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Caesalpinia Crista Seed Exhibits Strong Anticoagulant and Antiplatelet Activity

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Abstract: *Caesalpinia Crista Seeds stores robust phytochemicals responsible for several therapeutic efficacy, hence it has been using from ancient time in the folk medicine. Although, researchers putting effort to validate its stored health benefits, its role in thrombosis was least explored. Thus, current study investigate the anticoagulant, antiplatelet and clot lysis efficiency of Caesalpinia Crista Seed Aqueous Extract (CCSAE). The protein blue prints of CCSAE revealed similar banding pattern from 15-200 kDa on 10 % SDS-PAGE suggests that CCSAE reserves only monomeric proteins. CCSAE exhibited proteolytic activity by degrading casein and gelatin with the specific activity of 0.160 and 0.210 units/mg/min respectively at 37°C. The proteolytic activity was strongly inhibited by both IAA and 1,10 Phenanthroline, while PMSF and EDTA did not show inhibition, suggesting the presence of both cysteine and zinc dependent metallo protease in the seeds. CCSAE showcased strong anticoagulant effect as enhanced the clotting time of both PRP and PPP from control 180 s to 1367 s and 200 s to 1565 s respectively. Anticoagulation effect and its site of participation was also further strengthened by APTT and PT test. CCSAE appeared to interfere in intrinsic pathway of coagulation. CCSAE found to hydrolyze fibrin but not plasma proteins. Furthermore, CCSAE also exhibited strong antiplatelet activity by inhibiting both ADP and epinephrine agonists induced platelet function. The recorded inhibition percentage was found to be 44.0 % and 76.7 % respectively. Interestingly, CCSAE devoid of damaging blood vessels, edema and RBC lysis potency.*

***Keywords**: CCSAE, protease, anticoagulant, antiplatelet, clot lysis.*

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I.Introduction

The hyper activation of platelets and clotting factors leads to the thrombosis a pathological phenomenon. In addition, thrombin do play a key role in the progression of thrombotic disorders [1]. Thrombin not only involved in the conversion of fibrinogen to fibrin (clot) but also activates several proteins and platelets as well [2]. Thrombosis involved in the formation of abnormal clot in the artery (arterial thrombosis) and vein (venous thrombosis). Thrombotic disorders terrifically increases the mortality and morbidity rate both in developed and under developed countries [3]. Thus, controlling hyper activation of coagulation factors and platelets helps in the better management of thrombotic disorders. Although, anticoagulants (low-molecular-weight heparin; warfarin, rivaroxaban), thrombolytic agents (tissue plasminogen activator, streptokinase, urokinase) and antiplatelet (Clopidogrel, prasugrel, tcogrelor, tclopidine, abciximimab, epitifibatide, tirofiban, dabigatran, enoxapurine, fondaparinux, bivahrudin) agents have been extensively used. The life-threatening side effects such as internal bleeding, digestive complication and birth defects led to the parallel discovery of antiplatelet, anticoagulant and thrombolytic drugs in the treatment regime of thrombosis. Based on the facts discussed above identification of novel anticoagulant, antiplatelet and thrombolytic agents from the natural sources with least side effects complement the bottleneck phenomenon associated with thrombotic disorders. The extract prepared from the various parts of the plants have been using since ancient period. Hence, the extracts of plants and their purified compounds gaining much attention not only for their curative efficiency but also for their least side effects. Thus, understanding the pharmocognasy of biologicals is the fascinating field for the researchers. Seeds stores huge amounts of proteins/peptides and hydrolytic enzymes responsible for wide range of pharmacological effects. For instance, seeds of jackfruit, flax, bitter gourd, pea and tamarind display anticancer, antibacterial, antioxidant, antiarthrities, anticoagulant, antiplatelet and clot hydrolysis efficiency [4-8]. Caesalpinia crista Linn. ubiquitously growing plant, fall under the family caesalpiniaceae or leguminous consists of more than 500 species. The seeds of Caesalpinia crista are quit commonly used folk medicine espicailly in Asian countries [9].

According to the literature survey the seeds of Caesalpinia crista have been extensively used in the treatment of cancer, infectious and skin diseases, problems associated with digestive, respiratory, reproduction and circulatory systems [10]. Several secondary metabolites have been isolated and characterized from Caesalpinia crista seeds [11-12]. For instance, furanoditerpenes, phytosterinin, β-sitosterol, flavonoids, bonducellin, aspartic acid, arginine, citrulline and β-carotene were well characterized [13-14]. While, proteolytic enzymes have been least studied. Thus, the current study for the first time explores the anticoagulant, antiplatelet and thrombolytic activity triggered by cysteine/zinc dependent metallo protease present in the Caesalpinia Crista Seed Aqueous Extract and the results are presented.

II.Materials and methods

Reagents

PT and APTT reagents were purchased from AGAPPE diagnostic Pvt. Kerala, India. Human plasma fibrinogen was purchased from Sigma Chemicals Co. St. Louis, USA. Molecular weight markers were from Bangalore Genie Private limited, India. Phenyl Methyl Sulphonyl Fluoride (PMSF), Ethylene Di-Amine Tetra Acetic acid (EDTA), Iodo-Acetic Acid (IAA), 1,10 Phenanthroline and fat free casein were purchased from sigma chemicals company. All other chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the platelet rich plasma (PRP).

Preparation of Caesalpinia Crista Seed Aqueous Extract (CCSAE) and Protein estimation

Caesalpinia crista seeds were collected from Aswal village, Piryapatna Taluk, Mysore district. Seeds were separated from coat and homogenized in double distilled water and centrifuged at 1500 g for 20 min at 15° C. Supernatant was collected and proteins were precipitated using 30 % of ammonium sulphate. Precipitated proteins were again centrifuged at 1500 g for 20 min, pellet was subjected to dialyzed overnight. The protein sample obtained was stored at -20° C in freeze condition until further use. This extracted protein sample was used throughout the study and referred as CCSAE. Protein concentration was determined as described by Bradford et al, [15]. Using Bovine Serum Albumin (BSA) as standards.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel electrophoresis (SDS-PAGE) and Glycoprotein staining.

