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RESEARCH ARTICLE

EVIDENCES FOR THE PRESENCE OF PROTEOLYTIC ENZYME IN FINGER MILLET (*Eleusine coracana*) AND ITS BENEFICIAL ROLE IN THROMBOSIS

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ABSTRACT

The current study aims to examine the clot dissolving, anticoagulant and antiplatelet, activities of Finger Millet Extract (FME). FME exhibited proteolytic activity as it hydrolyzed casein with the specific activity of 0.212units/mg/min. FME was analyzed for plasma re-calcification time using both human platelet rich and poor plasma. Interestingly, it showed strong anticoagulant effect in both PRP and PPP by enhancing the clotting time from control 210s to 660s and 246s to 780s respectively at the concentration of 12µg. FME in unambiguously prolonged the clot formation process of only APTT but not PT. Furthermore, FME dose dependently hydrolyzed only Aα and Bβ chains of human fibrinogen without affecting γ chain. In addition, it also dissolved human fibrin clot but not other plasma proteins. Furthermore, FME inhibited the agonists such as ADP and epinephrine induced platelet and it did not hydrolyze RBC cells suggesting its nontoxic properties.

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INTRODUCTION

Finger millet *(Eleusine Coracana)* is a wonder plant, mostly found in the continents of Africa and Asia. It is a staple food for a large segment of the population in Africa and Asian countries (Palanisamy *et al.,* 2014). Finger millet commonly known as ragi or mandua provides major portion of nutritional supplements to the large segments of the population in developing and under developed countries of low income groups (Doraiswamy *et al.,* 1969). In India, Karnataka top the first place in the production of finger millet about 58% of its global production. While, only few Indians are conscious about the health and nutritional values of ragi. Finger millet is a naked caryopsis having seed coat with brick red or black color (Dinesh *et al.,* 2016). It has been commonly used in the preparation of traditional foods, such as unleavened breads or pancake, dumpling and thin porridge (Doesthate *et al.,* 1970). Studies by various research groups suggests that the regular consumption of finger millets have immense beneficial effects in curing cardiovascular diseases, type II diabetes, anti aging, anti bacterial and gastrointestinal cancers (McKeown, 2002). The said therapeutic efficacy of Finger millet could be due to the presence of wide range of phytoconstituents that includes macro/micro nutrients and secondary metabolites (Chethan and Malleshi, 2007).

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Finger millet shares about 70% carbohydrates, 5–8% protein, 20% dietary fibers, 3-4% minerals. It has highest amount of calcium content among all the cereals. In addition, Finger millet also found to contain least amount of phytates, polyphenols, tannins and trypsin inhibitory factors (Babu *et al.,* 1987). Thus, United States National Academies has considered Finger millet as the 'super cereal' (Anil et al, 2016). Although, several phytoconstituents and their therapeutic importance have been reported from of finger millet proteolytic enzymes are least explored. Chandrashekar *et al.* (1953) demonstrated that ragi during the time of germination has proteolytic activity while under un-germination no proteolytic activity (Chandrasekara and Swaminathan, 1953). Later on none of the studies claim the presence of proteolytic enzymes and their therapeutic applications as such in the ragi. Proteases or peptidases are a group of hydrolytic enzymes capable of hydrolyzing peptide bond in a proteins chain. Currently two types of peptidase are there based on site of action namely exopeptidases (acts near the terminal end of the protein chain) and endopeptidases (acts in between the protein chain). Whereas, based on the requirement for their catalytic function they can be divided into serine proteases (Serine residue in the active site), cysteine proteases (Cysteine residue in the active site), aspartic proteases (Asp residue in catalytic site), metalloproteases (needs metal ions for catalytic mechanism) (Lee *et al.,* 2010). Earlier serine proteases were considered to be uncommon in plants. While, in recent years serine proteases have been

extensively studied enzymes in various parts of the plants including seeds, latex and fruits (Sowmyashree *et al.,* 2015; Monti *et al.,* 2000; Kaneda *et al.,* 1997). To mention few, seeds of barley, maize, soybean, rice found to exhibit proteolytic activity (Messdaghi and Dietz, 2000). In view of this, the current study aims to identify the evidence of serine and cysteine proteases in the finger millet and its beneficial effect on thrombosis.

MATERIALS AND METHODS

All chemicals used were of analytical grade, Human plasma fibrinogen was purchased from Sigma Chemicals Co. St. Louis, USA. APTT and PT Reagents were purchased from AGAPPE diagnostic Pvt. Ernakulum, Kerala, 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 (St. Louis, USA). Molecular weight markers were from Bangalore Genie Private limited, India. Fresh human blood was collected from healthy donors for the preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP).

***Preparation of Finger Millet Extract (FME) and Protein estimation*:** Finger millets were purchased from the local market of Tumkur. It was homogenized using double distilled water and centrifuged at 5000g for 20min at 15°C. The supernatant was collected and proteins were precipitated using 30% of ammonium sulfate. The precipitated protein sample was again centrifuged at 5000g for 20min; the pellet was collected and dialyzed overnight. Upon dialysis process again centrifuged at 5000g for 20min, then the supernatant was collected and stored at 20°C until it was used for further studies. Protein concentration was determined as described by Bradford (1976) using bovine serum albumin (BSA) as standards.

