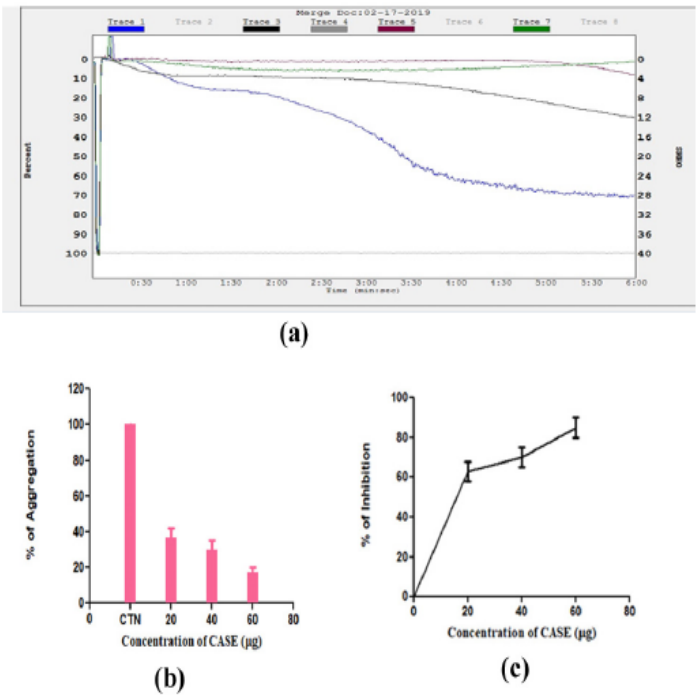

Fig 12. **Washed Platelet aggregation was initiated by adding Epinephrine as an agonist**. (a) Traces of platelet aggregation: Trace 1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM+20µg of CASE); Trace 3 (Epinephrine 5µM+40µg of CASE); Trace 4 (Epinephrine 5µM+60µg of CASE). The values represent of three independent experiments. **(**b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%.

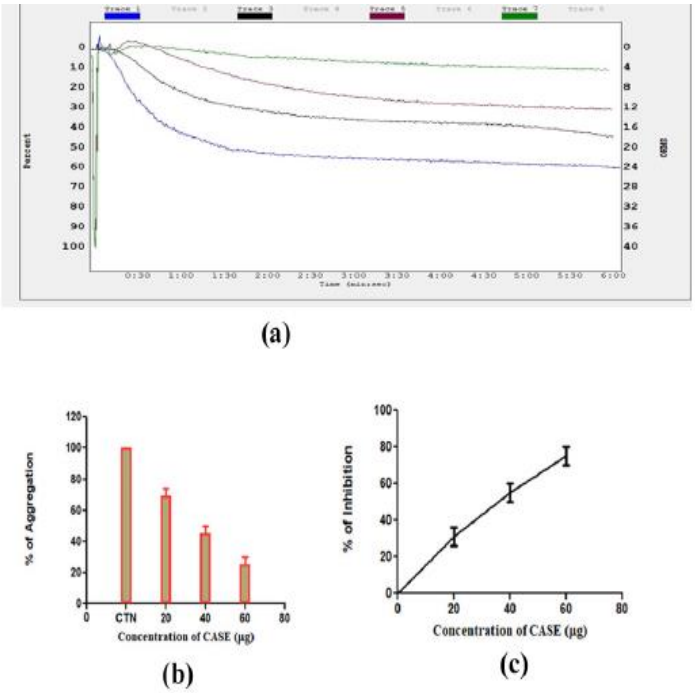

Fig 13. Washed Platelet aggregation was initiated by adding Thrombin as an agonist. (a) Traces of platelet aggregation: Trace 1 (Thrombin 2µM); Trace 2 (Thrombin 2µM+20µg of CASE); Trace 3 (Thrombin 2µM+40µg of CASE); Trace 4 (Thrombin 2µM+60µg of CASE). The values represent of three independent experiments. (b) Dose dependent platelet aggregation %. (c) Dose dependent platelet aggregation inhibition%.

