

**Research Article****Macrotyloma uniflorum seed aqueous extract exhibits anticoagulant, antiplatelet and clot dissolving properties**

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Received: 20 December 2018

Revised: 18 January 2019

Accepted: 9 February 2019

**Abstract**

**Objective:** The present work demonstrates the anticoagulant and antiplatelet property of *Macrotyloma uniflorum* Seed Aqueous Extract (MUSAE). **Materials and Methods:** The protein banding blueprint of MUSAE (100µg) was analyzed on SDS-PAGE. The proteolytic activity of MUSAE was analyzed by means of casein zymography at the concentration of 50 µg. Anticoagulant effect of MUSAE was tested using plasma recalcification time, mouse tail bleeding time, Activated Partial ThromboPlastin Time (APTT) and Prothrombin Time (PT) at concentrations of 0-120µg. Fibrinogen and fibrin clot degrading actions of MUSAE were analyzed on SDS-PAGE under reduced conditions. The non-toxic property of MUSAE was tested by edema, hemorrhage and direct hemolytic activities. **Results:** MUSAE showed similar protein banding pattern in both reduced and non-reduced conditions on SDS-PAGE. MUSAE exhibited proteolytic activity as it hydrolyzed casein with the specific activity of 0.121units/mg/min. while, the proteolytic activity of MUSAE was totally eradicated by 1, 10-Pheanthroline and PMSF but EDTA and IAA did not; confirms the presence of serine and zinc metallo protease in MUSAE. MUSAE delayed the clotting time of human citrated plasma against the control 184sec to 407sec suggesting its anti-coagulant property. Interestingly, MUSAE delayed the clot formation process of only APTT, suggesting its participation in an intrinsic pathway of blood coagulation cascade. Furthermore, MUSAE hydrolyzed human fibrinogen, fibrin clot without hydrolyzing other plasma proteins. In addition, MUSAE exhibited antiplatelet aggregation property by inhibiting agonists ADP and Epinephrine induced platelet aggregation. The percentage of inhibition was found to be 75% and 72% in PRP. MUSAE was also inhibited the washed platelet aggregation induced by thrombin, ADP, collagen, arachidonic acid and epinephrine in washed platelet. The observed inhibition percentage was found to be 98%, 81%, 54%, 50% and 48% respectively. Moreover, MUSAE was nontoxic as there was no hemolytic, hemorrhagic and edema forming activities were observed. **Conclusion:** MUSAE exhibited anticoagulant, antiplatelet and clot dissolving properties, hence, it could be promising agent in the management of thrombotic disorders.

**Keywords:** *Macrotyloma uniflorum*, Blood coagulation cascade, intrinsic pathway, platelet aggregation

**Introduction**

*Macrotyloma uniflorum* is a robust pulse crop of semi-arid tropics that has been least studied. It is a novel legume belongs to

the family Fabaceae and the phylum Leguminosae (Kumar, 2006). It is an underutilized food crop largely grown in India, Africa, Australia, Burma, Malaysia & Srilanka. Despite high quality of nutritional supplements there are embed prejudice against the usage of *Macrotyloma uniflorum* in the population (Nezamuddin, 1970). The reason is, *Macrotyloma uniflorum* has been considered as a low grade food (Kadam et al., 1985; Ambasta, 1986; Smartt,1985). In spite of such prejudice, *Macrotyloma uniflorum* (horsegram) is the fifth most extensively growing pulse in India.

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DOI: <https://doi.org/10.31024/ajpp.2019.5.3.23>

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*Macrotyloma uniflorum* seeds store high quantity of macro (Protein, carbohydrates and lipids) micro (vitamins and minerals), secondary metabolites and negligible amount of antinutritional factors. Phytic acid, tannins & phenolic acids are the major secondary metabolites identified so far. In addition, the occurrence of flavonoids, terpenoids, glycosides, tannins, steroids, and saponins has high influence on anti-bacterial, antifungal, cytotoxic and anti-oxidant activities (Tiwari et al., 2013; Pritha Chakraborty et al., 2016). Kaempferol-3-O- $\beta$ -D-glucoside,  $\beta$ -sitosterol, stigmasterol Dolichin A & B, Pyroglutaminyl glutamine are the few isolated compounds from *Macrotyloma uniflorum* seeds (Handa et al., 1990; Kawsar et al., 2009). Although, there is little scientific validation, many researchers have been highlighted the medicinal usage of *Macrotyloma uniflorum* seeds in the treatment several diseases. From the ancient time *Macrotyloma uniflorum* has been widely used in the treatment of kidney stones, flatulence menstrual bleeding, urinary diseases, piles, common cold, throat infection and fever (Phillips, 1993; Ravishankar et al., 2012; Kennedy et al., 1993; Perumal et al., 2007; Koratkar et al., 1997). Although, *Macrotyloma uniflorum* highest amount of protein content yet therapeutic role of them have been least explored.

Bacterial infection, inflammation, vascular injury, oxidative stress and cancer increases the risk of thrombosis, a process of formation of blood clot in an artery or vein in living individual (Falanga et al., 2005; Wannamethee et al., 2005). Arterial thrombosis blocks supply of oxygen and nutrition to the different parts of the body, while, venous thrombosis blocks return of deoxygenated blood to the heart. Thus, thrombosis causes myocardial infarction and stroke in most of all the population worldwide. Although, several anticoagulant and antiplatelet agents have been extensively used in the treatment of thrombosis. The life threatening side effects limits their usage. Hence, thrombosis remains one of the burning issues in both developed and under developed countries. Therefore, identifying anticoagulant, antiplatelet and clot dissolving agents from natural sources with least side effects helps in the better management of thrombotic disorders. Even though, MUSAE stores large amount of proteins, their beneficial role in coagulation and platelet function is not identified. Thus, the current study aims to explore the role of *Macrotyloma uniflorum* seed proteins on thrombolysis, plasma coagulation time and platelet aggregation.

#### Materials and methods

Phenyl Methyl Sulphonyl Fluoride (PMSF), Ethylene Di-Amine Tetra Acetic acid (EDTA), Ethylene Glycol-N, N, N', N'-Tetra Acetic acid (EGTA), 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.

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).

#### Ethics statement

Human blood was collected from healthy adult volunteers with transcribed informed consent according to the procedures of Institutional Human Ethical Committee, University of Mysore, Mysore. All the experimentations were conducted in accordance with the ethical guidelines and were approved by the Institutional Human Ethical Committee (IHEC-UOM No. 47Res/2014-15), University of Mysore, Mysore. Conducting animal experiments were permitted by the Institutional Animal Ethical Committee (UOM/IAEC/02/2016), University of Mysore, Mysore. The animal handling were proceeded in accordance with the guidelines of the Committee for the Purpose of monitoring and Supervision of Experiments on Animals (CPCSEA)

#### Extraction of plant material

*Macrotyloma uniflorum* seeds were purchased from local market Tumkur. The seeds were washed with distilled water and thoroughly chopped and homogenized using double distilled water and centrifuged at 4000g for 2min at 4°C. The supernatant was collected and proteins were precipitated using 30% of ammonium sulphate. The precipitated protein sample was again centrifuged at 6,000g 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 *Macrotyloma uniflorum* Seed Aqueous Extract (MUSAE).

