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Research Article

EFFECT OF AQUEOUS EXTRACT OF TENDER MANGO SEED ON BLOOD COAGULATION, FIBRINO(GENO)LYTIC ACTIVITIES AND ON PLATELET FUNCTION

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ABSTRACT

This study high lights on the anticoagulant, fibrino (geno) lytic and antiplatelet activities of Aqueous Extract of raw Tender Mango Seed (RGMS). RGMS showed varied molecular weight proteins bands from the range 65kDa to 14.3 kDa on SDS-PAGE under reduced and non-reduced condition in a similar way. When, RGMS was analyzed for its probable carbohydrate content using PAS-staining, only single protein at the low molecular weight region taken up the PAS stain. RGMS digested casein with the specific activity of 1.106 units/mg/min at 37°C. The proteolytic activity was inhibited by only 1, 10, Phenanthrolene but IAA, PMSF, EDTA and EGTA did not affect the activity, suggesting the presence of zinc dependent metalloprotease. Furthermore, RGMS showed strong anticoagulant effect in plasma coagulation time by extending the clotting time from control 250 sec to 1200sec. RGMS hydrolyzed only Aα chain of the fibrinogen without affecting Bβ and γ chains. In addition, RGMS degraded all the chains of fibrin clot in a dose dependent manner. Above all, RGMS also exhibited antiplatelet activity by inhibiting agonists ADP and epinephrine induced platelet aggregation of platelet-rich plasma. The platelet aggregation inhibition was an extent of 80% 96% respectively. Moreover, RGMS was non toxic to Red Blood Cells. Taken an account of said properties, RGMS could be further exploit in the treatment of thrombotic disorders.

Keywords: Metallo protease, Anticoagulant, Fibrino(geno) lytic , Antiplatelet activity

1. INTRODUCTION

Management of life style and infectious diseases by using plant based therapeutics is an ancient practice [1]. The overwhelming interest is on natural product could be due to their high efficacy with least side effects [2]. The plant extract alone and/or in combination with other plant extract (in order to have the synergistic effect) and/or purified components have been extensively used in the treatment of said diseases [3-4]. Thus, natural products have been gaining much importance in recent time. Different parts of the plants such as fruits, seeds, barks, roots, leaf and latex have been widely used in the treatment of several diseases. The key reason could be due to the presence of plethora of phytochemicals such as proteins, lipids, carbohydrates, vitamins and secondary metabolites [5-7]. In view of this, *Mangifera indica* seed (Mango) was analyzed for its beneficial effect on thrombotic disorders.

Mangifera indica belongs to the family *Anacardiaceae* that consists of about 30 different species. It has been cultivated worldwide, while, it is the national fruit of India, Philippines and Bangladesh [8-9]. Tender Mango/fruit is consumed due to its sourness/delicacy, but the seeds have been thrown. It is to state that mango seeds are richest source of macro and micro nutrients. Several researchers explored the anti-diabetic, anti-oxidant, anti-inflammatory, anti-viral, properties of mango seeds. Antibacterial, anti-cancer, anti-fungal, antihelmintic, antiparasitic, antibone resorption, hepatoprtective, antidiarrheal, gastrprotective, immunomodulative and antipyretic [10-25] properties have also been reported from several research groups. Despite the long list of therapeutic applications of mango seed, the role of its seed proteins on thrombotic disorders are least explored.

Thrombotic disorders are due to imbalance in blood hemostasis. Hemostasis is a highly regulated physiological response which prevents blood loss during vascular injury. However, the genetic and environmental factors may imbalance the hemostatic pathway that eventually leads to thrombosis [26]. Thrombosis enhances the risk of cardiovascular/ cerebrovascular complications and represents the major health problem worldwide. For the prevention of thromboembolic disorders, Anticoagulant and antiplatelet agents play a essential role in, although the currently available anticoagulant and antiplatelet therapies have lot of constraints to offer safety against thromboembolic disorders [3, 7]. Thus, identification of target-specific anticoagulants from the natural sources could help in the better management of the thrombotic. Thus, the present study focused and evaluated the effect of Aqueous Extract of Tender Mango Seed (RGMS) on anticoagulant, fibrino(geno)lytic and antiplatelet properties .