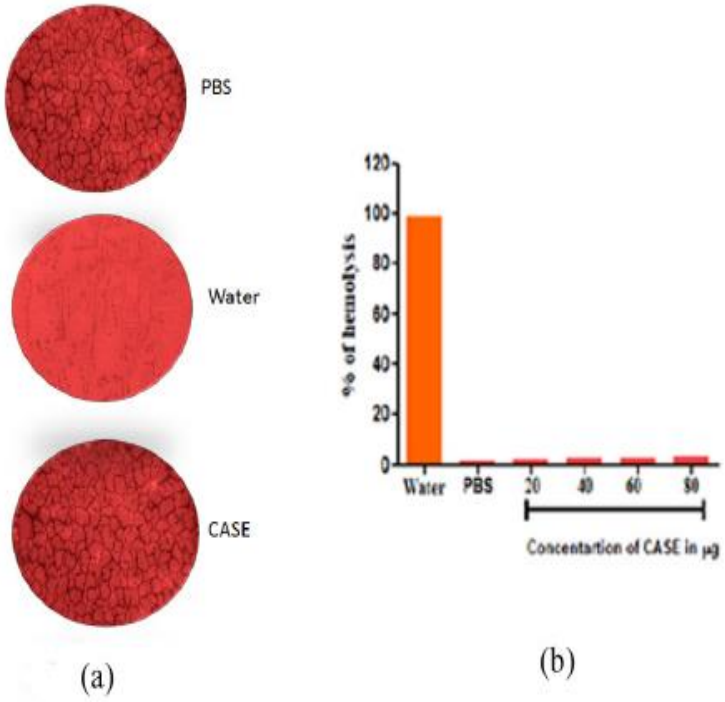

Fig. 14 Direct Hemolytic assay. (a)Microscopic image of RBC**:** RBC treated with PBS, RBC treated with Water, RBC treated with CASE 50µg.(b)The different concentration of CASE (0-100 µg) was incubated independently for 1 hr at 37° C with the 1 ml of suspension made with packed human erythrocytes and phosphate buffered saline (PBS) 1:9 v/v. 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. CASE: Cicer arietinum Seed Extract

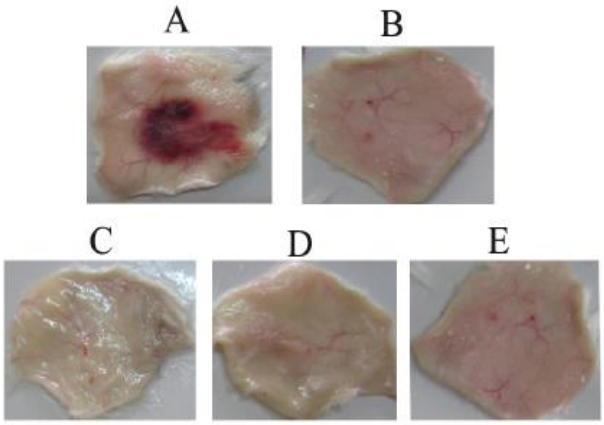

Fig. 15Dose-dependent hemorrhagic activity of CASE. (a) Saline, (b) positive control 2 MDH venom, (c) 200µg of CASE was injected independently into mice in a total volume of 50 µl intradermal. CASE: Cicer arietinum Seed Extract

Table 1 : Effect of Inhibitors on the Proteolytic Activity of CASE

Inhibitor (5mM each)	Activity/residual activity (%)
None	100
EDTA	95.05
1,10-Phenanthroline	90.90
IAA	81.10
PMSF	20.21

Table 2: Dose dependent effect of CASE on clotting time of normal human plasma

CASE (µg)	PT clotting time in seconds	PT (INR Value)	APTT clotting time in seconds	APTT ratio
0	11.10±0.02	0.93±0.02	33.40±0.05	1.22±0.02
10	11.10±0.03	0.91±0.03	36.51±0.02	1.25±0.01
20	11.40±0.02	0.92±0.01	37.42±0.07	1.37±0.04
30	11.20±0.01	0.90±0.01	39.41±0.03	1.51±0.05
40	12.40±0.02	1.44±0.01	41.10±0.01	1.78±0.02
50	12.10±0.01	1.50±0.01	45.01±0.03	1.91±0.01
60	13.10±0.03	1.37±0.02	48.22±0.05	2.15±0.03
70	12.25±0.01	1.50±0.02	51.50±0.01	2.67±0.02
80	13.20±0.01	1.47±0.03	52.10±0.04	2.72±0.01
90	13.15±0.04	1.40±0.01	52.40±0.02	2.91±0.03
100	13.05±0.01	1.38±0.05	52.52±0.01	2.95±0.02