#### Protein estimation

Protein concentration was determined as described by Bradford et al., 1976 Using Bovine Serum Albumin (BSA) as standards.

#### Gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by the method of Laemmle et al., 1970. The crude MUSAE (100 $\mu$ g) was prepared under both reduced and non-reduced conditions. The electrophoresis was carried out using 25mM Tris, 192mM glycine and 0.1% SDS for 120min at room temperature. Gels were stained after electrophoresis with 0.1% CBB R-250 then de-stained with 40% ethanol in 10% acetic acid and water (40:10:50v/v) for detection of protein bands. Standard molecular markers were used.

### **Glycoprotein (PAS) staining**

To perform PAS staining, method of Leach et al., 1980 was carried out. The gel was fixed in acetic acid solution (7.5%) and stored at room temperature for 60min after electrophoresis. Then the gel was washed with nitric acid solution (1%) and kept in aqueous periodic acid solution (0.2%) and stored at 4°C for 45min. Finally, the gel was placed in Schiff's reagent at 4°C for 24h to visualize the pink colour band.

### **Proteolytic activity**

#### **Colorimetric Estimation**

Proteolytic activity was determined according to the method of Satake et al., 1963 using 2% casein in 0.2M Tris HCL buffer, pH 8.5. The MUSAE (50µg) was incubated separately with 0.4mL of substrate in a total volume of volume of 1ml for 2.30h at 37°C. Undigested casein was precipitated by adding 1.5ml of 0.44M/l Trichloroacetic acid (TCA). The reaction mixture was then centrifuged at 2000g for 10min. Sodium carbonate (2.5ml, 0.4M/l) and Folin–Ciocalteu's reagent were added sequentially to 1ml of the supernatant and the color developed was read at 660nm. 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 MUSAE (50µg) for 30min with 5mM/l each of EDTA, 1,10-phenanthroline, PMSF and IAA. In all the cases, appropriate controls were kept.

#### **Zymography**

Zymogram was carried as described previously, briefly MUSAE (50µg & 100µg) prepared under non-reduced condition was loaded onto polymerized 2% casein in resolving gel. After electrophoresis gels were washed with 10mM sodium phosphate buffer containing 2.5% of Triton X-100 with constant agitation for 1h to remove SDS. The gel was incubated overnight at 37°C in Tris–HCl buffer (50mM) pH 7.6 containing 50mM CaCl<sub>2</sub> and 40mM NaCl. Gel was then stained to observe the translucent activity bands. For inhibition studies, a similar reaction was performed independently after pre incubating the crude MUSAE (50µg) for 20min with 5mM/l 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 as described by Ardlie and Han et al., 1974. The platelet concentration of PRP was adjusted to  $3.1 \times 10^8$  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.

#### **Plasma re-calcification time**

The method of Quick et al., 1935 was followed for plasma

recalcification time assay. Briefly, the crude MUSAE (0–120µg) 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<sub>2</sub> was added to the pre-incubated mixture and clotting time was recorded.

#### **Tail Bleeding Time Assay**

The bleeding time assay was performed as described previously. Concisely, MUSAE (0–50µg) 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. Instantly, 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.

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

Briefly, 100µl of normal citrated human plasma and MUSAE (0–120µg) were pre-incubated for 1min. For APTT, 100µl reagent (LIQUICELIN-E Phospholipids preparation derived from Rabbit brain with ellagic acid), which was activated for 3min at 37°C was added. The clotting was initiated by adding 100µl of 0.02M CaCl<sub>2</sub> and the clotting time was measured. For PT, the clotting was initiated by adding 200µl 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 identical period of time.

### **Fibrin (ogeno)lytic activity**

Fibrinogenolytic activity was determined as described previously by Ouyang and Teng et al., 1976. MUSAE (0–25µg) 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 analyzed by 10% SDS-PAGE. For inhibition studies, MUSAE (10µg) was pre incubated for about 20min with 5mM each of PMSF, IAA, EDTA and 1,10-Phenanthroline.

#### **Clot Degradation Assay**

##### **Fibrin clot-hydrolyzing activity by calorimeter**

Fibrin clot degrading property was assayed by the method of Rajesh et al., 2005. Concisely, 100µl of citrated human plasma was mixed with 20µl of 0.2M CaCl<sub>2</sub> and incubated

for 120min at 37°C. The obtained clot was washed thoroughly with PBS and re-suspended in 400µl of 0.2M Tris buffer (pH 8.5). To the above washed fibrin clot varied amounts of MUSAE (0–100µg) was added in 100µl of saline and incubated for 2.30h 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 1:3 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/h at 37°C.

#### **Fibrinolytic activity by SDS-PAGE:**

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

#### **Human plasma protein hydrolyzing activity**

Human plasma protein hydrolyzing activity was carried out according to the method of Kumar et al., 2010. The MUSAE (0–100µg) was incubated with the 100µg of plasma proteins for 24h at 37°C in a reaction volume of 40µl 10mM Tris (pH 7.4) containing 10mM NaCl, 0.05% sodium azide. The reaction was stopped by adding 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**

Washed platelets were prepared as described by the method of Born 1962. 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<sub>2</sub>, 6H<sub>2</sub>O and this suspension was taken for platelet aggregation study.

#### **Platelet aggregation**

For platelet aggregation assay turbid metric method of Born 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 MUSAE (0–60µg) in 0.25ml reaction volume. The aggregation was initiated independently by the addition of agonists, such as ADP, epinephrine, thrombin, arachidonic acid and collagen then followed for 3 min.

#### **Direct hemolytic activity**

Briefly, packed human erythrocytes and PBS (1:9v/v) were mixed; 1ml of this suspension was incubated independently with the various concentration of MUSAE (0-150µg) for 60min at 37°C. The reaction was terminated 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.

#### **Edema inducing activity**

Edematic activity was performed as described by vishwanath et al., 1987. Groups of five mice were injected separately into the right foot pads with different doses (0-200µg) of MUSAE 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. Briefly, MUSAE (0 - 200µg) was injected (intradermal) independently into the groups of five mice in 30µl saline. After 3h, mice were anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for hemorrhage 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. Significant difference between the groups were considered if P < 0.01.

#### **Results and Discussion**

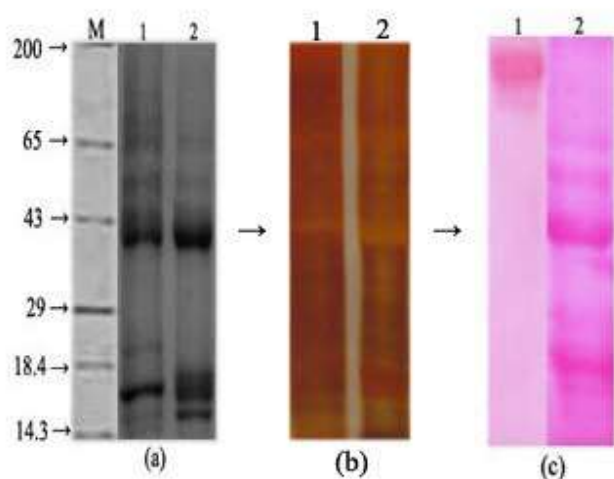
Present study demonstrates the thrombolytic, anticoagulant and antiplatelet activities of *Macrotyloma Uniflorum* seed Aqueous extract (MUSAE). Protein banding pattern of MUSAE was analyzed using SDS-PAGE. It showed similar

kind of protein banding pattern under reduced and non-reduced conditions, suggesting the existence of majority of monomeric proteins. (Figure.1a&1b). MUSAE was analyzed for the probable carbohydrate content in its stored proteins using Periodate Schiff (PAS) staining. Interestingly, most of all the proteins of MUSAE range from 200 to 14kDa taken up the PAS stain that were compared with the positive control fibrinogen (Figure.1c). Glycoproteins found to involve in diversified functions namely, fertilization, neuronal development, hormone activities, immune surveillance, inflammatory responses, blood clotting and platelet activity (Brighton et al., 1996).