2. MATERIAL AND METHODS

2.1. Material

Fat free casein, phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA), ethylene glycol-N, N, N', N'- tetra acetic acid (EGTA), iodoacetic acid (IAA), 1, 10, phenonthrolene were purchased from Sigma Chemicals Company.(St.Louis, USA). Molecular weight markers were from Bangalore Genei Private limited, India. All other chemicals used were of analytical grade. Chemicals Company, St. Louis, USA. UNIPLASTIN, LIQUICELIN-E and FIBROQUANT were purchased from Tulip Diagnostics Pvt. Ltd., Goa, India. All other chemicals and reagents used were analytical grade. Fresh blood sample was collected from healthy human donors.

2.2. Methods

2.2.1. Preparations of Aqueous Extract of Tender Mango Seed (RGMS) and Protein estimation

Tender Mangos were purchased from local market Tumkur and seeds were collected from the mango. The outer coat was removed, thoroughly chopped, homogenized using double distilled water and centrifuged at 5000 *g* for 20min at 4°C. The supernatant was collected and proteins were precipitated using 40% of ammonium sulphate. The precipitated protein sample was again centrifuged at 10,000 *g* for 20min; supernatant was collected dialyzed over night. The protein sample obtained was stored at -20°C until use it for the further studies. Concentration of protein was determined by Lowry method [27] using bovine serum albumin (BSA) as standards.

2.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried by the method of Laemmili [28] under both reduced and non-reduced conditions. Resolving gel (10%) was prepared by mixing 1.67ml of monomeric acrylamide solution (30% acrylamide, 0.8% N'-N' bisacrylamide made up 100ml with distilled water), 1.25ml of 4X separation gel buffer (1.5M Tris-HCl buffer, pH 8.8), 0.1ml of 10% SDS, 0.1ml of 10% ammonium per sulfate (APS) and distilled water and made up to the volume of 5 ml. The mixture was deaerated and 20µl of TEMED was added. The contents were poured into a vertical slab gel plate to form 1mm thick gel slab. Stacking gel (4.5%) was prepared by mixing 0.75ml monomeric acrylamide solution, 1.25ml 4X stacking gel buffer (0.5M Tris-HCl buffer, pH 6.8), 0.1ml of 10% SDS, and 0.1ml of 10% APS and 2.89 ml of distilled water. The mixture was deaerated and 20µl TEMED was added and mixed. The contents were overlaid on top of the resolving gel. Molecular weight markers (prestained) (14.3-200kDa) and orange seed extract, 100µg was prepared with equal volumes of reducing and non-reducing sample buffers (4% SDS, 2% Glycerol, with and without 10% β-mercaptoethanol in 0.125M Tris-HCl buffer pH 6.8) and kept in boiling water bath for 3 to 5 min. The sample was then cooled to room temperature and a suitable amount of bromophenol blue was added as a tracking dye. The mango seed extract sample was loaded into each well and electrophoresis was carried out using Tris-Glycine buffer (0.25M Tris and 0.192M Glycine pH 8.3 containing 0.1% SDS) at a constant current of 100 volts for 2hr. The gels were stained for protein with 0.1% (w/v) Coomassie brilliant blue R-250 and destained using methanol, acetic acid and water (30:10:60 v/v).

2.2.3. Anticoagulant activity

Recalcification time was determined according to the method of Quick [29] by incubating different concentration of the mango seed extract in a total volume of 0.2ml of citrated human plasma with of 10Mm Tris HCl buffer pH 7.4 for 1 min, and then clotting time was determined by adding 20µl of 0.25M $CaCl_2$.

2.2.4. Proteolytic activity

Proteolytic activity was determined according to the method of Satake [30] using 2% casein in 0.2M Tris-HCl

buffer, pH 8.5 the mango seed extract (50 μg protein) was incubated separately with 0.4mL of substrate in a total volume of 1ml for 2hr and 30 min at 37°C. Undigested casein was precipitated by adding 1.5ml of 0.44M trichloroacetic acid. The digested casein in supernatant (1ml) was determined using Folin-Ciocalteu's reagent. One unit of activity was defined as the amount of enzyme required to cause an increase in O.D. by 0.01 at 660nm/min. For inhibition studies of protease inhibitors such as 1,10, Phenonthroline, Iodo acetic acid, PMSF, EDTA and EGTA were used.