***Sodium Do-decyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)*:** Laemmlii (1970) method was adopted to carry out the 10% SDS-PAGE. The crude FME (100µg) was prepared under reducing and non-reducing conditions for SDS-PAGE. The electrophoresis was carried out for 2hr at room temperature using Tris (25mM), glycine (192mM) and SDS (0.1%). There after 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.

Proteolytic activity: Proteolytic activity was performed according to the method of Satake *et al.* (1963). Briefly, 50µg of FME crude was incubated with fat-free casein (0.4mL, 2% in 0.2M Tris–HCl buffer, pH 7.6) in a total volume of 1mL for 2hr and 30min at 37°C. Add 1.5mL of 0.44M Trichloroacetic acid in order to precipitate the undigested casein and left to stand for 30min at room temperature. Upon that the reaction mixture was centrifuged at 2000g for 10min then 2.5mL of 0.4M sodium carbonate and Folin-ciocalteu's reagent (1:2) were added to 1mL of the supernatant and the color developed was read at 660nm. 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 660nm/min. The specific activity was expressed as units/min/mg of protein. For inhibition studies, a similar reaction was performed independently after pre-incubating the crude FME (50µg) for 30min with 5mM each of EDTA, 1,10-phenanthroline, PMSF and IAA. In all the cases, appropriate controls were kept.

***Preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP)*:** PRP and PPP were prepared according to the method of Ardlie and Han (1974). The platelet concentration of PRP was adjusted to 3.1×10^8 platelets/mL with PPP. Both PRP and PPP were carried out using plastic wares or siliconized glass wares.

***Plasma re-calcification time*:** The plasma re-calcification time was assayed as described by Quick *et al.* (1935). Briefly, the FME crude (0-75µg) was pre-incubated with 0.2mL of citrated human plasma along with 10mM Tris HCl (20µL) buffer pH 7.4 for 1min at 37°C. Clotting time was recorded after the addition of 20µL $CaCl_2$ (0.25M).

***Fibrinogenolytic activity*:** Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng (1976). FME (0–100µg) was incubated with the human plasma fibrinogen (50µg) in a total volume of 40µL of 10mM Tris–HCl buffer pH 7.4 for 4hr at 37°C. Prolong the incubation time for 24hr at 37°C for time dependent assay. After the incubation period, reaction was terminated by adding 20µL denaturing buffer containing 1M urea, 4% SDS and 4% β-mercaptoethanol. It was then analyzed by 10% SDS-PAGE.

***Whole blood clot lysis assay*:** Whole blood clot lysis assay was performed as per the method described by Prasad *et al.* (2006). Briefly, freshly drawn healthy human blood was transferred to pre-weighed sterilized micro-centrifuge tubes (500µl/tube) and incubated for 45min at 37°C. Serum was removed after the formation of whole blood clot and each tube was weighed again. FME (50-200µg) was added individually to all the tubes thereafter incubated for 90min at 37°C. The obtained fluid was removed after incubation period and calculated the clot lysis percentage.

***Fibrin clot-hydrolyzing activity by colorimeter*:** Fibrin clot-hydrolyzing activity was assayed based on the method described by Rajesh *et al.* (2005). Fibrin clot was generated by treating 100µL of citrated human plasma with 20µL of 0.2M $CaCl_2$ and incubated for 2hr at 37°C. Generated clot was washed several times with PBS and suspended in 400µL of 0.2M Tris–HCl buffer (pH 8.5). Various amount of FME (0-100 µg) was added to 100µL of saline in order to initiate the reaction and incubated for 2hr and 30min at 37°C. By adding 750µLl of 0.44M TCA the undigested clot was precipitated and it further allowed to stand for 30min and centrifuged for 15min at 1500g. Relocated the 0.5mL of obtained supernatant to a clean glass tubes and followed by addition of 1.25mL of 0.4M sodium carbonate and 0.25mL of 1:2 diluted Folin–Ciocalteu's phenol reagent. After the incubation period of 30min the obtained color was measured at 660nm. 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.

***Degradation of human plasma proteins*:** Degradation of human plasma protein was determined as described by Kumar *et al.* (2010). Briefly, Plasma proteins (100µg) were incubated with FME (0–100µg) for 24hr at 37°C. Finally, addition of 20µL denaturing buffer terminates the reaction. After boiling for 5min it was analyzed on a 7.5% SDS-PAGE under non-reduced condition.

***Platelet aggregation*:** Platelet aggregation was analyzed according to the method of Born (1962) by using lumi aggregation system (Model-700). FME (0-150µg) was pre incubated with PRP in 0.25mL reaction volume. Process of platelet aggregation was initiated independently by the addition of agonists ADP and epinephrine then followed for 6min.

***RBC lysis activity*:** RBC lysis activity was assayed according to the method previously described by Devaraja *et al.* (2010). Briefly, 1mL of PBS treated packed erythrocytes were incubated with FME (0-150µg) for 1hr at 37°C. By adding 9mL of cold PBS reaction was terminated and centrifuged at 1000g for 10min at 37°C. The percentages of RBC lysis were evaluated based on the amount of hemoglobin released and it was measured at 540nm. PBS served as negative control and 100% of water serves as positive control.