MUSAE hydrolyzed casein with the specific activity of

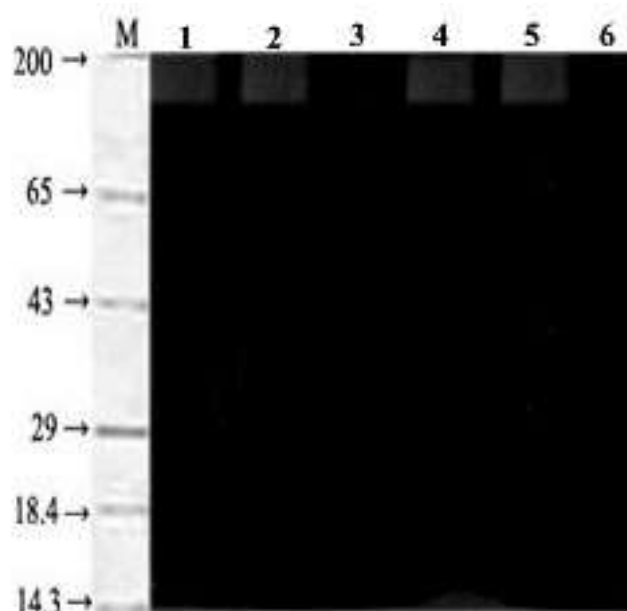
**Table 1.** Effect of inhibitors on the proteolytic activity of *Macrotyloma uniflorum*

| Inhibitor (5mM each) | Activity/residual activity % |
|----------------------|------------------------------|
| None                 | 100                          |
| EDTA                 | 99.5                         |
| 1,10-Phenanthroline  | 11.10                        |
| IAA                  | 94.00                        |
| PMSF                 | 15.80                        |



**Figure 1.** (a) SDS-PAGE 10% (b) Silver staining (c) Glycoprotein staining. (a) MUSAE (100µg) under non-reduced (a1) and reduced conditions (a2), (b) MUSAE (30µg) under non-reduced (b1) and reduced conditions (b2), (c) Positive control fibrinogen (c1) and MUSAE (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, MUSAE: *Macrotyloma uniflorum* Seed Aqueous Extract.

0.121units/mg/min at 37°C, suggesting its proteolytic activity. The proteolytic activity of MUSAE was also genuinely identified by using casein zymogram. MUSAE showed translucent activity band at the region 200 kDa (Figure 2). The proteolytic activity of MUSAE was completely obliterated by PMSF and 1, 10, Phenanthroline but not IAA and EDTA, suggesting the presence of serine and zinc dependent metallo-protease in the MUSAE (Table 1). Despite, hydrolytic activity on polypeptide chain, proteases hold great promise as a growing class of drugs. They have been gaining much attention as they are useful tool in the treatment of cardiovascular diseases, sepsis, digestive disorders, inflammation, cystic fibrosis, retinal disorders, psoriasis and other diseases (Abbenante et al., 2005). t-PA and streptokinase have been widely employed in the treatment of thrombotic complications (Diwedi et al., 2005). Snake venoms, bacteria, fleas and ticks, contain a treasure of proteases (Kumar et al., 2010; Lynn et al., 1985). Germinating cotyledons from *Macrotyloma uniflorum* found to exhibit cysteine proteolytic activity. Serine, cysteine and metallo proteolytic enzymes were detected from the seeds such as, *Araucaria angustifolia*, jackfruit, bitter gourd, pea and maize (Sowmyashree et al., 2015; Flávio Lopes Alves et al., 2016; Bhagyalakshmi et al., 2014; Chethana et al., 2017; Takafumi et al., 2000).



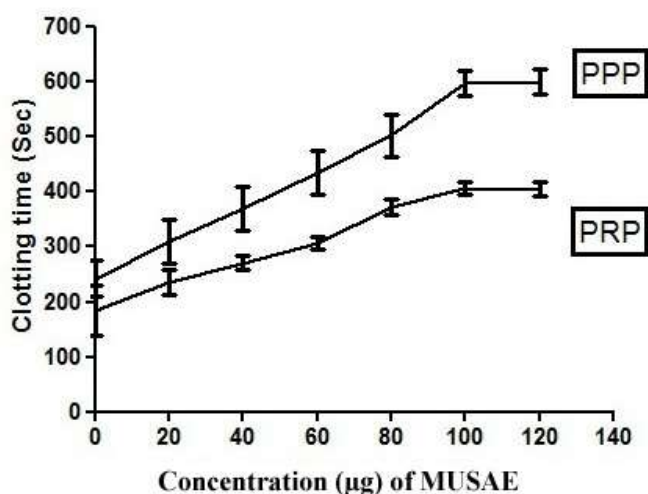
**Figure 2.** Casein Zymogram. MUSAE 50µg (1) and 100µg (2) under non-reduced conditions. MUSAE 50µg was pretreated with 5mM PMSF (3), MUSAE 50µg was pretreated with 5mM IAA (4), MUSAE 50µg was pretreated with 5mM EDTA (5), MUSAE 50µg was pretreated with 5mM 1,10,Phenanthroline (6) under non-reduced conditions.

**Table 2.** Clotting time of PT and APTT

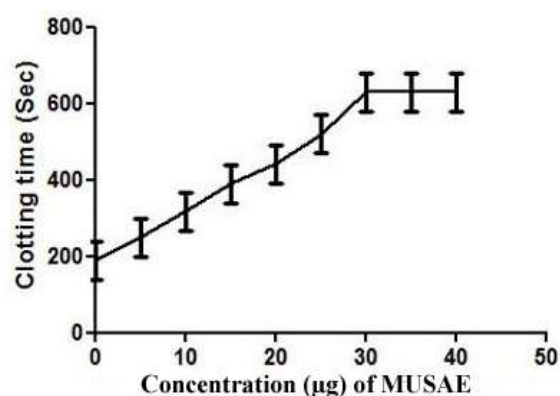
| MUSAE (µg) | PT clotting time in sec | PT (INR value) | APTT clotting time in sec | APTT ratio |
|------------|-------------------------|----------------|---------------------------|------------|
| 0          | 11.6±0.06               | 0.84±0.02      | 34.9±0.04                 | 1.01±0.01  |
| 20         | 12.7±0.02               | 0.095±0.01     | 51.2±0.01                 | 1.45±0.05  |
| 40         | 12.8±0.01               | 0.101±0.03     | 68.0±0.04                 | 1.56±0.02  |
| 60         | 12.6±0.04               | 0.098±0.05     | 74.4±0.03                 | 1.68±0.04  |
| 80         | 12.9±0.03               | 0.112±0.08     | 82.4±0.02                 | 1.71±0.03  |
| 100        | 13.0±0.02               | 0.116±0.04     | 91.2±0.05                 | 1.75±0.06  |
| 120        | 12.7±0.05               | 0.099±0.06     | 99.3±0.08                 | 1.89±0.09  |