2.2.5. Fibrinolytic activity

Fibrinogenolytic activity was determined as described by Ouyang and Teng [31], EDTA (2 μg/ml) treated blood was centrifuged for 15min at 500g to separate platelet poor plasma. Plasma (100μl) was mixed with equal volume of 0.25M $CaCl_2$ for 15min at 37°C to get the soft fibrin clot. The fibrin clot was washed thoroughly 5-6 times with phosphate buffered saline (PBS) and suspended and incubated with the various concentration of mango seed extract in a final volume 40μl of 10mM Tris-HCl buffer pH 7.4 at 37°C for 5h. The reaction was stopped by adding 20μl of sample buffer containing 4% SDS, 1M urea and 4 % β-mercaptoethanol. The samples were kept on boiling water bath for 3min and centrifuged to settle the debris of plasma clot. An aliquot of 20μl supernatant was analyzed in 7.5 % SDS-PAGE for fibrin degradation study.

2.2.6. Estimation of fibrin clot-hydrolyzing activity by colorimeter

Fibrin clot-hydrolyzing activity was determined as described by Rajesh [32]. Briefly, 100ml of citrated human blood/plasma was mixed with 30 ml of 0.2mol/l $CaCl_2$ and incubated for 2h at 37°C. The clot obtained was washed thoroughly for 5-6 times with PBS and suspended in 400ml of 0.2mol/l Tris–HCl buffer (pH 8.5). The reaction was initiated by adding varied amounts of RGMS (0-30μg) in 100μl of saline and incubated for 2h and 30min at 37°C. The undigested clot was precipitated by adding 750μl of 0.44 mol/l 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 0.4 mol/l sodium carbonate and 0.25 ml of 1: 3 diluted Folin-Ciocalteu's phenol reagent. The color developed was read at 660nm 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/h at 37°C.

2.2.7. Direct hemolytic activity

Direct hemolytic activity was determined by Henkelman et, al [33] using washed human erythrocytes. Briefly, packed human erythrocytes and phosphate buffered saline (PBS) (1:9 v/v) were mixed; 1mL of this suspension was incubated independently with the various concentration of RGMS (0-200μg) for 1h at 37°C. The reaction was stopped by adding 9ml of ice cold PBS and centrifuged at 1000 × g for 10min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540nm. Activity was expressed as percentage 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.

2.2.8. Preparation of platelet rich plasma (PRP) and platelet poor plasma

Nine volumes of human blood from healthy donors (who were non-smokers and non-medicated at least for the previous 15 days) in to one volume of acid citrate dextrose (93mM sodium citrate, 7mM citric acid and 140mM glucose pH 6.5) followed by centrifugation at 90 g for 10 min at room temperature. The supernatant was called platelet rich plasma (PRP) [34]. The remaining blood was centrifuged at 500g for 15min and the supernatant obtained was the platelet poor plasma (PPP). 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 2h. All the above preparations were carried out using plastic (polypropylene tubes) wares or siliconized glass wares.

2.2.9. Platelet aggregation

The turbidometric method was followed as described by Ardlie and Han [35] for using a Chronolog dual channel aggregometer connected to an omniscrible dual pen recorder to record the light transmission as a function of time. Aliquot of PRP (0.45ml) was pre-incubated with the RGMS (0-20μg) for 3min in a cylindrical glass cuvette under constant stirring. The aggregation was initiated by the addition of agonist such as collagen, followed for 8 min along with aliquots of PPP (0.45ml). As platelets aggregate in response to an added agonist, light transmission decreases progressively producing an aggregation trace on the recorder. The aggregation trace

was the plot of light transmission between platelet rich plasma (PRP) and platelet poor plasma (PPP) base line, which represent 0% and 100% aggregation respectively.

3. RESULTS AND DISCUSSION

This study reports on the anticoagulant, fibrin (ogeno) lytic and antiplatelet activities of raw mango seed extract(RGMS).SDS-PAGE pattern of mango seed extract under non-reduced and reduced condition. The protein bands are distributed in the broad molecular weight range from 200Kda to 14.5Kda and showed low molecular weight glycoprotein (Fig.1a and b).