***Edema inducing activity*:** Edema inducing activity was assayed based on the method of Sannanaik *et al.* (1987). FME (10-200µg) was injected separately for right foot pads of mice whereas 20µL of saline injected to left foot pads which served as control. After 1hr mice were anaesthetized by diethyl ether inhalation and hind limbs were removed at the ankle joint then weighed. Minimum edema dose (MED) was expressed as the amount of protein required to cause an edema ratio of 120%.

***Hemorrhagic activity*:** Hemorrhagic activity was described based on the method Kondo *et al.* (1969). FME (0-200µg) was injected (intradermal) independently to the mice in 30µL saline. Mice received saline alone served as negative control and mice received venom (2MHD) served as positive control. After the reaction time of 3hr, individually mice were anaesthetized by inhalation of diethyl ether. Upon that dorsal patch of mice skin surface was carefully removed and observed for hemorrhage and this was compared against saline injected control mice. The minimum hemorrhagic dose (MHD) was defined as the amount of the protein producing 10mm 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 $P < 0.01$.

RESULTS AND DISCUSSION

Current study demonstrates the clot dissolving, anticoagulant and antiplatelet activities Finger millet Extract (FME). Initially protein banding pattern was analyzed on SDS-PAGE and visualized by comassieve brilliant blue. Interestingly, FME exhibited dissimilar protein banding pattern from the range 200kDa to 14kDa on 10% SDS-PAGE under reduced and non-reduced conditions (Fig.1). Suggesting the presence of both monomeric and oligomeric proteins with varied molecular mass. In addition, FME exhibited proteolytic activity as it hydrolyzed casein with the specific activity of 0.202units/mg/min at 37°C. Furthermore, proteolytic activity of FME was inhibited by PMSF and IAA were capable of inhibiting proteolytic activity, while EDTA, 1,10, Phenanthroline were insensitive. Proteolytic inhibition studies suggest that FME has both serine and cysteine proteases in it but not metallo proteases. Indeed, FME is the richest source for proteins yet proteolytic enzymes have been not identified. The study by Chandrashekar *et al.* (1953) suggests that ragi (*Eleusine coracana*) expresses proteolytic enzymes only during germination, while in ungerminated state there was no proteolytic activity. On the other hand, proteolytic enzymes namely serine, metallo and cysteine proteases have been extensively studied in other seeds such as bitter gourd, Jackfruit, peanut, Araucaria angustifolia, citrulluscolocynthis, maize and sorghum seeds (Bhagyalakshmi *et al.,* 2014; Kumar *et al.,* 2010; Yadav *et al.,* 2006; Ramachandraiah *et al.,* 2017). Proteolytic enzymes play a vital role in plant growth and development. Apart from this plant based proteases have been extensively used in food industry and medical field as a wound healing agent (Devaraja *et al.,* 2008; Reis *et al.,* 2001; Devaraja *et al.,* 2011). FME showed strong anticoagulant in both PRP and PPP *in vitro*. FME anticoagulant effect in PRP was found to be 660s against control 210s (Fig 2). While, in case of PPP 780s against the control 246s. The anticoagulant elicited by FME was further rationalized by using mouse tail bleeding assay *in vivo*. The injection of FME radically delayed the bleeding time in a dose dependent manner. The documentated bleeding time was >480s ($P < 0.01$) at the concentration of 20µg against the PBS treated control of 186s with the IC_{50} values 10µg/mL (Fig 3). Furthermore, FME in unambiguously prolonged the clot formation process of only APTT but not PT, revealed the observed anticoagulation by the FME could be due to the interference in intrinsic pathway of blood coagulation cascade (Fig 4). Blood coagulation cascade is a physiological phenomenon that could be activated in order to arrest the bleeding following an injury (Drake *et al.,* 1989). It basically involves the zymogen activation of several serine proteolytic enzymes along with the platelet-mediated primary hemostasis (Kitchen *et al.,* 2014). Thus coagulation has evolved as chief defense mechanism against bleeding in response to rupture of endothelium (Denson, 1969).

The fibrin clot generated after series of cascades reaction takes place in the coagulation system is been coordinated with the activated platelets (plug) primarily occludes the vascular lesion (Zanetti *et al.,* 2002). Blood coagulation cascade is a highly regulated phenomenon as it involves the synchronization of natural anticoagulant, procoagulant and fibrinolytic forces (Astrup, 1958). Often genetic and acquired factors cause instability of natural balance between the procoagulant, anticoagulant and fibrinolytic systems due to genetic or acquired factors may perhaps results in bleeding or thrombotic diseases (Crawley and Lane, 2008). Thrombosis involves the development of unusual clot in the arteries and veins and most common underlying pathology of the three major cardiovascular disorders namely, ischemic heart disease (acute coronary syndrome), stroke and Venous Thrombo Embolism (VTE) (Asadi *et al.,* 2014). The mortality and morbidity rate has been increasing even more than the cases of cancer (Lu *et al.,* 2005). Although, the anticoagulant and antiplatelet therapy currently available, they themselves cause life threatening side effects (Rembrandt, 2000). FME exhibited anticoagulant effect thus; FME could be a better candidate to impede the unusual clot formation. Anticoagulant from snake venom (Ancrod), fungi aspergilla's oryzae (Brinase), coumarin derivative from sweet clover, hirudin derivative from saliva of leach are currently being used to treat thrombotic disorders (Larsson *et al.,* 1988; Veiga *et al.,* 2000; Amiral *et al.,* 1997). Bitter gourd, flaxseed and Jackfruit seed extract found to exhibit the anticoagulants effect (Nandish *et al.,* 2018). Furthermore, FME cleaved Aα and Bβ chains of human fibrinogen without affecting γ chain at the concentration of 20µg at 24h of incubation period (Fig 5).