MUSAE exhibited strong anticoagulant effect in both Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP). MUSAE increased the clotting time of PRP from the control 184 to 407 sec, where as in case of PPP the observed clotting time was 600 sec against the control 242 sec. In both the cases the maximum concentration utilized was 120µg and remain unchanged upon increased dose up to 140 µg (Figure 3). Furthermore, the anticoagulant effect of MUSAE was also strengthened using *in-vivo* mouse tail bleeding activity. The intra venous injection of MUSAE significantly prolonged the bleeding time in a dose dependent manner and the obtained bleeding time was more than 600 sec ( $p < 0.01$ ) at the concentration of 30 µg against the PBS treated control 180±5 sec (Figure 4). Site of action of MUSAE for its anticoagulation was identified using APTT and PT assays. MUSAE in particular delayed the clotting time of only APTT without altering the PT, revealed the triggered anticoagulation by MUSAE could be due to

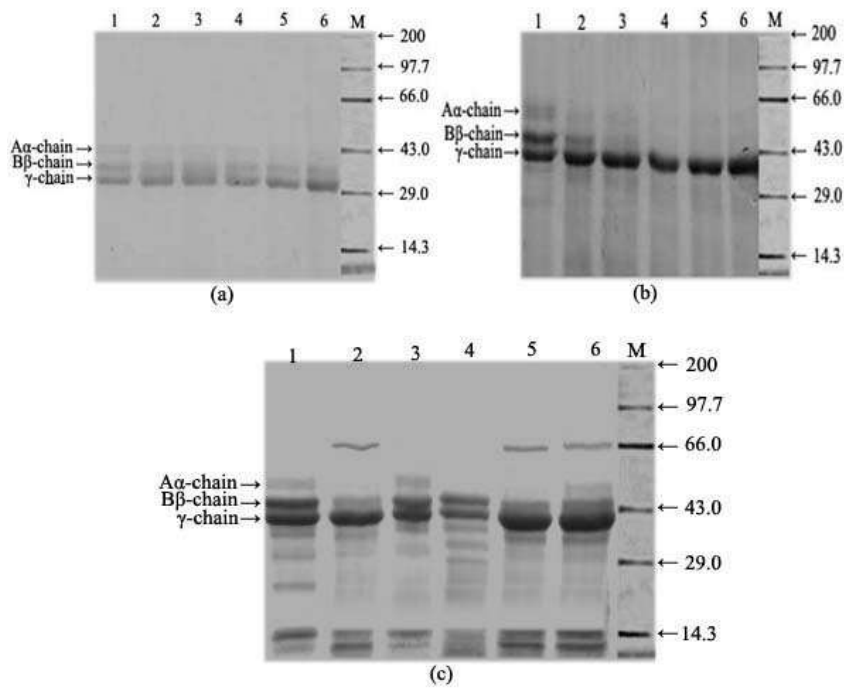
interference in intrinsic pathway of coagulation cascade (Table 2). Strong anticoagulant agents from plants and animal source with least side effects help in the better management of thrombotic disorders. Numerous novel proteases those exhibits anticoagulant activity were identified in snake and spider venoms, earthworm secretions, dung beetles, microorganisms, marine creatures and fermented food products of e Japanese Natto and Korean Chungkook-Jang soy sauce (Hrzenjak et al., 1998; Chudzinski-Tavassi et al., 1998; Matsui et al., 2000; Devaraja et al., 2010). Even though, several anticoagulant proteases have been reported from various research groups, snake venom (Ancord), fungi aspergillusoryzae (Brinase), coumarin derivative from sweet clover (acenocoumarol), hirudin derivative from saliva of leech (Bialirudin) are currently available in the market and they are widely used to treat cardio/cerebrovascular complications caused due to thrombosis (Zanetti et al., 2002; Devaraja et al., 2008; Rachidi et al., 2013; Mulenga et al., 2013; Crawley et al., 2008).



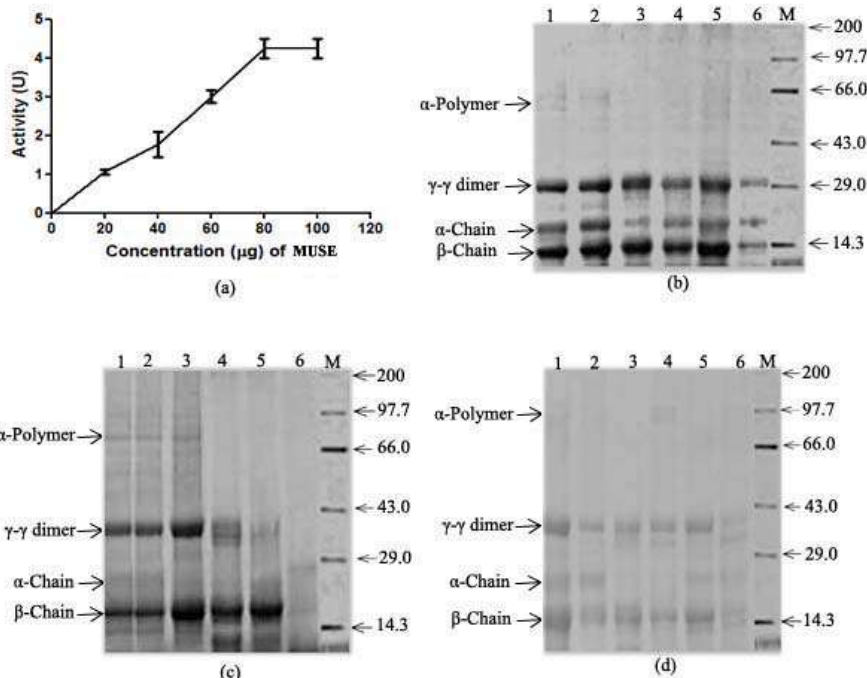
**Figure 3.** Effect of MUSAE on Plasma re-calcification time. (a) MUSAE (0–120µg) was pre-incubated with 0.2ml of citrated human plasma PRP/PPP in the presence of 20µl 10mM Tris–HCl buffer (pH 7.4) for 1 min at 37°C. 20µl of 0.25M CaCl<sub>2</sub> was added to the pre-incubated mixture and clotting time was recorded.