10 % SDS-PAGE was assayed based on the method described by Laemmli [16]. CCSAE crude (100 µg) was treated in both reduced and non-reduced reagents and electrophoresis was performed by using Tris (25 mM), glycine (192 mM) and SDS (0.1 %) for 2 hr at room temperature. Upon that gels were stained with 0.1 % Coomassie brilliant blue R-250 thereafter de-stained with 40 % ethanol in 10 % acetic acid and water (40:10:50 v/v) to visualize the protein bands. Molecular weight standards were used from 200 kDa to 14.3 kDa. Similarly PAS staining was assayed based on the method described by Leach et al., [17]. But, in PAS staining after electrophoresis the gels were fixed in solution of 7.5 % acetic acid and incubated for 1 hr at room temperature. Then after the gels were washed with the solution of 1 % nitric acid and stored at 4° C for 45 min with 0.2 % aqueous periodic acid solution. Then gel was treated with Schiff's reagent for 24 hr at 4° C and de-stained with 10 % acetic acid in order to observe the pink color band.

High Performance Liquid Chromatography (HPLC)

HPLC analysis was analyzed by using shimadzu LC-20AD prominence HPLC instrument with PDA detector. CCSAE (20 µl) was injected to C18 column (150 mm × 4.60 mm, particle size 5 µm) which was pre-equilibrated with 0.1 % Trifluoro Acetic Acid (TFA) in water by linear gradient method from 100 % of solution A (0.1 % TFA in water) to 100 % solution B (0.1 % TFA in acetonitrile). The column was eluted for 40 min and at the flow rate of 1 ml/min then the detector wavelength was monitored at 280 nm.

Proteolytic activity

Analysis of proteolytic activity was assayed based on the method described by Satake et al., [18]. CCSAE crude 50 µg was incubated with fat-free casein (0.4 ml, 2 % in 0.2 M Tris–HCl buffer, pH 7.6) at 37° C for 2 hr and 30 min. Add 1.5 ml of 0.44 M Tri-chloro Acetic acid (TCA) in order to precipitate undigested casein and allowed to stand for 30 min. There after centrifuged at 2000 g for 10 min. Upon that add 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of Folin–Ciocalteu's reagent (1:2) to 1 ml of the supernatant and the color developed were measured 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 (OD) of 0.01 at 660 nm/min at 37° C. The specific activity was expressed as units/min/mg of protein. The inhibition studies was performed independently by pre-incubating the crude CCSAE (50 µg) for 30 min with 5 mM each of EDTA, 1,10-phenanthroline, PMSF and IAA.

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

PRP and PPP were prepared based on the method described by Ardlie and Han [19]. 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 hr. Both PRP and PPP were prepared using plastic wares or siliconized glass wares.

Plasma re-calcification time

The plasma re-calcification time was assayed based on the method described by Quick et al., [20]. CCSAE crude (2-14 µg) was pre-incubated 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.

Activated Partial Thromboplastin Time (APTT) and Partial Thromboplastin Time (PTT)

APTT was assayed by treating 100 µl of normal citrated human plasma with CCSAE crude (0–6 µg) for 1 min, after that add 100 µl of APTT reagent (Derived from Rabbit brain with ellagic acid) followed for 3 min at 37° C. 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.

Fibrin clot-hydrolyzing activity by colorimeter and SDS-PAGE

Fibrin clot-hydrolyzing activity was assayed based on the method described by Rajesh et al., [21]. Concisely, 100 µl of citrated human plasma was mixed with 20 µl of 0.2 M $CaCl_2$ and incubated for 2 hr at 37 °C. The clot obtained was washed thoroughly for 5–6 times with PBS and suspended in 400 µl of 0.2 M Tris–HCl buffer (pH 8.5). The reaction was initiated by adding varied amounts of CCSAE (0–50 µg) in 100 µl of saline and incubated for 2 hr and 30 min at 37° C. The undigested clot was precipitated by adding 750 µl of 0.44 M TCA and allowed to stand for 30 min and centrifuged for 15 min at 1500 g. The aliquots of 0.5 ml supernatant was transferred to clean glass tubes and it was followed by the addition of 1.25 ml of 0.4 M 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. For SDS-PAGE analysis the clot obtained was incubated with the various concentrations of CCSAE in a final volume of 40 µl of 10 mM Tris–HCl buffer (pH 7.4) at 37° C for 6 hr. The reaction was stopped by adding 20 µl of sample buffer containing 4 % SDS, 1 M 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.

Blood clot lysis study

The blood clot lysis study was determined according to the method of prasad S. et al [22]. Freshly drawn blood was transferred to pre-weighed sterile micro-centrifuge tubes (500 µl/tube) and incubated at 37° C for 45 min. After the clot formation serum was completely removed and weighed again to know the clot weight. Different concentration of CCSAE was added to all tubes and incubated at 37° C for 90 min then observed for clot lysis. After incubation time the obtained fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The clot lysis was expressed in percentage.

Human plasma proteins degradation assay

Degradation of human plasma protein was assayed according to the method of Kumar et al., [23]. CCSAE (0–10 µg) was incubated with the 100 µg of plasma proteins for 24 hr at 37° C. The reaction was terminated by adding 20 µl of denaturing buffer containing 4 % SDS and boiled for 5 min. It was then analyzed on 7.5 % SDS-PAGE under non-reduced condition.