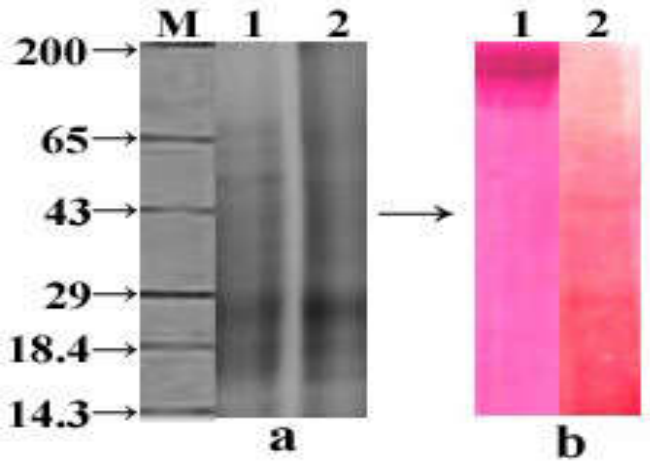

Fig. 1. (a) *SDS-PAGE 10% (b) Glycoprotein staining.* (a) FME as shown in SDS-PAGE (10%): FME (100µg) under non-reduced (a1) and reduced conditions (a2), (b) PAS staining of FME: positive control fibrinogen (b1) and FME (b2). M represents the molecular weight marker in kDa from top to bottom: myosin-H-chain (200), BSA (65), ovalbumin (43), carbonic anhydrase (29), lactalbumin (18.4) and lysozyme (14.3) BSA: bovine serum albumin, FME: *Eleusine coracana* Extract

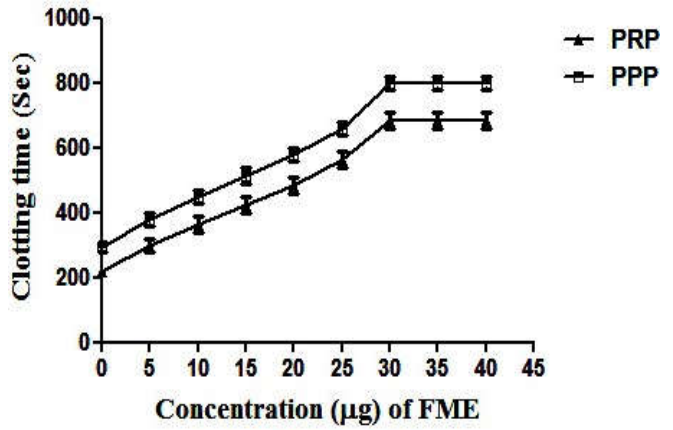

Fig. 2. Effect of FME on Plasma recalcification time. (a) FME (0–120µg) was pre-incubated with 0.2mL of citrated human plasma PRP/PPP in the presence of 20µL 10mM Tris–HCl buffer (pH 7.4) for 1min at 37°C. 20µL of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded

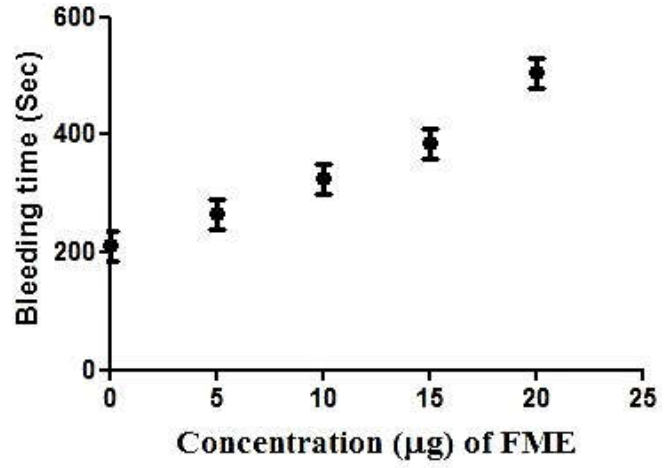

Fig. 3. Tail bleeding time. Tail bleeding time was measured 10min after intravenous administration of PBS or various doses of FME. Each point represents the mean ± SD of three independent experiments (P<0.01). Bleeding time longer than 800s was expressed as above 800s

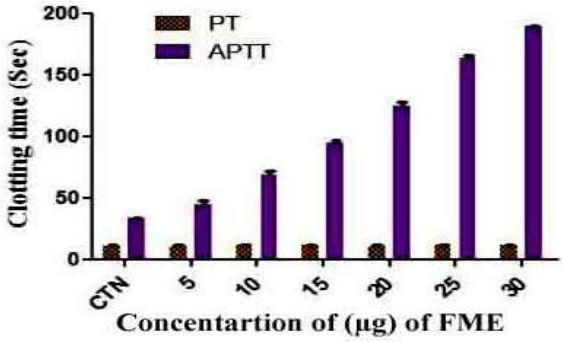

Fig. 4. PT and APTTFME. (0-30µg) was pre-incubated with 0.2mL of citrated human plasma PRP in the presence of 100µl of APTT and 200µl of PT reagent of 0.25M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded. The time taken for the visible clot was recorded in seconds. The APTT ratio and the international normalized ratio (INR) for PT at each point was calculated from the values of control plasma incubated with the buffer for identical period of time