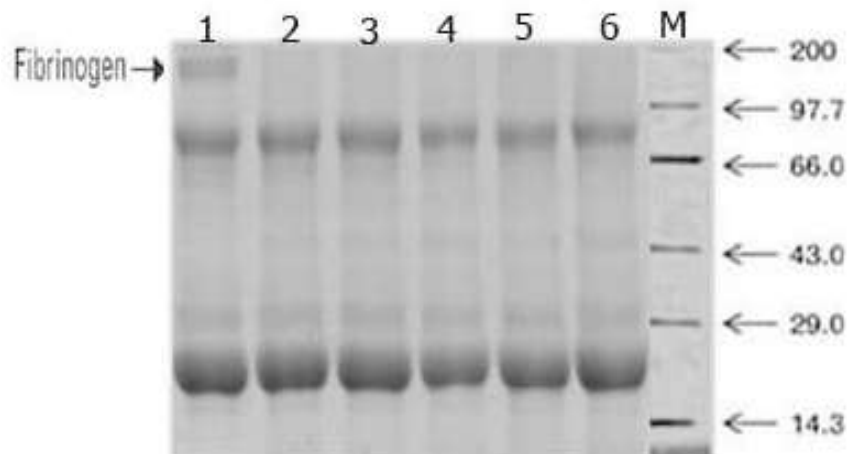
**Figure 4.** Tail bleeding time. Tail bleeding time was measured 10 min after intravenous administration of PBS or various doses of MUSAE. Each point represents the mean ± SD of three independent experiments ( $P < 0.01$ ). Bleeding time longer than 800 s was expressed as above 800 s



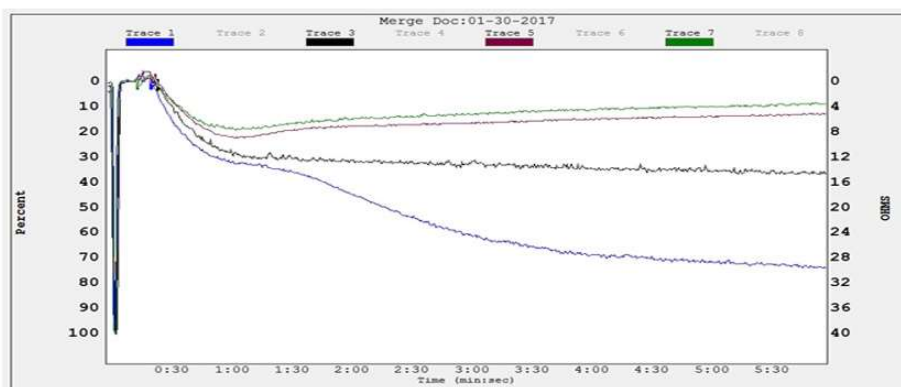
**Figure 5.** Effect of MUSAE on Fibrinogenolytic activity. (a) Dose-dependent effect: fibrinogen alone 50µg (a1), fibrinogen treated with 5µg (a2), 10µg (a3), 15µg (a4), 20µg (a5), 25µg (a6) of MUSAE respectively, incubated for 4hr at 37°C and then separated on 10% SDS-PAGE under reduced condition. (b) Time-dependent effect: MUSAE 10µg was incubated with fibrinogen 50µg for 0hr (b1), 4hr (b2), 8hr (b3), 12hr (b4), 16hr (b5) and 24hr (b6) respectively at 37°C. (c) Inhibition study: MUSAE 10µg was pre-incubated with protease inhibitors for 30min at 37°C. Further reaction was initiated by adding 50µg of fibrinogen and incubated for 4hr, fibrinogen alone (c1), MUSAE 10µg (c2), fibrinogen 50µg and MUSAE 10µg with 5mM PMSF (c3), fibrinogen 50µg and MUSAE 10µg with 5mM IAA (c4), fibrinogen 50µg and MUSAE 10µg with 5mM EDTA (c5), fibrinogen 50µg and MUSAE 10µg with 5mM 1,10,phenanthroline (c6).



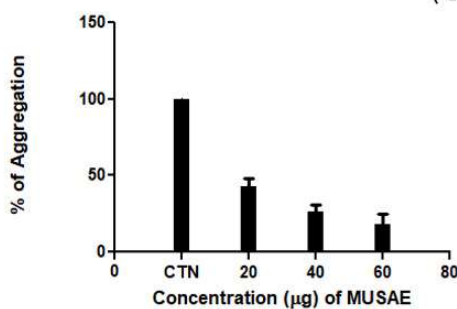
**Figure 6.** Effect of MUSAE on Fibrinolytic activity: a) Colorimetric assay Washed plasma clot was incubated with 0–100µg of MUSAE for 2.30hr and then the OD was measured at 660nm. (b) Dose-dependent effect; Washed plasma clot was incubated for 12hr and then separated on SDS-PAGE (7.5%), washed plasma clot alone (b1), plasma clot treated with 20µg (b2), 40µg (b3), 60µg (b4), 80µg (b5) and 100µg (b6) of MUSAE respectively. (c) Time-dependent effect; MUSAE 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 MUSAE. (d) Inhibition study: MUSAE 40µg was pre-incubated with protease inhibitors for 30min at 37°C. Further reaction was initiated by adding fibrin clot and incubated for 12hr, fibrin clot alone (d1), MUSAE 40µg (d2), fibrin clot and MUSAE 40µg with 5mM PMSF (d3), fibrin clot and MUSAE 40µg with 5mM IAA (d4), fibrin clot and MUSAE 40µg with 5mM EDTA (d5), fibrin clot and MUSAE 40µg with 5mM 1,10,phenanthroline (d6).



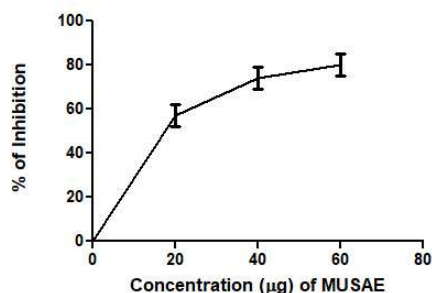
**Figure 7.** Effect of MUSAE on Degradation of plasma proteins. Plasma protein (100µg) was incubated with MUSAE in 40µl of 10mM Tris-HCl buffer (pH 7.4) at 37°C and then analyzed on 7.5% SDS-PAGE under non-reduced condition. Plasma protein (100µg) alone (1), plasma protein treated with 20µg (2), 40µg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



(a)



(b)

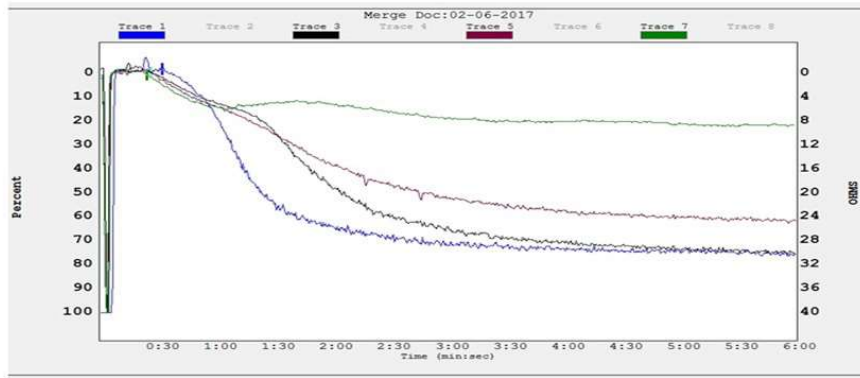


(c)

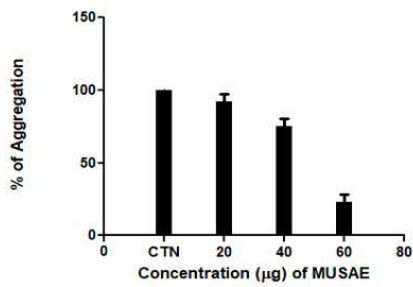
**Figure 8.** Platelet aggregation was initiated by adding ADP as an agonist. (a) Traces of platelet aggregation: Trace 1 (ADP 10µM); Trace 2 (ADP 10µM+20µg of MUSAE); Trace 3 (ADP 10µM+40µg of MUSAE); Trace 4 (ADP 10µM+60µg of MUSAE). The values represent  $\pm$ SD of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.