Platelet aggregation

Platelet aggregation was analyzed based on the method described by Born [24] in lumi aggregation system (Model-700). PRP was pre-incubated with various concentrations of CCSAE (0–30 µg) in 0.25 ml reaction volume. The aggregation was initiated independently by the addition of agonists such as ADP and epinephrine followed for 6 min.

Direct hemolytic activity

Packed human erythrocytes and phosphate buffered saline (PBS) (1:9 v/v) were mixed; 1 ml of this suspension was incubated independently with the different concentrations of CCSAE (10-100 µg) for 1 hr at 37° C. The reaction was terminated 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 (positive) and phosphate buffered saline taken as negative controls.

Edema inducing activity

Viswanathan et al., [26] method was followed. Mice were injected separately into the right foot pads with different doses (10 to 200 µg) of CCSAE in 20 µl saline. The left foot pads received 20 µl 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., [27]. A different concentration of CCSAE (0 to 200 µg) was injected (intradermal) independently into the groups of five mice in 30 µl saline. Mice received saline alone serves as negative control and mice received venom (2 MHD) as positive control. After 3 hr, mice were anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for hemorrhage against saline injected controlled mice. The diameter of hemorrhagic spot on the inner surface of the skin was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of the protein producing 10 mm of hemorrhage in diameter.

Statistical analysis

The data are presented as mean ± SD. Statistical analyses were performed by Student's
T-test. A significant difference between the groups were considered if $P < 0.01$.

III. Results

Current study evaluates the anticoagulant and antiplatelet and blood/fibrin clot dissolving ability of CCSAE. CCSAE showed the similar protein banding pattern arranged between 15 and 100 kDa on 10 % SDS-PAGE under reduced and non-reduced conditions (Fig. 1a). Furthermore, when CCSAE was subjected to basic PAGE (Fig. 1b) it exhibited maximum acidic proteins. In addition, CCSAE was positive for PAS staining as all the proteins taken up the stain that was compared with the fibrinogen a positive control (Fig. 1c). When CCSAE sample was subjected to RP-HPLC-PDA instrument at the flow rate of 1 ml/min. Four peaks were resolved with the retention time of 1.9, 3.2, 6.2, 9.6 at the wave length 280 nm (Fig. 2). CCSAE exhibited proteolytic activity by degrading casein and gelatin as a substrate with the specific activity 0.160 and 0.210 units/mg/min at 37° C respectively. Proteolytic activity of CCSAE was completely neutralized by both IAA and 1,10, Phenanthroline. While, PMSF and EDTA, have not inhibited the proteolytic activity of the extract (Table. 1). CCSAE when analyzed for its anticoagulant effect it increased the clotting time of citrated human PRP and PPP from control 180 s to 1367 s and 200 s to 1565 s respectively, at the maximum concentration of 12 µg after this dose it reached saturation (Fig. 3). CCSAE was able to prolong Activated Partial Thromboplastin Time (APTT) and did not show significant effect on Prothrombin Time (PT) in a dose-dependent manner. The delaying in APTT of CCSAE was almost 369 s at the concentration of 4 µg but the control was just 32 s Table. 2). CCSAE hydrolyzed both blood clot and fibrin clot suggesting its clot dissolving ability. When whole blood clot was incubated with CCSAE (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 52 % at the concentration of 200 µg of CCSAE against the positive control streptokinase 66.8 %. (Fig. 4). Furthermore, The fibrin clot dissolving ability of CCSAE was carried out by colorimetric method. CCSAE hydrolyzed the fibrin clot in a dose dependent manner with the specific activity of 4.6 units/mg/min at 37° C (Fig. 5a). SDS-PAGE analysis further confirmed the fibrinolytic activity of CCSAE. At the concentration of 40 µg, it hydrolyzed only α-polymer of fibrin clot without affecting β-chain, α-chain and γ- γ-dimer in a dose dependent manner (Fig. 5b). On the other hand, when CCSAE was incubated for 24 hr it hydrolyzed α-polymer, β and α-chains of fibrin clot without affecting γ- γ-dimer (Fig. 5c). Interestingly, fibrinolytic efficiency of CCSAE was completely abolished by both IAA and Phenanthroline (Fig. 5d). CCSAE did not hydrolyze other plasma proteins except the fibrinogen (Fig. 6). CCSAE at the concentration of 60 µg invisibly dissipate the fibrinogen band, where as other plasma proteins were not affected by proteolysis of the CCSAE activity, indicated the limited substrate specificity of CCSAE. In addition, The potential antiplatelet aggregation activity of CCSAE was investigated by antiplatelet aggregation assays using ADP and epinephrine as agonists. CCSAE inhibited the agonists such as ADP (Fig. 7) and epinephrine induced platelet aggregation in a dose dependent manner (`Fig. 8). The observed platelet aggregation inhibition percentage was found to be 44.0 % and 76.7 % at the concentration of 30 µg, respectively. Moreover, CCSAE did not hydrolyze RBC up to the concentration of 200 µg when compare to the water positive control and PBS taken as a negative

control. This has been concluded that, CCSAE is non-toxic to RBC (Fig. 9). CCSAE did not cause hemorrhage and edema in experimental mice up to the concentration of 200 µg, while positive control Daboia russelli venom induced hemorrhage and edema in experimental mice, suggesting its nontoxic property (Fig. 10).