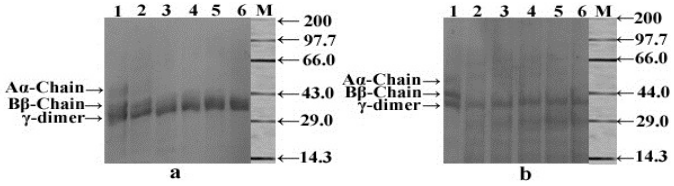

Fig. 5. Effect of FME on Fibrinogenolytic activity. (a) Dose-dependent effect: fibrinogen alone 50µg (a1), fibrinogen treated with 20µg (a2), 40µg (a3), 60µg (a4), 80µg (a5), 100µg (a6) of FME respectively, incubated for 4hr at 37°C and then separated on 10% SDS-PAGE under reduced condition. (b) Time-dependent effect: FME 40µg was incubated with fibrinogen 50µg for 0hr (b1), 4hr (b2), 8hr (b3), 12hr (b4), 16hr (b5) and 24hr (b6) respectively at 37°C

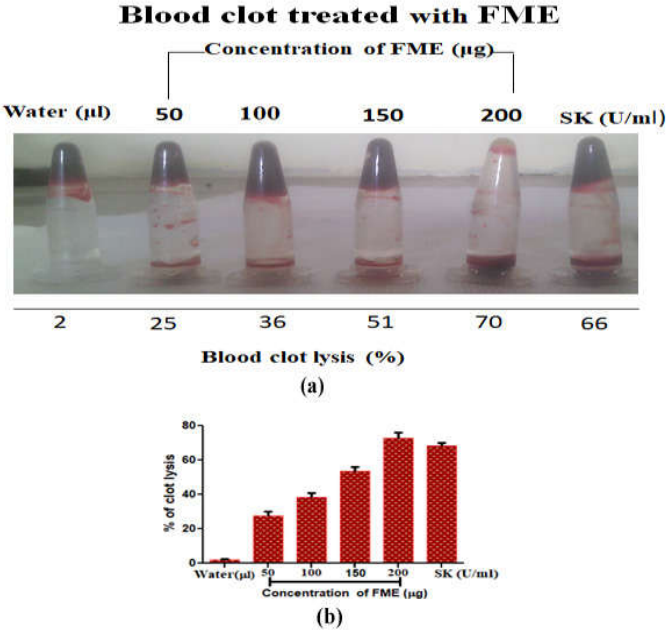

Fig. 6. Whole blood clot lysis. (a) Whole blood clot lysis image (b) Percentage of whole blood clot lysis. (a) Freshly drawn healthy human blood was transferred to pre-weighed sterilized microcentrifuge tubes (500µL/tube) and incubated for 45min at 37°C. Serum was removed after the formation of whole blood clot then each tube was weighed again. FME (50-200µg) was added individually to all the tubes thereafter incubated for 90min at 37°C and calculated the clot lysis percentage. (b) Percentage of whole blood clot lysis