Furthermore, MUSAE was examined for its hydrolytic efficiency on human fibrinogen. Amazingly, MUSAE hydrolyzed only A $\alpha$  chain in a dose dependent manner at the concentration of 25µg for the 4h incubation time at 37°C (Figure.5a). On the other hand, MUSAE hydrolyzed both A $\alpha$  and

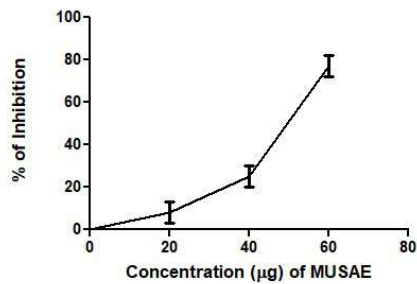
B $\beta$  chains of human fibrinogen in a time dependent manner at the concentration of 10µg for 24h incubation time at 37°C (Figure.5b). Fibrinogenolytic activity of MUSAE was inhibited by both PMSF and 1,10 Phenanthroline but not IAA and EDTA, suggests the role of serine and zinc metallo



(a)

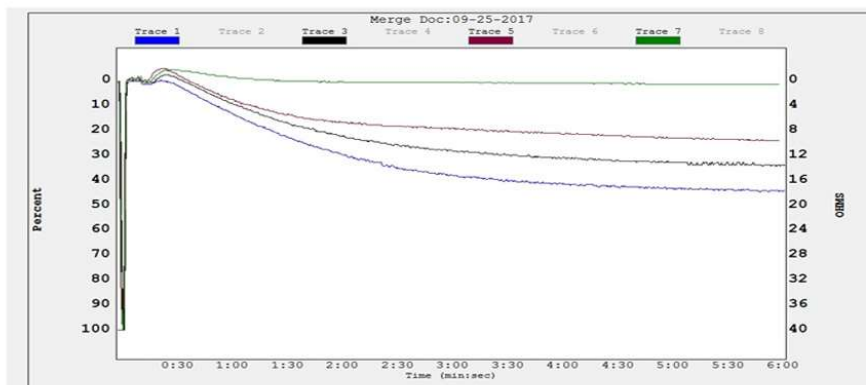


(b)

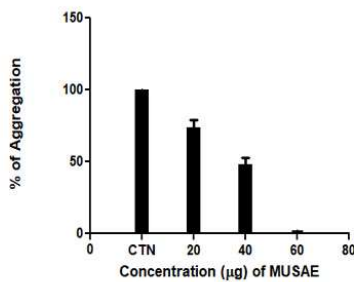


(c)

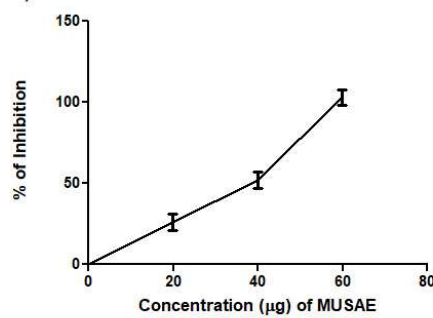
**Figure 9.** Platelet aggregation was initiated by adding Epinephrine as an agonist. (a) Traces of platelet aggregation: Trace 1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM+20µg of MUSAE); Trace 3 (Epinephrine 5µM+40µg of MUSAE); Trace 4 (Epinephrine 5µM+60µg of MUSAE). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.



(a)

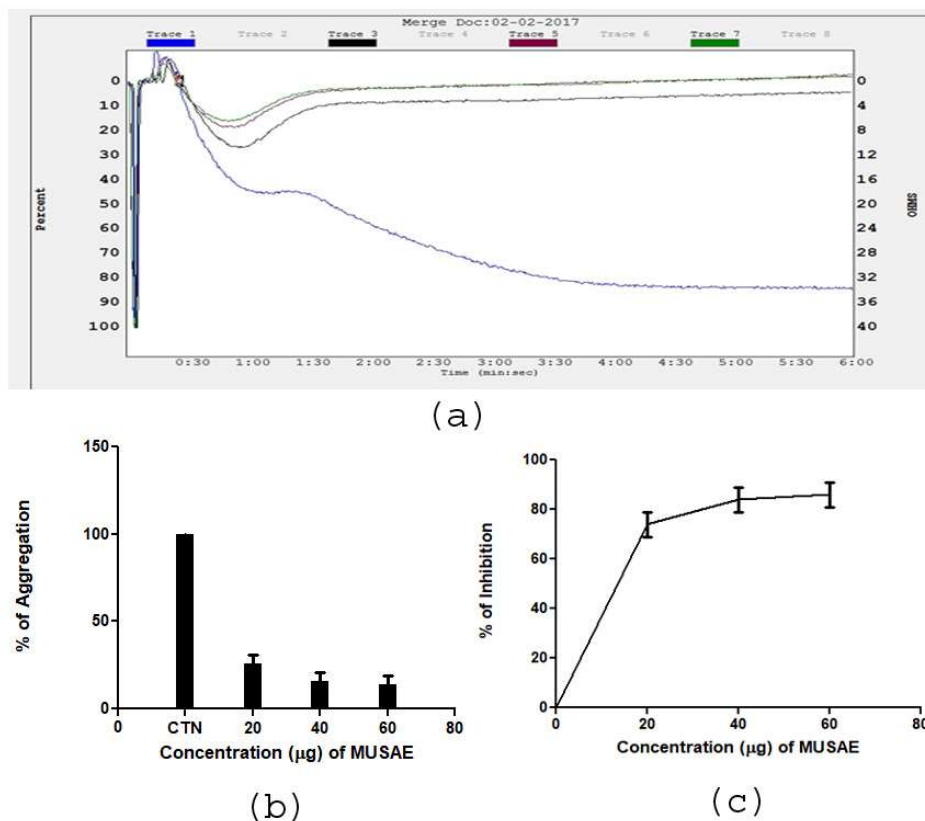


(b)



(c)

**Figure 10.** 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 MUSAE); Trace 3 (Thrombin 2µM+40µg of MUSAE); Trace 4 (Thrombin 2µM+60µg of MUSAE). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.



**Figure 11.** 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 MUSAE); Trace 3 (ADP 10µM+40µg of MUSAE); Trace 4 (ADP 10µM+60µg of MUSAE). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.

proteases in the observed fibrinolysis (Figure.5c). Fibrinolytic enzymes those cleave A $\alpha$  and B $\beta$  chains of fibrinogen from N-terminal end by producing fibrinopeptide A and B are thrombin like enzymes results in procoagulation. However, proteases those degrade fibrinogen from C-terminal end and generates truncated fibrinogen with lack of polymerization potential results in anticoagulation (Devaraja et al., 2010). Metallo, serine and cysteine proteolytic enzymes those degrades fibrinogen were reported from the seeds of bitter gourd, jackfruit, flaxseed and pea (Sowmyashree et al., 2015; Bhagyalakshmi et al., 2014; Chethana et al., 2017; Sharath Kumar et al., 2018).