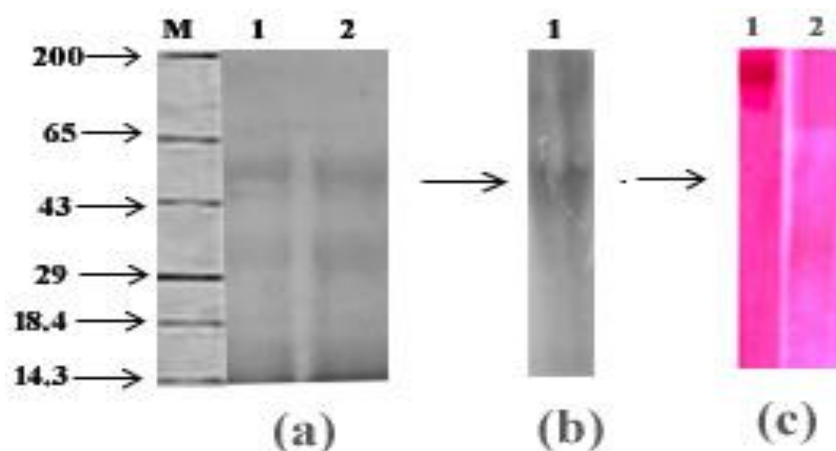

Fig. 1 (a) SDS-PAGE 10 % (b) Native (basic) PAGE (c) Glycoprotein staining. (a) CCSAE as shown in SDS-PAGE (10 %): CCSAE (100 µg) under non-reduced (a1) and reduced conditions (a2), **(b) Native basic PAGE of CCSE:** CCSAE (100 µg) under non-reduced conditions (b1). **(c) PAS staining of CCSAE:** positive control fibrinogen (c1) and CCSAE (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, CCSAE: Caesalpinia Crista Seed Aqueous Extract.

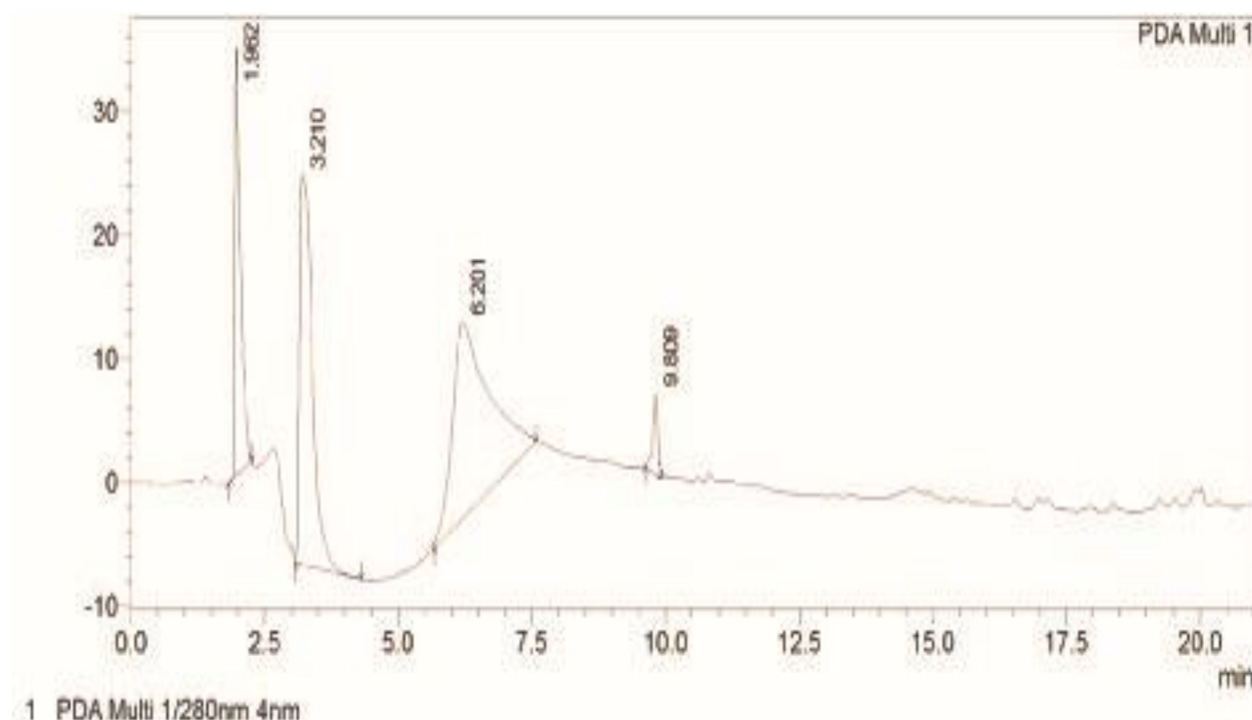

Fig. 2 RP-HPLC. RP-HPLC profile of CCSAE (5 µg) with C_{18} Column (5 mm, 0.21X25 cm) which was pre-equilibrated with 0.1 % Trifluoro Acetic Acid (TFA) in water. The column was eluted with increasing concentration (0-100 %) of acetonitrile with 0.1 % TFA for 40 min at a flow rate of 1 ml/min and monitored at 280 nm.

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

Inhibitor (5mM each)	Activity/residual activity (%)
None	100
PMSF	99.50
IAA	9.0
EDTA	98.10
1,10-Phenanthroline	10.22