DISCUSSION

Hemostasis is a complex physiological process that requires the participation of platelets, coagulation factors and extracellular matrix proteins. The hemostatic system is usually activated upon injury to the blood vessels, which disrupts endothelial cells exposing thrombogenic substances in the extravascular space. Clot formation is typically initiated by vascular injury, in which a platelet plug forms and is strengthened with fibrin produced via the extrinsic pathway. Accordingly, Impaired blood coagulation cascade intensify the risk of thrombosis, an unusual clot formation in arteries &veins. Anti-thrombotic and antiplatelet agents are the best-known therapy to treat thrombosis. While the currently available synthetic anticoagulant drugs have numerous side effects that may lead to health issues. Therefore, identifying the natural anticoagulant and antiplatelet agents helps to manage thrombotic disorders. Thus the current study displays the anticoagulant, antiplatelet and fibrino(geno)lytic activities of *Cicer arietinum*. CASE portrayed similar banding pattern under both reduced and non-reduced conditions revealed the presence of monomeric proteins. CASE was positive to PAS staining as it took up PAS stain signifying CASE withhold maximum number of glycoproteins. Glycoproteins play an important role in several physiological aspects like cell signalling, neuronal development, immune response, fertilization, hormonal activities and transportation across the membrane [29-33]. CASE hydrolysed casein signifying its proteolytic activity. Curiously, proteolytic activity of CASE was completely neutralized by PMSF suggests the existence of serine protease in the extract. Several serine proteases were explored from latex, seeds, leaves, and earthworms, caterpillar, snake venom, spider and honeybees [34]. Interestingly, CASE exhibits anticoagulant property in both in vitro and in vivo experiments.

CASE only prolonged the clotting process of APTT but it did not interfere in the clotting process of PT, revealing the triggered anticoagulation by the extract could be due to its interference in the intrinsic pathway but not in the extrinsic pathway of blood coagulation cascade. The in-vitro and in-vivo anticoagulant effect was completely inhibited by PMSF, a serine protease inhibitor but not 1,10-phenanthroline, EDTA & IAA. Numerous effective anticoagulants proteins were identified in marine creatures, herbal medicines, snake venoms, earthworm secretions, dung beetles, food-grade, microorganisms and fermented food products like Japanese Natto and Korean Chungkook-Jang soy sauce [35]. Several anticoagulant proteases have been characterized from seeds such as Horse gram, flax seed, Bitter gourd, Pea and jackfruit [36-40]. CASE hydrolyzed Aα and Bβ chains of fibrinogen without affecting γ chain. Thrombin specifically hydrolyzed Aα and Bβ chains of fibrinogen from N-terminal end and generates fibrinopeptide A and B. Proteolytic enzymes those degrade fibrinogen similar to thrombin like enzymes results in procogulation. However, the enzymes those degrade Aα and Bβ chains of fibrinogen from C-terminal end that results in the truncated structure which lacks polymerization potential leads to the anticoagulation. Though, the CASE hydrolysed Aα and Bβ chains of fibrinogen it might have hydrolysed from the C-terminal rather N-terminal. Thus, the fibrinogenolytic activity of CASE further strengthened its anticoagulant property. The fibrin(ogen)olytic activity was completely neutralized by only PMSF, again strengthening the role of serine proteolytic activity of the extract. Numerous serine proteases exhibit fibrin(ogen)olytic were reported from plant latex, earthworms, caterpillar, venoms of snake, spider, and honey bees [41]. Furthermore, CASE has efficiently dissolved both blood clot and fibrin clot strengthened its clot lysis activity along with anticoagulant potential. It hydrolyzed all the chains of human fibrin clot revealed the beneficial role in thrombotic disorders. Fibrinolysis is an important event in the haemostasis that helps in clot dissolution. Plasmin play a key role in the fibrinolysis [42]. The fibrinolytic activity was evidently abolished by PMSF, strengthening the role of serine proteolytic activity of the extract. CASE degraded only plasma fibrinogen but did not degrade other plasma proteins under nonreduced condition. Furthermore, CASE inhibited the agonists ADP, Epinephrine, collagen, thrombin, arachidonic acid induced platelet aggregation in PRP and washed platelets. Seeds of Horse gram, Bitter gourd, Jackfruit, Pea and flax seed exhibited antiplatelet property [36-40]. CASE was nontoxic to experimental mice as it did not show hemolytic activity, edema and hemorrhage in experimental mice.