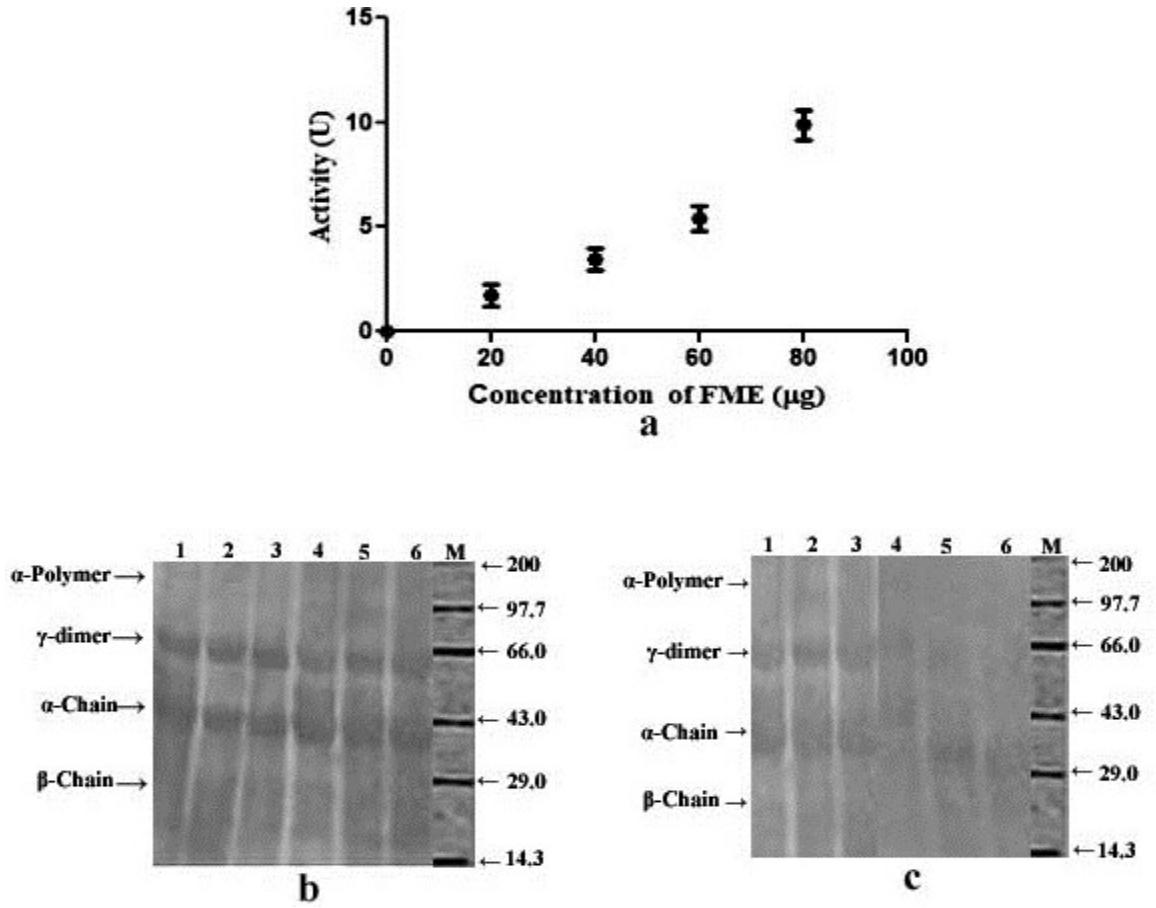

Fig.7. Effect of FME on Fibrinolytic activity: a) Colorimetric assay Washed plasma clot was incubated with 0–100µg of FME for 2.30hr and then the OD was measured at 660nm. (b) Dose-dependent effect; Washed plasma clot was incubated for 12hr and then separated on SDS-PAGE (7.5%), washed plasma clot alone (b1), plasma clot treated with 20µg (b2), 40µg (b3), 60µg (b4), 80µg (b5) and 100µg (b6) of FME respectively. (c) Time-dependent effect; FME 40µg was incubated with fibrin clot at 37°C, fibrin clot alone (c1), 0hr (c2), 6hr (c3), 12hr (c4), 18hr (c5) and 24hr (c6) of FME. (d)

Fibrinogenolytic enzymes have been extensively studied in plant latex, venoms of snake and spider (Rajesh *et al.,* 2006). In addition, bitter gourd, jackfruit, flaxseed and garlic found to hydrolyze human fibrinogen (Jain, 1977). Interestingly FME dissolved both whole blood clot and fibrin clot as well. When whole blood clot was incubated with FME (0-200µg) against positive control streptokinase (60 U/ml) it able to dissolve 75% of the whole blood clot (Fig 6). FME also dissolves fibrin clot, initially the clot dissolving property was analyzed using colorimeter and the obtained specific activity was found to be 4.98units/mg/min (Fig 7a). fibrinolytic activity of the FME was also further strengthened by analyzing on 7.5% SDS-PAGE, it degraded only α polymer chain in a dose dependent manner at the concentration of 100µg (Fig 7b). But when incubation time was prolonged at the concentration of 40µg FME was able to cleave all the chain of fibrin clot at the incubation time of 24hr (Fig 7c). In normal condition, once the fibrin clot has been formed immediately clot is dissolved by fibrinolytic enzyme the plasmin (Chauhan *et al.,* 1982). While, in case of thrombosis the clot remains in the arteries and veins. At present, urokinase, streptokinase, plasminogen activator (t-PA) have been extensively used as thrombolytic agents (Schwartz *et al.,* 1973). However, their high cost and side effects there is an increasing demand for thrombolytic agents with least side effects. FME dissolved both blood clot and fibrin clot strengthened its clot lysis activity along with anticoagulant potential. Thrombolytic agents have been characterized from microorganisms, earthworms, snake venoms, centipede venoms, insects, and leeches (Lewis *et al.,* 1985; Hatano *et al.,* 1996; Metzig *et al.* 1999; Smyth *et al.,* 1962). Interestingly, FME did not hydrolyze plasma proteins except fibrinogen present in the plasma when incubated at 37°C for 12h at the concentration of 50µg, suggesting its substrate specificity (Fig 8). Above all, FME strongly inhibited the platelet aggregation triggered by the agonists such as ADP and epinephrine in platelet-rich plasma (Fig 9 and 10).

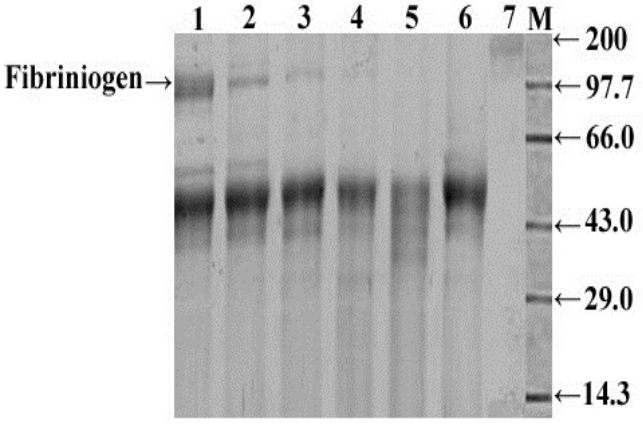

Fig.8 Effect of FME on Degradation of plasma proteins. Plasma protein (100µg) was incubated with FME in 40µL of 10mM Tris–HCl buffer (pH 7.4) at 37°C and then analyzed on 7.5% SDS-PAGE under non-reduced condition. Plasma protein (100µg) alone (1), plasma protein treated with 20µg (2), 40µg (3), 60µg (4), 80µg (5), 100µg (6) of FME and 20µg of fibrinogen as control (7).