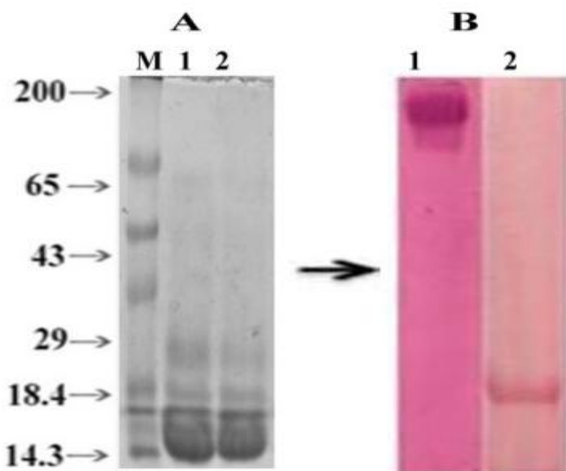

Fig. 1: (a) SDS-PAGE, and (b) glycoprotein staining

(a) RGMS as shown in SDS-PAGE (10%): 1, RGMS (100µg) under nonreduced and 2, reduced conditions. (b) PAS staining of RGMS (1 and 2) positive control fibrinogen and RGMS, respectively (2). 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). aqueous Tender mango seed extract; RGMS.

The mango seed extract hydrolyzed casein suggesting the proteolytic activity and the specific activity was found to be 1.106units/µg/min at 37°C. The proteolytic activity was inhibited by only 1,10,Phenanthrolene result showed residual activity but IAA, PMSF, EDTA and EGTA did not affect the activity suggesting presence of metallo protease in the RGMS as shown in Table 1. In order to evaluate effect on plasma recalcification was done by using platelet rich human plasma and interesting, the extract was found to interfere in plasma coagulation cascade. It found to be anticoagulant in nature as it increased the clotting time from control 250-1200sec at the concentration of 50µg (Fig.2).

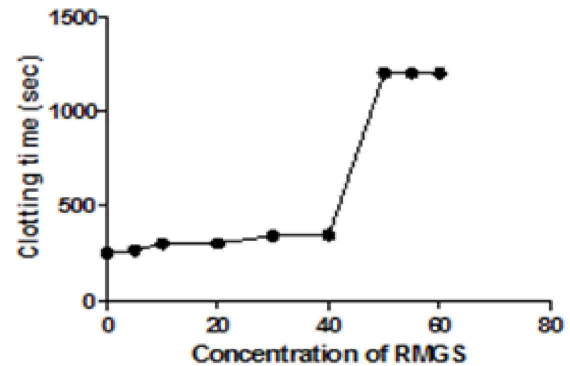

Fig. 2: Plasma Recalcification time

RGMS (0-60µg) was preincubated with 0.2ml of citrated human plasma in the presence of 20ml 10 0mmol/1 Tris-HCl buffer (pH 7.4) for 1min at 37°C. Twenty microliter of 0.25 mol/1 $CaCl_2$ was added to the preincubated mixture and clotting time was recorded

Furthermore, RGMS specifically hydrolysed Aα chain of the fibrinogen in a dose dependent manner without affecting the rest of the two chains such as, Bβ and γ in time dependent manner both Aα and Bβ was cleaved. Fascinatingly with different protease inhibitors only with 1,10, Phenanthrolene there is no hydrolysis of any chain of fibrinogen (Fig.3.a, b and c). In addition, mango seed extract hydrolysed all the subunits of fibrin clot in a dose dependent manner (Fig.4 a and b). Platelet aggregation was analyzed using agonists such as ADP and epinephrine using platelet-rich plasma. RGMS inhibited both agonists induced platelet aggregation, inhibition up to 80% for ADP and for epinephrine 96% at the concentration of 20µg (Fig.5 and 6).