In conclusion, this study reports on the anticoagulant, fibrinogenolytic, fibrinolytic and anti-aggregation properties of CASE. The said activity of CASE is due to its serine proteolytic activity. Thus, purification and its characterization is of great interest.

DECLARATION OF CONFLICT OF INTEREST

The authors declared no potential conflict of interest with respect to the authorship and publication.

ACKNOWLEDGMENT

Devaraja Sannaningaiah and Chethana Ramachandraiah thank the Department of Science and Technology, Government of India, New Delhi and Vision Group on Science and Technology, Government of Karnataka, Bangalore for financial assistance.

REFERENCES

1. Gupta AK, Sharma M. Reviews on Indian Medicinal Plants, V- 6, Medicinal Plants Unit Indian Council of Medical Research. 178-236, (2008).
2. Tucakov J. Healing with plants – phytotherapy. Beograd: Culture. 180–90, (1971).
3. Jayaweera DMA. Medicinal Plants (Indigenous & Exotic) used in Ceylon (Part 3). The National Science Council.1-297, (1981).
4. Singh K, Ahlawat S and Patra A. Pharmacognostical evaluation of Cicer arietinum Linn. Phcog Net. 1(3): 195-200, (2013).
5. Arora M, Singh S, Kaur P. Pharmacognostic & phytochemical evaluation of selected seeds of 'Cicer arietinum' Linn. seeds from oopnagar Punab. International Journal of Pharmaceutical Science Invention. 2(11):18-29, (2013).
6. Doppalapudi S, Sandya L, Reddy KC, Nagarjuna S, Padmanabha RY, Saba S. Anti- inflammatory activity of Cicer arietinum seed extracts. Asian Journal of Pharmaceutical & Clinical Research.5:64-68, (2012).
7. Valligatla Sukanya SG, Gayathri G. Variability in the distribution of daidzein and genistein in legume sprouts and their anticancer activity with MCF-7 breast cancer cells. Academic Journal of Cancer Research.7 (3): 173-178, (2014).
8. Chu KT, Liu KH, Ng TB. Cicerarin, a novel antifungal peptide from the green chickpea. Peptides.24: 659-663, (2003).
9. Pitkin RM. Folate and neural tube defects. Am J Clin Nutr.85:285S-288S, (2007).
10. Cummings JH, Stephen AM, Branch WJ Implications of dietary fibre breakdown in the human colon. In Banbury Report 7 Gastrointestinal Cancer. 71–81, (1981)
11. Mathers JC. Pulses and carcinogenesis: potential for the prevention of colon, breast and other cancers. Br J Nutr 88. Suppl. 3. S273–S279, (2002).
12. Porkodi k, Usha. A comparative study on phytochemical and biochemical analyses between Cicer arietinum brown and white seeds. IJPRBS. 3(4): 40-63, (2014).
13. Zhang T, Jiang B, Wang Z. Nutrition and application of chickpea (in Chinese). *Cereals Oils* **7**.18–20, (2007).
14. Giovannucci E, Ascherio A, Rimm EB, et al. Intakes of carotenoids and retinal in relation to risk of prostate cancer. J Natl Cancer Inst 87.1767–1776, (1995).
15. Yanagihara K, Ito A, Toge T, et al. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. Cancer Res 53.5815–5821, (1993).
16. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin reagent. J Biol Chem.193:265-275, (1951).
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685, (1970).
18. Leach BS, Collawn JF, Fish WW. Behavior of glycopolypeptides with empirical molecular weight estimation methods, In sodium dodecyl sulfate. Biochemistry9: 5734–5741, (1980).
19. Satake M, Murata Y, Suzuki. Studies on snake venoms XIII Chromatographic separation and properties of three proteinases from Agkistrodonhalysblomhoffii venom. J Biochem 53: 483–497, (1963).
20. Ardlie NG, Han P. Enzymatic basis for platelet aggregation and release: the significance of the 'platelet atmosphere' and the relationship between platelet function and blood coagulation. J Haematol 26: 331–56, (1974).
21. Quick AJ, Stanley-Brown M, Bancroft FW. A study of the coagulation defect in hemophilia and in jaundice. Am J Med Sci 190: 501–11, (1935).
22. Ouyang C, Teng CM. Fibrinogenolytic enzymes of Trimeresurusmucrosquamatus venom. Biochim Biophys Acta 420: 298–308, (1976).
23. Rajesh R, Raghavendra Gowda CD, Nataraju A. Procoagulant activity of Calotropis gigantea latex associated with fibrin(ogen)olytic activity. Toxicon 46: 84–92, (2005).
24. S Prasad, RS Kashyap, JY Deopujari, HJ Purohit, GM Taori, HF Daginawala. Development of an in vitro method to study clot lysis activity of thrombolytic drugs, Thrombosis Journal 4(14): 1- 4, (2006).
25. Kumar MS, Devaraj VR, Vishwanath BS, Kemparaju K. Anticoagulant activity of a metalloprotease: further characterization from the Indian cobra (Najanaja) venom. J Thromb Thrombolysis 29: 340–348, (2010).
26. Born GV. Aggregation of blood platelets by adenosine di-phosphate and its reversal. Nature 194: 27–9, (1962).
27. Sannanaik V, Manjunatha Kini R, Veerabasappa Gowda T. Characterization of three edema-inducing phospholipase A2 enzyme from habu (Trimeresurus flavoviridis) venom and their interaction with the alkaloid aristolochic acid. Toxicon, 25:501-515, (1987).
28. Kondo H, Kondo S, Itezawa H, Murata R, Ohasaka A. A study on the quantitative method from determination of hemorrhagic activity of Habu snake venom. Jpn J Med Sci Biol 13:43–51, (1969).
29. Rajesh R, Nataraju A, Gowda CD,Frey FJ,Vishwanath BS.Purification and characterization of a 34-kDa,heat stable glycoprotein from Synadenium grantii