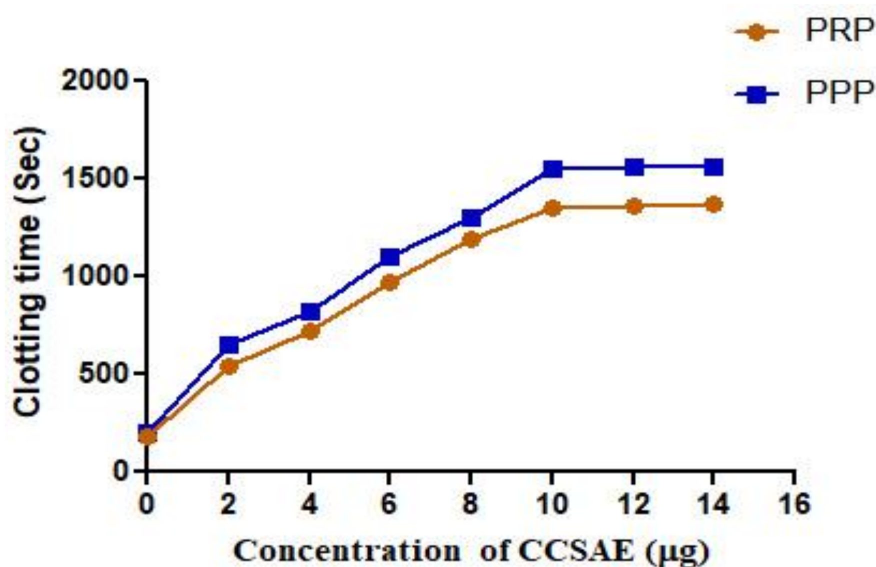

Fig. 3 Plasma re-calcification time. CCSAE (0–14 μg) was pre-incubated with 0.2 ml of citrated human plasma PRP/PPP in the presence of 20 μl 10 mM Tris–HCl buffer (pH 7.4) for 1 min at 37° C. 20 μl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.

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

CCSAE (μg)	APTT clotting time in seconds	APTT ratio	PT Clotting time in seconds	PT (INR values)
0	32.00 ± 0.04	0.55 ± 0.03	17.05 ± 0.03	1.04 ± 0.01
1	80.20 ± 0.01	2.92 ± 0.01	20.20 ± 0.02	1.23 ± 0.08
2	110.10 ± 0.05	4.01 ± 0.04	25.00 ± 0.04	1.52 ± 0.02
3	205.20 ± 0.02	8.30 ± 0.02	24.04 ± 0.02	1.53 ± 0.03
4	369.00 ± 0.04	≥10 ± 0.06	22.10 ± 0.08	1.34 ± 0.04

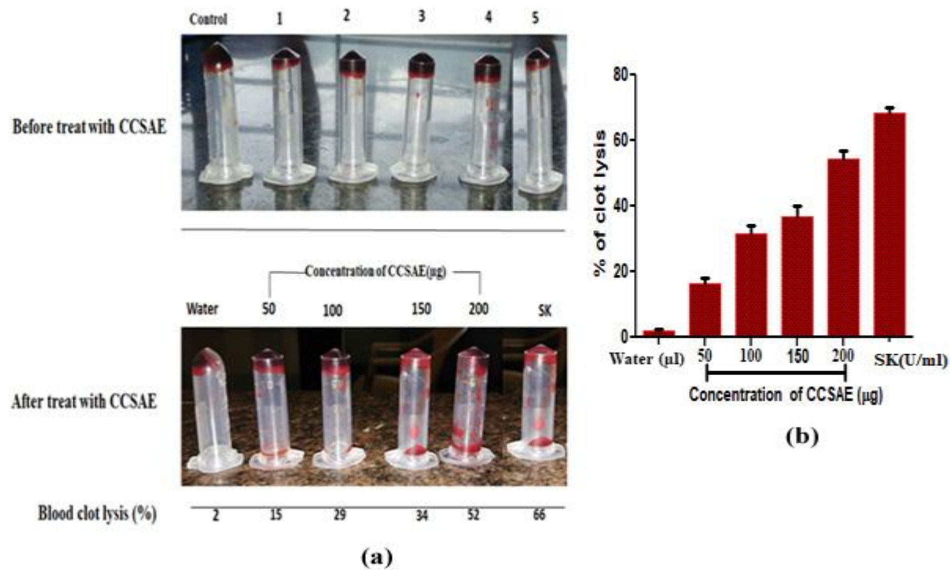

Fig. 4 Blood clot lysis study. (a) Photographic image of blood clot lysis assay (b) Percentage of blood clot lysis. (a) Venous blood was transferred to different pre-weighed sterile microcentrifuge tubes (500 µl/tube) and incubated at 37° C for 45 min. After clot formation, serum was completely removed and each tube with clot was again weighed. The 50 to 200 µg of CCSAE was added to the microcentrifuge 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.