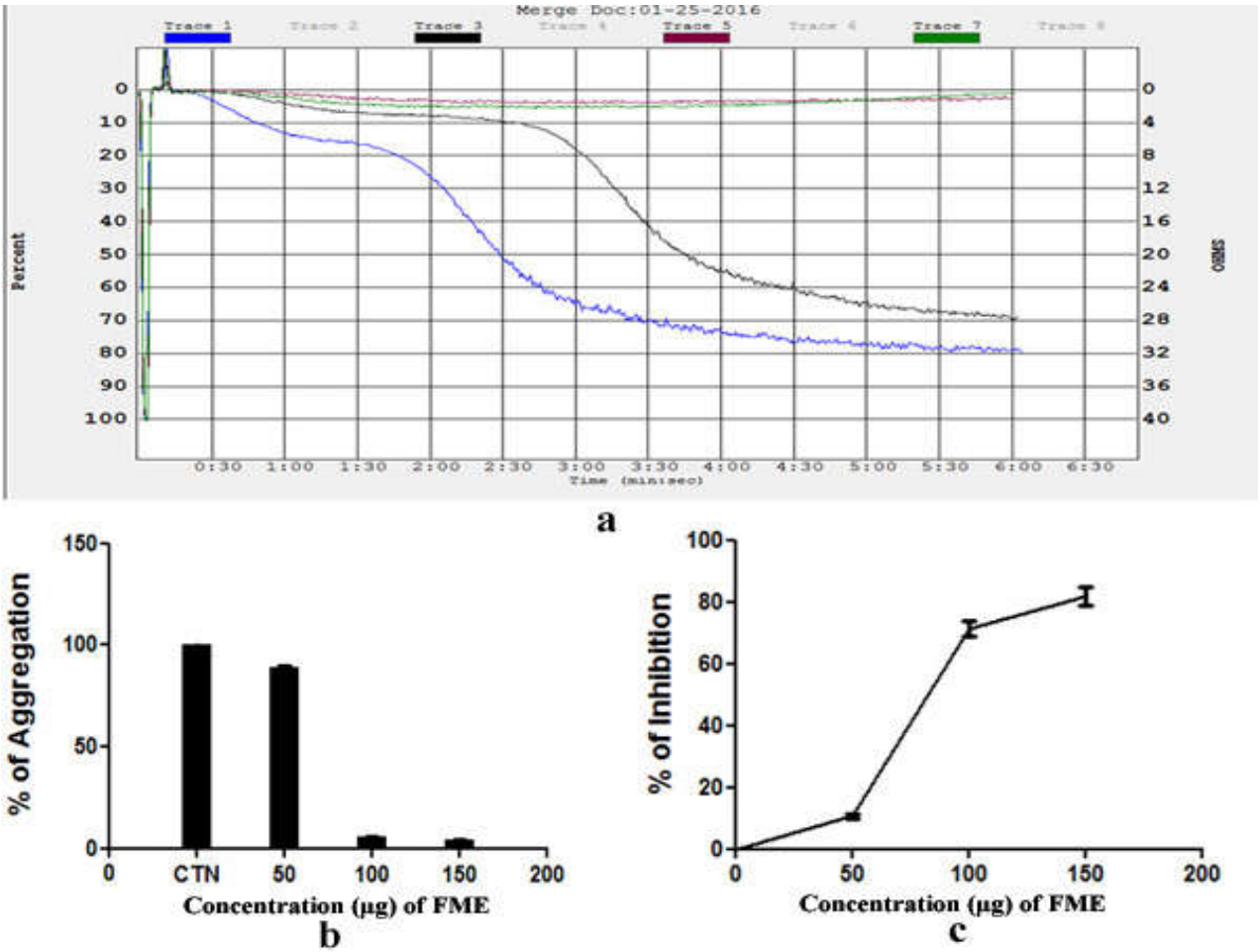

Fig. 10. Platelet aggregation was initiated by adding Epinephrine as an agonist. (a) Traces of platelet aggregation: Trace 1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM+50µg of FME); Trace 3 (Epinephrine 5µM+100µg of FME); Trace 4 (Epinephrine 5µM+150µg of FME). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.