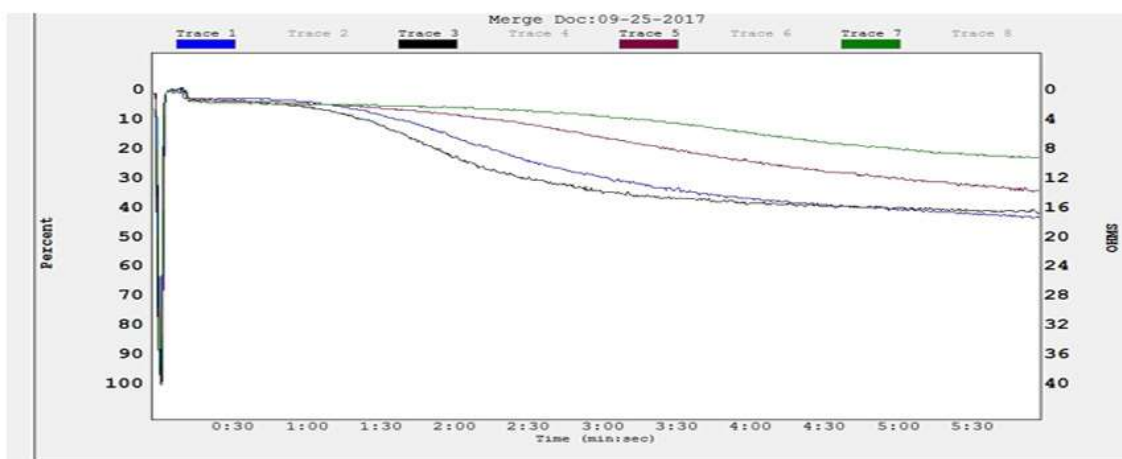
In physiological conditions, coagulation and fibrinolysis are perfectly controlled by the participation of substrates, receptors, activators, inhibitors and cofactors (Esmon et al., 1999). Highly coordinated and regulated molecular interaction between said systems permits the timely removal of localized fibrin deposits (Degen, 2001). Imbalance in the fibrinolytic system results in the deposition of fibrin clot in the arteries and veins. Fibrinolytic enzymes are the better therapeutic tools in the management of thrombosis. Thus, MUSAE was analyzed for its fibrinolytic

activity. Initially, clot dissolving property of MUSAE was analyzed using colorimeter; it showed specific activity of 0.12units/mg/min (Figure 6a). Fibrinolytic activity of MUSAE was further confirmed on SDS-PAGE. MUSAE degraded only  $\alpha$ -polymer chain in a dose dependent manner at the concentration of 100µg (Figure 6b). When the incubation time was prolonged for about 24hr at 37°C, MUSAE hydrolyzed all the chains of cross linked fibrin clot (Figure 6c). Moreover, fibrinolytic activity was inhibited by both PMSF and 1, 10, Phenanthroline, suggest the role of serine/zinc dependent metallo-proteases (Figure.6d). Fibrinolytic enzymes have significant therapeutic potency in curing thrombotic disorders. They have been well characterized from the plant latex, seeds of jackfruit, peas, flax and venoms of snakes and spiders (Sowmyashree et al., 2015; Chethana et al., 2017; Sharath kumar et al., 2018; Devaraja et al., 2010). Importantly, MUSAE did not hydrolyze the other plasma proteins except the plasma fibrinogen up to the incubation time of 12hr at the concentration of 20µg at 37°C. This suggests that substrate specificity of MUSAE on human plasma proteins which could be compared with positive control fibrinogen band alone (Figure 7).

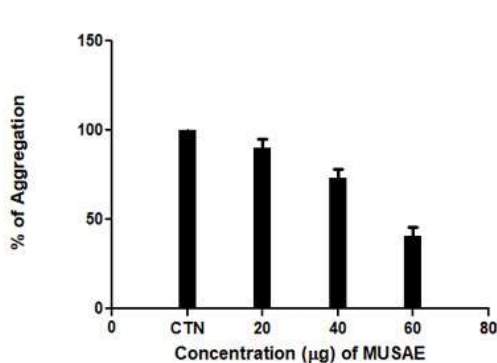
Above all, MUSAE was found to interfere in the platelet function of both platelet rich plasma and washed human platelets. MUSAE strongly inhibited the agonists such as ADP and epinephrine induced platelet aggregation. The identified percentage of platelet aggregation inhibition was found to be 75% and 72% respectively at the concentration of 60 $\mu$ g (Figure 8 and, 9). MUSAE was also Inhibited the platelet aggregation of washed platelets induced by thrombin, ADP, collagen, arachidonic acid and epinephrine. The observed percentage of inhibition was found to be 98%, 81%, 54%, 50% and 48% respectively at the concentration of 60 $\mu$ g. The order of inhibition observed among agonists examined was ADP>Epinephrine induced aggregation in platelet rich plasma. Thrombin>ADP>collagen>arachidonic acid>epinephrine induced platelet aggregation in washed platelets (Figure 10-14). Platelets play the major role in clot formation under the

circumstance of both physiological and pathological condition by forming a platelet plug along with fibrin clot at the site of injury. Physiological agonists namely ADP, epinephrine, thrombin, thromboxane, arachidonic acid and collagen activates platelets up on binding to specific receptor on the platelet. Hyper activation of platelets also a major contributor of thrombotic disorders such as arterial thrombosis, atrial fibrillation, myocardial infarction/heart attack, unstable angina, deep vein thrombosis, pulmonary embolism and cerebral stroke (Leng et al., 1996). Several antiplatelets were identified from both natural sources and synthetic compounds (Bharathwaj et al., 2018). MUSAE showed antiplatelet activity could be useful in the treatment of thrombotic disorders.

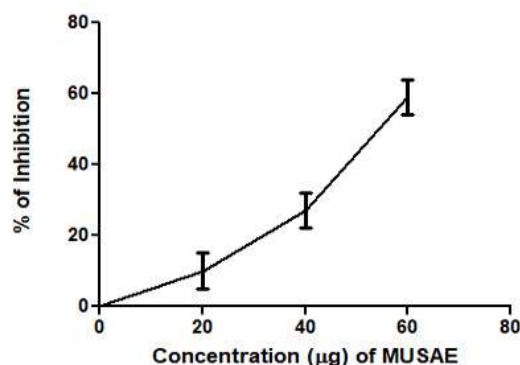
Moreover, MUSAE was non-toxic as it did not hydrolyze RBC up to the concentration of 300 $\mu$ g and it did not cause



(a)

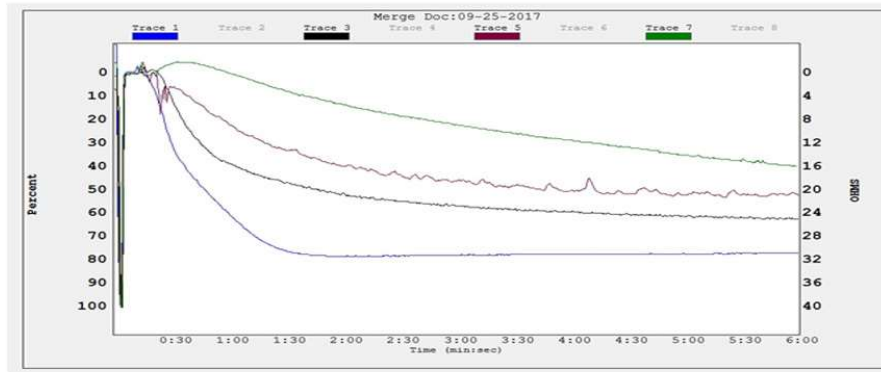


(b)