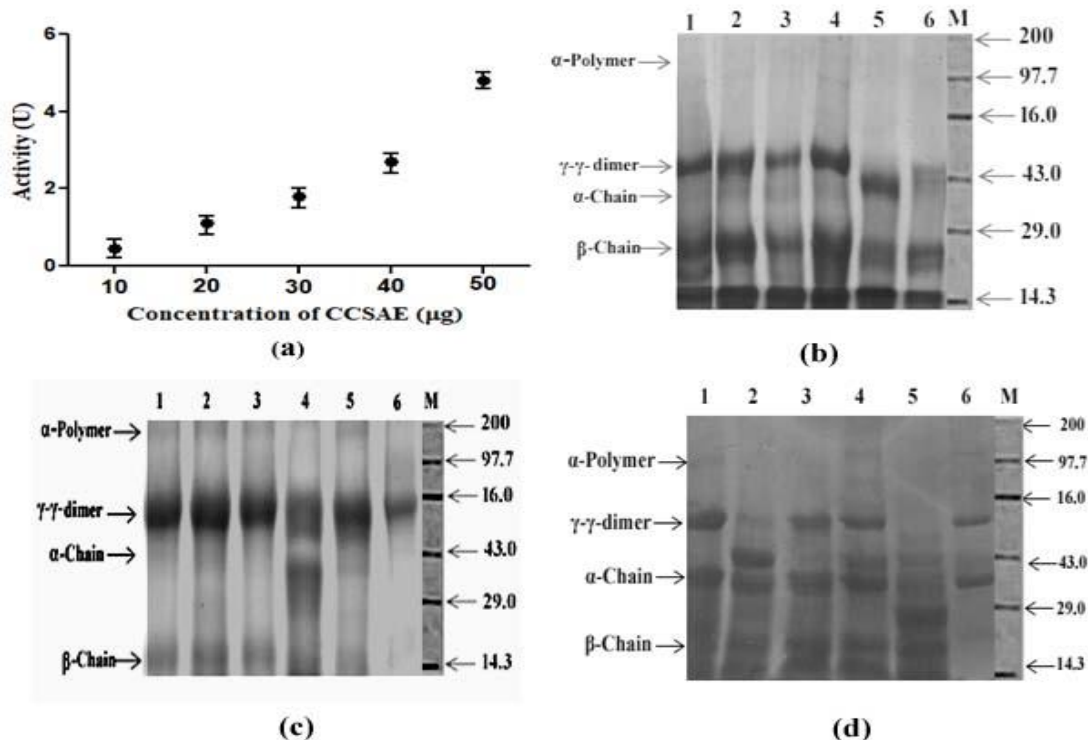

Fig. 5 Fibrinolytic activity. (a) Colorimetric assay (b) Dose-dependent effect (c) Time-dependent effect. (a) Washed plasma clot was incubated with 0–100 µg of CCSAE for 2.30 hr and then the OD was measured at 660 nm. **(b) CCSAE Dose-dependent effect:** Washed plasma clot was incubated for 12 hr and 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 CCSAE respectively. **(c) CCSAE Time-dependent effect:** CCSAE 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 CCSAE. **(d) CCSAE Inhibition study:** CCSAE 40 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), CCSAE 40 µg (d2), fibrin clot and CCSAE 40 µg with 5 mM PMSF (d3), fibrin clot and CCSAE 40 µg with 5

mM IAA (d4), fibrin clot and CCSAE 40 µg with 5 mM EDTA (d5), fibrin clot and CCSAE 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, CCSAE: Caesalpinia Crista Seed Aqueous Extract.

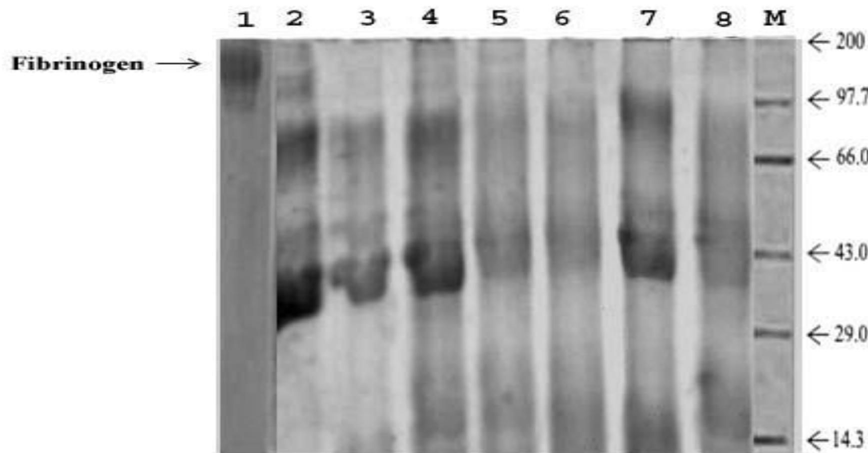

Fig. 6 Degradation of plasma proteins. Plasma protein (100 µg) was incubated with CCSAE in 40 µl of 10 mM Tris–HCl buffer (pH 7.4) at 37° C and then analyzed on 7.5 % SDS-PAGE under non-reduced condition. 20 µg of fibrinogen as control (1), plasma protein (100 µg) alone (2), plasma protein treated with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

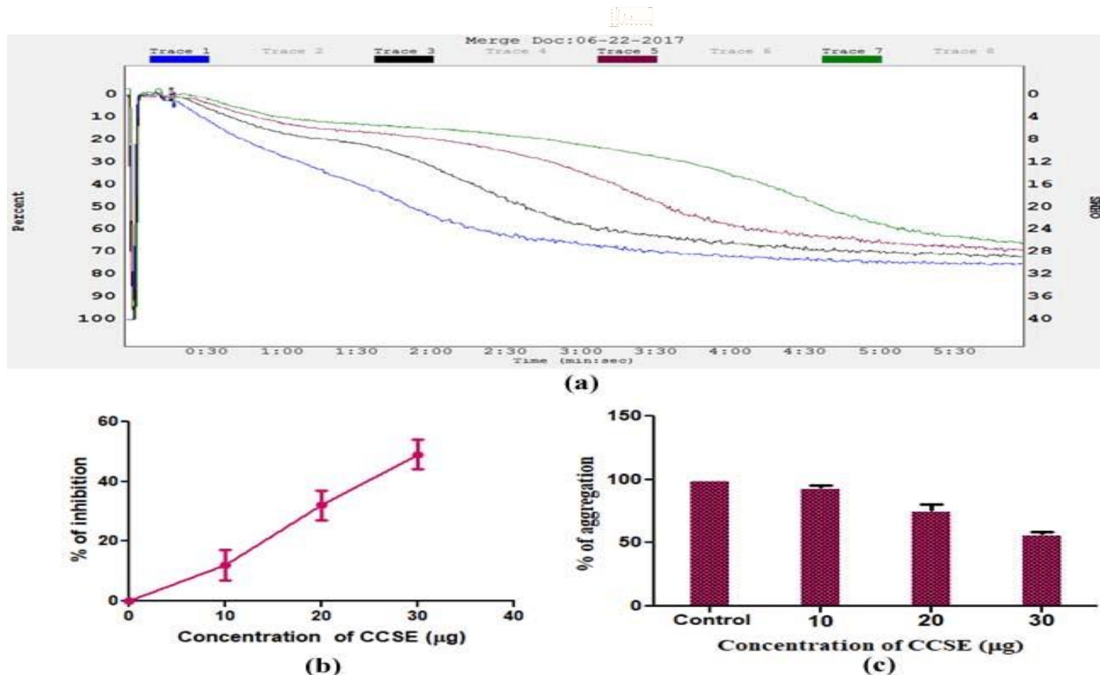

Fig. 7 Platelet aggregation was initiated by adding ADP as an agonist. (a) Traces of platelet aggregation: Trace 1 (ADP 10 µM); Trace 2 (ADP 10 µM + 10 µg of CCSAE); Trace 3 (ADP 10 µM + 20 µg of CCSAE); Trace 4 (ADP 10 µM + 30 µg of CCSAE). The values represent ±SD of three independent experiments**. (b) Dose dependent platelet aggregation inhibition % (c) Dose dependent platelet aggregation %.**