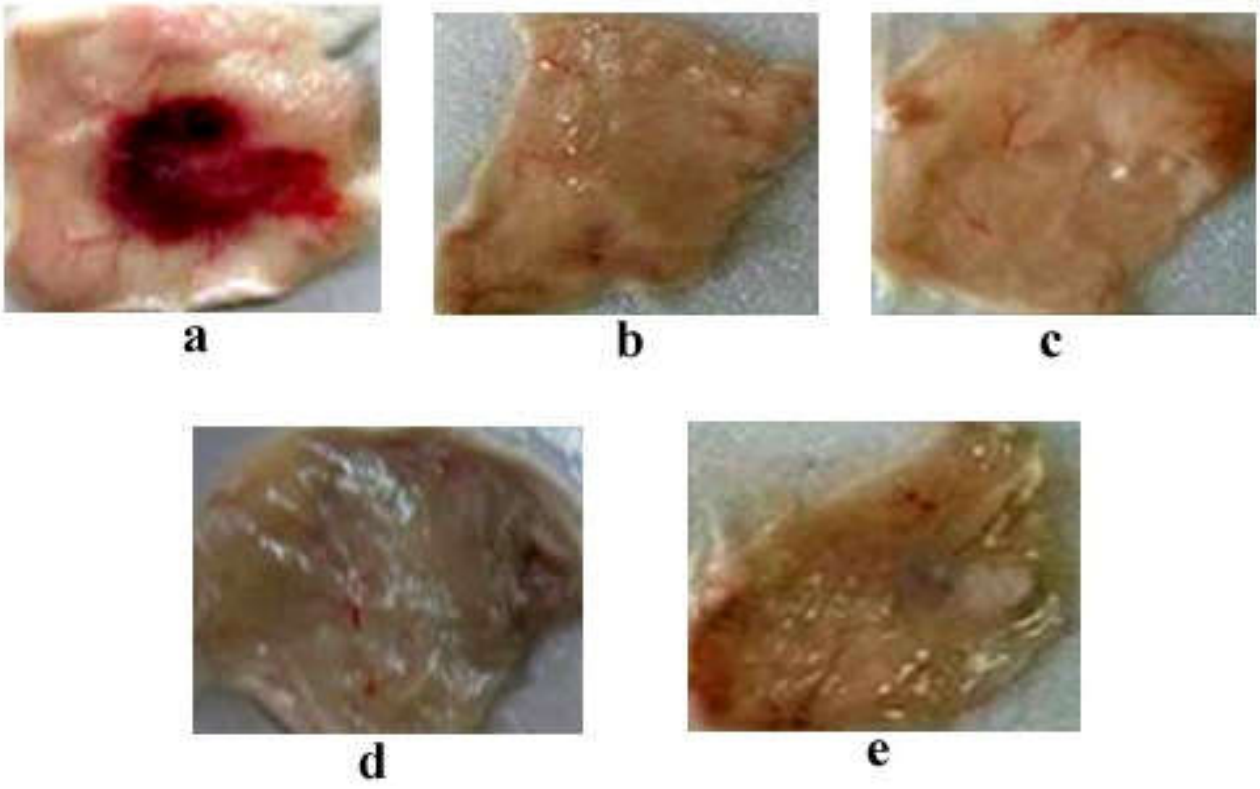

Fig. 11. Dose-dependent hemorrhagic activity of FME. (a) Saline, (b) positive control 2 MDH venom, (c) 50µg, (d) 100µg and (e) 200µg of FME were injected independently into mice in a total volume of 50µL intradermal.

Table 1. Effect of Inhibitors on the Proteolytic Activity of ECE

Inhibitor (5mM each)	Activity/residual activity (%)
None	100
EDTA	91.25
1, 10-Phenanthroline	95.10
IAA	85.06
PMSF	10.22

The observed platelet aggregation inhibition for both ADP and epinephrine was found to be 89.7% and 85.0% respectively at the concentration of 150µg. Accumulation of platelets at the site of injury is believed to be the first signal of hemostasis along with coagulation factors (Kahn *et al.,* 1998). Several physiological agonists namely ADP, epinephrine, thrombin, thromboxane, arachidonic acid and collagen, activates platelets (Sims *et al.,* 1989). Activated platelets play a prominent task in a series of sequential events during the hemostasis and thrombosis as they are actively involved in cell based thrombin generation (Stenberg *et al.,* 1985). Auto/alloimmune response, genetic abnormalities and dengue fever causes thrombocytopenia contribute to thrombosis (Giesen *et al.,* 1999). In addition, if thrombotic events occur in patients suffering from atherosclerosis the ruptured atherosclerotic plaque end up in heart attack or stroke. Recent investigations also demonstrated the contribution of platelets in the initiation of atherosclerosis (Metzig *et al.,* 1999). Above all, platelets do respond to the fibrinogen via interacting with integrin αIIbβ3 and GPIIb/IIIa surface fibrinogen receptor.

Hence, hyper activation of platelets not only linked to thrombosis but also inflammation, immune responses, lymphatic vessel development, angiogenesis, tumor metastasis and atherosclerosis as well (Brantl *et al.,* 2013). Thus, antiplatelet agents do play a key role in inhibiting hyper activation of platelets. Several antiplatelet agents have been characterized from nanoparticles, plants and animal sources (Livio *et al.,* 1978; Papapanagiotou *et al.,* 2016; Marulasiddeshwara *et al.,* 2017). Eptifibatide, derivative from rattle snake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently using in the treatment of coagulation disorders (Poon and d'Oiron, 2000). FME showed strong antiplatelet effect corroborate its therapeutic efficiency in thrombosis. FME was found to be non toxic to RBC cells as it did not cause hemolysis. Moreover, it was devoid of hemorrhage and edema in experimental mice up to the concentration of 150µg, while positive control *Daboia russelli* venom induced hemorrhage and edema in experimental mice (Fig 11). In conclusion, this study for the first time demonstrates the evidences for the presence of serine and cysteine proteolytic activity of FME responsible for clot dissolving, anticoagulant, and antiplatelet properties.

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