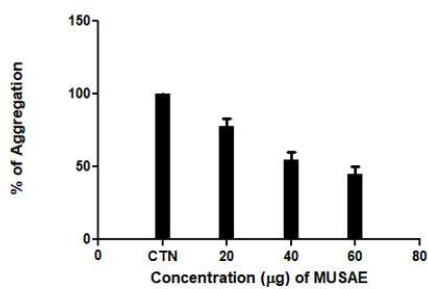


(c)

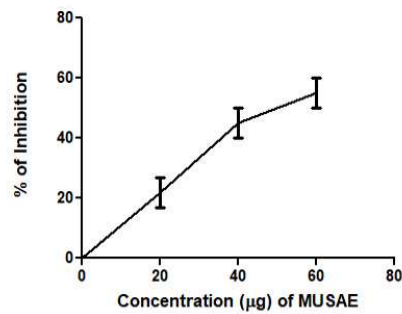
**Figure 12.** Washed Platelet aggregation was initiated by adding Collagen as an agonist. (a) Traces of platelet aggregation: Trace 1 (Collagen 5 $\mu$ M); Trace 2 (Collagen 5 $\mu$ M+20 $\mu$ g of MUSAE); Trace 3 (Collagen 5 $\mu$ M+40 $\mu$ g of MUSAE); Trace 4 (Collagen 5 $\mu$ M+60 $\mu$ g of MUSAE). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.



(a)

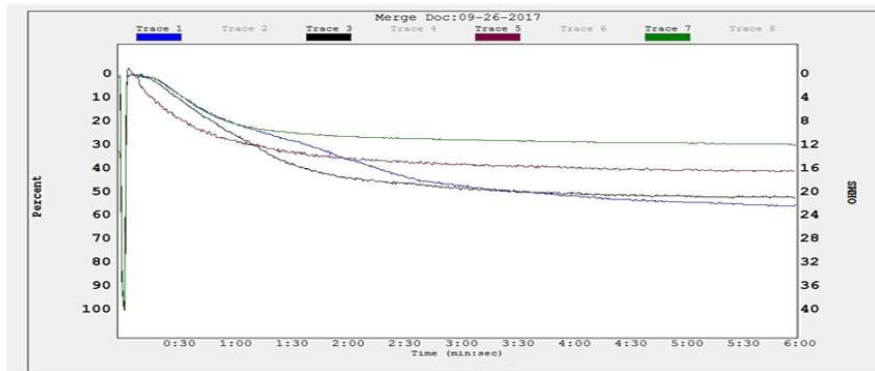


(b)

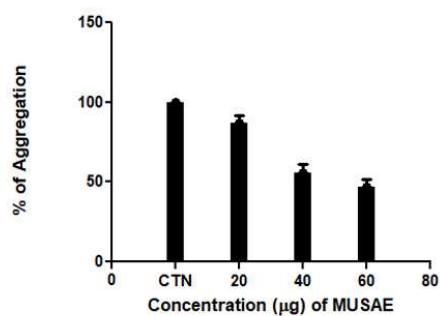


(c)

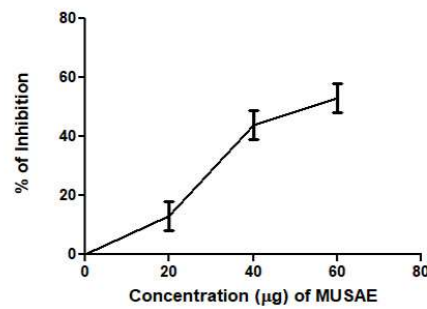
**Figure 13.** Washed Platelet aggregation was initiated by adding Arachidonic acid as an agonist. (a) Traces of platelet aggregation: Trace 1 (Arachidonic acid 5µM); Trace 2 (Arachidonic acid 5µM+20µg of MUSAE); Trace 3 (Arachidonic acid 5µM+40µg of MUSAE); Trace 4 (Arachidonic acid 5µM+60µg of MUSAE). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.



(a)

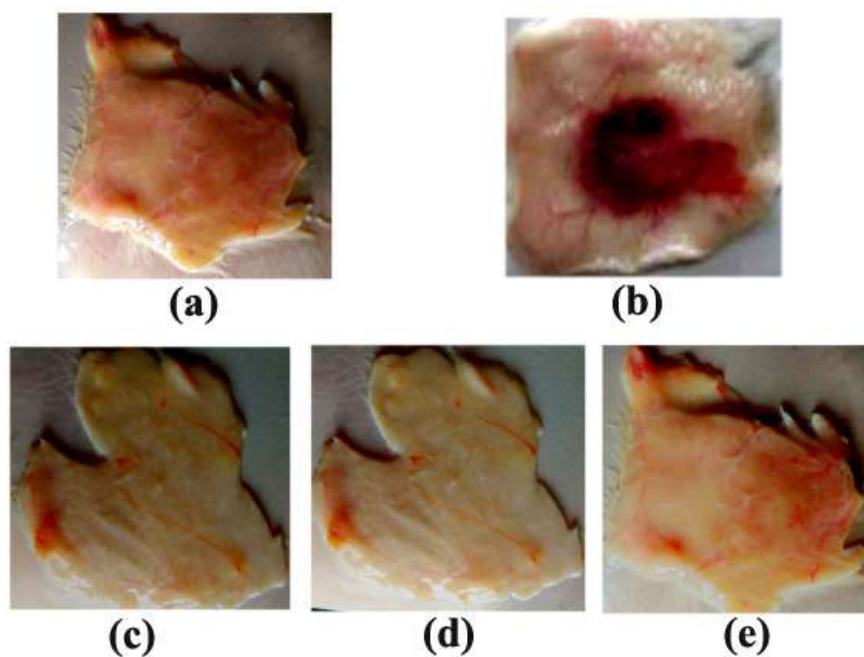


(b)



(c)

**Figure 14.** 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 MUSAE); Trace 3 (Epinephrine 5µM+40µg of MUSAE); Trace 4 (Epinephrine 5µM+60µg of MUSAE). The values represents of three independent experiments. (b) Platelet aggregation inhibition% (c) Platelet aggregation%.



**Figure 15.** Dose-dependent hemorrhagic activity of *Macrotyloma Uniflorum* seed Extract. (a) Saline, (b) positive control 2 MDH venom, (c) 50 $\mu$ g, (d) 100 $\mu$ g and (e) 200 $\mu$ g of MUSAE were injected independently into mice in a total volume of 50 $\mu$ l intradermal.

hemorrhage and edema in experimental mice up to the concentration of 100 $\mu$ g while positive control *Daboiarusselli* venom induced hemorrhage and edema in experimental mice (Figure 15). In conclusion, MUSAE exhibited anticoagulant, anti-platelet, and clot dissolving properties. Thus, purification and characterization of protease/s from MUSAE sounds interesting.

#### Acknowledgments

D.S and C.R thankful to 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.

#### Declaration of Conflict of Interest

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

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