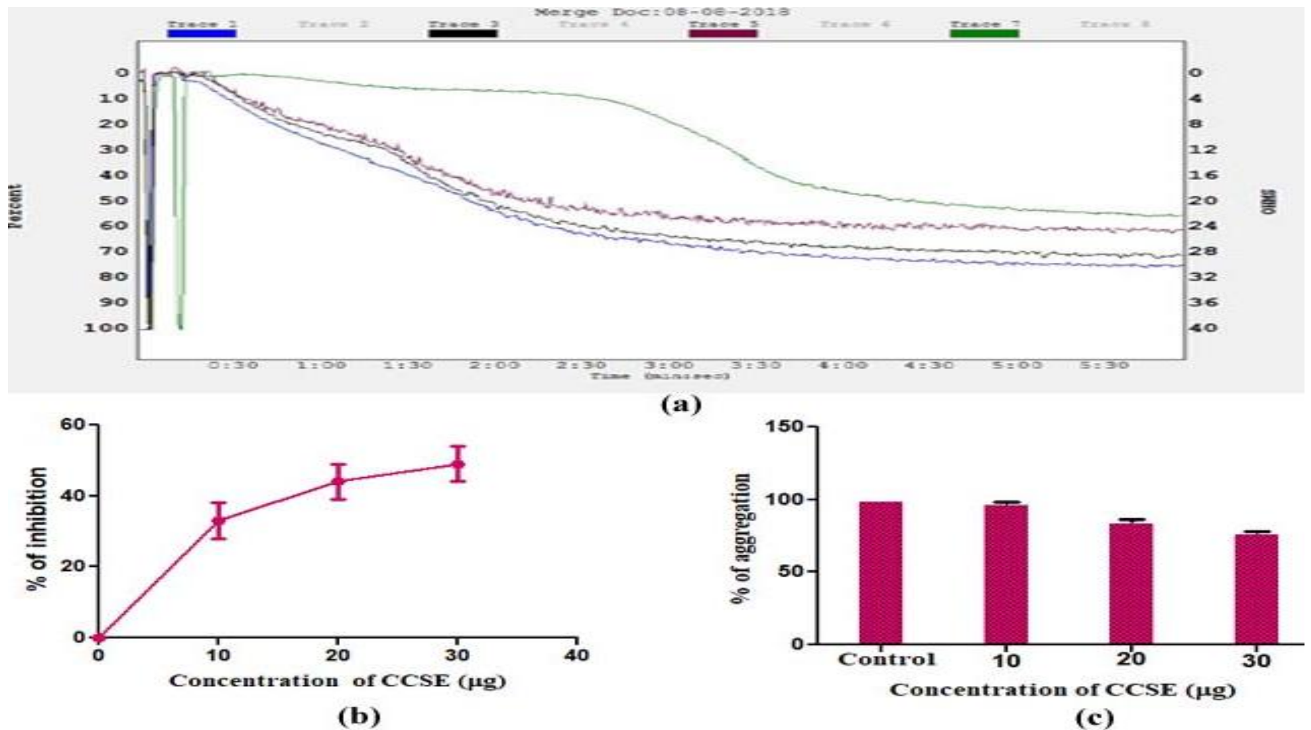

Fig. 8 Platelet aggregation was initiated by adding Epinephrine as an agonist. (a) Traces of platelet aggregation: Trace 1 (Epinephrine 5 µM); Trace 2 (Epinephrine 5 µM + 10 µg of CCSAE); Trace 3 (Epinephrine 5 µM + 20 µg of CCSAE); Trace 4 (Epinephrine 5 µM + 30 µg of CCSAE). The values represents of three independent experiments. **(b) Dose dependent platelet aggregation inhibition % (c) Dose dependent platelet aggregation %.**