latex:action on human fibrinogen and fibrin clot.Biochimie.88:1313-22,(2006).
30. Dwek RA. Glycobiology: Towards understanding the function of sugars. Chem Rev.96(2):683-720, (1996).
31. Rudd PM, Elliot T, Cresswell P, Wilson IA, Dwek RA. Glycosylation and the immune system. Science.291:2370-6, (2001).
32. Talbot P, Shur BD, Myles DG. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions of ERK and JNK in CD4+T Cells.J Leukoc Biol.68:1-9, (2003).
33. Varki A. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3:97-130, (1993).
34. Hrzenjak T, Popovic M, Bozic T, Grdisa M, Kobrehel D, Tiska-Rudman L. Fibrinolytic and anticoagulative activities from the earthworm Eisenia foetida. Comp Biochem Physiol 119: 825–832, (1998).
35. Mulenga A, Kim T, Ibelli AM. Amblyommaamericanum tick saliva serine protease inhibitor 6 is a cross-class inhibitor of serine proteases and papain like cysteine proteases that delays plasma clotting and inhibits platelet aggregation. Insect Mol Biol. 22:3306–3319, (2013).
36. Chethana Ramachandraiah, Sharath Kumar M. Nandish, Jayanna Kengaiah, et al. Macrotyloma uniflorum seed aqueous extract exhibits anticoagulant, antiplatelet and clot dissolving properties. Asian Journal of Pharmacy and Pharmacology.5(3):589-603, (2019).
37. Sowmyashree G, Bhagyalakshmi M, Girish KS, Kemparaju K, Rangaiah SM, Jane HP, et al. Jackfruit (Artocarpusheterophyllus) seed extract exhibits fibrino(geno)lytic activity. Phcog J 7: 171-7, (2015).
38. Bhagyalakshmi Manjappa, Sowmyashree Gangaraju, Kesturu Girish S, Kempaiah Kemparaju, Sathish J, Gonchigar d, et al. Blood Coagulation and Fibrinolysis 25: 00–00, (2014).
39. Ramachandraiah C, Nandish SKM, Kengaiah J.et al. Evaluation of Anticoagulant and Antiplatelet Activity of Pisum sativum Pod Extract. Journal of Blood Research and Hematological Disorders, Vol.2:1, (2017).
40. Nandish SK, Kengaiah J, Ramachandraiah C Et al. Anticoagulant, antiplatelet and fibrin clot hydrolyzing activities of flax seed buffer extract. Pharmacognosy Magazine, 14: S175-83, (2018).
41. Devaraja S, Girish KS, Devaraj VR, Kemparaju K. Factor Xa-like and fibrin(ogen)olytic activities of a serine protease from Hippasa agelenoides spider venom gland extract. J Thromb Thrombolysis 29: 119–126, (2010).
42. Castillino FJ. Recent advances in the chemistry of the fibrinolytic system. Chem. Rev 431-446, (1981).