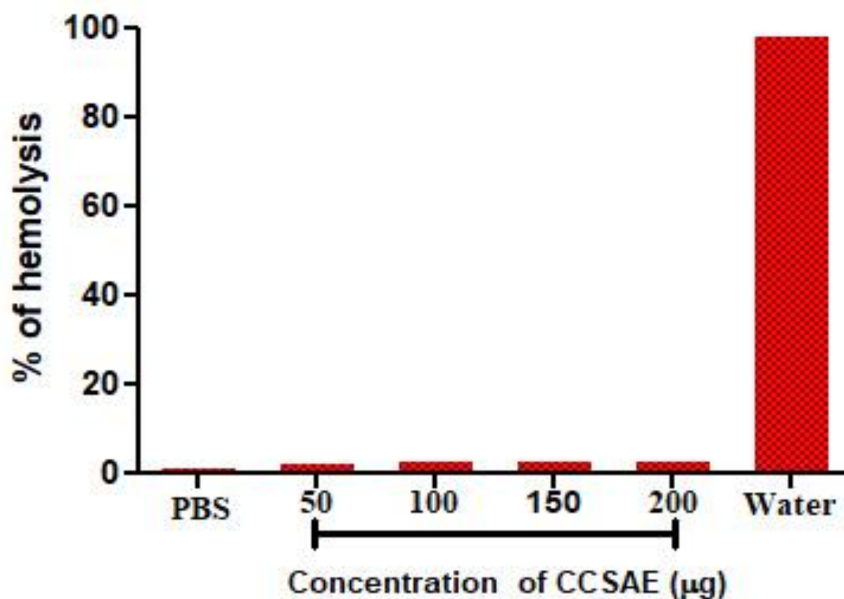

Fig. 9 Direct Hemolytic assay. The different concentration of CCSAE (10-100 µg) was incubated independently for 1 hr at 37° C with the 1 ml of suspension made with packed human erythrocytes and phosphate buffered saline (PBS) 1:9 v/v. The reaction was stopped by adding 9 ml of ice cold PBS and centrifuged at 1000 g for 10 min at 37° C. The amount of hemoglobin released in the supernatant was measured at 540 nm.

Fig. 10 Dose-dependent hemorrhagic activity of CCSAE. (a) Saline, (b) positive control 2 MDH venom, (c) 200μg of CCSAE was injected independently into mice in a total volume of 50 μl intradermal. CCSAE: Caesalpinia Crista Seed Aqueous Extract.

IV.Discussion

Thrombosis is a process of generation of unusual clot in the arties and veins leads deleterious effect on normal individual as it involved in the cardiovascular and cerebro vascular complications. Thus, in the treatment regime of thrombosis anticoagulant, antiplatelet and thrombolytic agents have been employed. Although, the said drugs provides marginal protection against the thrombotic disorders. Yet their life threatening side effects (birth defects, internal bleeding) limits their usage boundary. Thus, usage of herbal decoctions as medicines to cure both infectious and life style disorders has been gaining much importance due to their least side effects. In this context, Caesalpinia Crista Seeds have been examined for their anticoagulant, antiplatelet and fibrinolytic potential. Interestingly CCSAE showed protein banding pattern under both reduced and nonreduced condition in a similar manner, suggesting the presence of monomeric protein. Biological sources stores large array of proteins. Proteins perform diversified biological functions right from birth to death. Based on the polypeptide chains, they may be oligomeric or monomeric in nature. CCSAE showed only two proteins bands on basic PAGE, suggesting rest of the proteins are basic in nature. CCSAE was positive for PAS staining since three bands taken up the stain reveals the presence of glycoproteins in the seeds. CCSAE hydrolyzed both casein and gelatin revealed its proteolytic activity. The proteolytic activity of the extract was inhibited by IAA and 1, 10, Phenanthroline, suggesting the presence of both cysteine and zinc dependent metallo protease in the extract. Proteases from Caesalpinia Crista Seeds are least studied enzymes, however, proteases inhibitors were explored (28-30). proteases are well characterized in microbes, plant latex, seeds of flax and jackfruit (31-32,4), venoms of snake, spider and scorpions (33-35). Proteases are widely used in food and pharma industries (36-39). Currently, proteases like papain, bromelin and ficin are not only used in beverages and cheese industries but also used to soften meats [40]. In addition, proteases from snake venom with anticoagulant and antiplatelet potential are being used to treat thrombotic disorder (41). Thus, Demand for the proteolytic enzyme in the global market has been increasing day by day. CCSAE showed strong anticoagulant effect in both PRP and PPP. In addition, CCSAE specifically prolonged the clotting time of APTT without altering the PT clotting time, suggesting its interaction with intrinsic coagulation factors such as VIII, IX, XI, XII, and V or Willebrand's factor. Blood coagulation encompasses intrinsic, extrinsic and common pathways. Intrinsic pathway involves the participation of factor XII, XI, IX, VIII, and V, while the extrinsic pathway, involving tissue factor and factor VII [42-43]. Both these pathways converge to activate factor X and lead to conversion of prothrombin into thrombin that intern convert fibrinogen into fibrin (clot). In addition, thrombin may also activate platelets as well [45-46]. Factor Xa and thrombin inhibitors have been extensively used in the treatment of thrombotic disorders. A protease inhibitor from Caesalpinia echinata (pau-brasil) seeds inhibited plasmin and factor XIIa [30]. CCSAE appears to interfering in intrinsic pathway of blood coagulation cascade; hence it can be exploiting in the management of thrombotic complications. In normal condition, once the fibrin clot has been formed, immediately clot is dissolved by fibrinolytic enzyme the plasmin [46]. While, in case of thrombosis the clot remains in the arteries and vein leads to thrombosis. Currently, plasminogen activator (t-PA), urokinase, streptokinase have been extensively used as thrombolytic agents [47-48]. However, they cause excessive bleeding, short half-life and are very costly to produce [49]. Thus, there is an increasing demand for thrombolytic agents with least side effects. CCSAE dissolved both blood clot and fibrin clot strengthened its clot lysis activity along with anticoagulant potential. Thrombolytic agents have been characterized from microorganisms [50-51], earthworms [52], snake venoms [53], centipede venoms [54], insects [55], and leeches. Hyper activation of platelets also a major contributor of thrombosis [56]. The interactions between platelets and

various adhesive proteins, such as collagen and soluble agonists such as ADP provide potential targets for developing antiplatelet agents [57-58]. CCSAE evaluated the potential antiplatelet activity by inhibiting the agonist such as ADP and epinephrine induced platelet aggregation of human platelet rich plasma confirms its antiplatelet activity as well. Moreover, CCSAE did not cause hemolysis, platelet apoptosis when it was treated with RBC and platelets. It did not cause hemorrhage and edema in experimental mice suggesting its non-toxic nature. In conclusion, this study for the first time explores the anticoagulant, antiplatelet and thrombolytic activities of protease present in the CCSAE. Thus it could be better contender in the management of thrombotic disorders.

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Declaration of Conflict of Interest

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

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