All assays showed the crude Raw mango seed extract exhibited metalloprotease activity, Several metallo and serine proteases exhibits fibrin(ogen)olytic were reported from plant latex seed extract , earthworms, caterpillar, venoms of snake, spider, and honey bees [28-29]. RGMS displayed strong anticoagulation property by extending plasma recalcification time by delaying clot formation process in PRP and anticoagulant effect was completely inhibited by 1,101,10, Phenanthrolene in both in-vitro and in-vivo, suggesting the triggered anticoagulant effect is due to the metalloprotease of RGMS. Numerous potent anticoagulants with immense therapeutic applications on thrombosis were identified from earthworms, caterpillar, and venoms of snake, spider, marine creatures, herbal medicines, fermented food products like Japanese Natto, Korean Chungkook-Jang soy sauce and honey bees [36-40]. Although, the said

have been gaining much importance but yet requires validation.

To further reinforce the observed anticoagulation of RGMC, fibrinogen degradation ability could be taken into consideration.

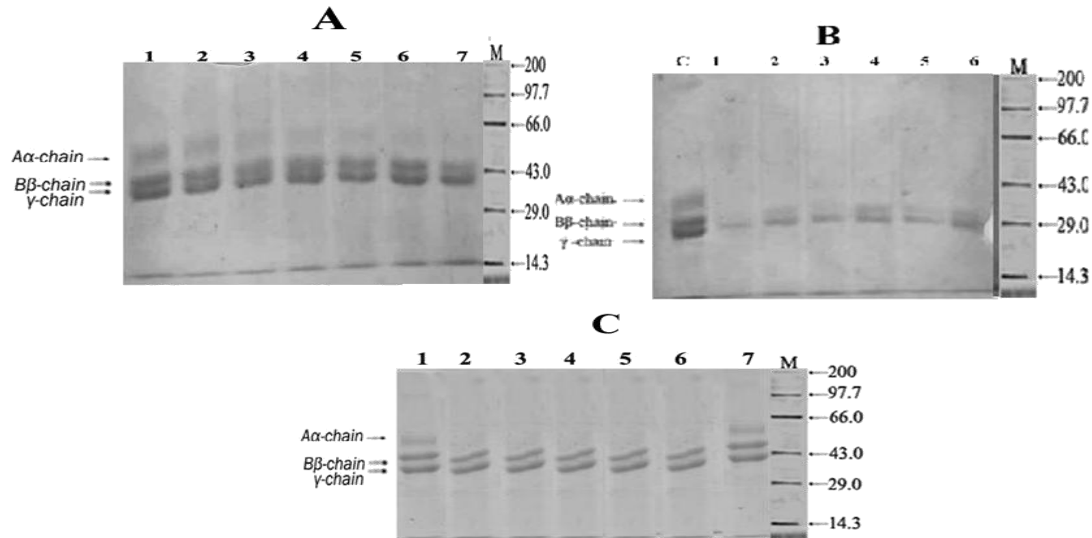

Fig. 3: Fibrinogenolytic activity

*(**a**)Fibrinogen (50 µg) incubated for 4 h at 378C and then separated on 10% SDS-PAGE under reduced condition. (a)effect: fibrinogen alone (1) and fibrinogen treated with 1µg (2), 2 0µg (3), 4 0µg (4), 8 µg (5), 12µg (6) and 20µg (7) of RGMS. (**b**) Time dependent effect: RGMS (16µg) was incubated with fibrinogen (50 µg) at 378C for 0 h (1), 2h (2), 4h (3), 8h (4), 14h (5), 18h (6) and 24h (7). (**c**) Inhibition of fibrinogenolytic activity: RGMS (2020 µg) was preincubated with and without protease inhibitors for 30 min at 37^{0}C. Further reaction was initiated by adding 50µg of fibrinogen and incubated for 4 h (a), fibrinogen alone (1), (a) and (b), 16µg RGMS (2), (a) and (b), and 5mmol/l 1,10- phenanthroline (3), (a) and (b), 5mmol/l PMSF (4), (a) and (b), 5mmol/l EDTA (5), (a) and (b) 5mmol/l EGTA (6), (a) and (b), and 5mmol/l IAA (7). 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; EGTA, ethylene glycol-N,N,N0,N0-tetra acetic acid; IAA, iodoacetic acid; , phenyl methyl sulphonyl fluoride ;,PMSF, RGMS, Raw mango seed extract*

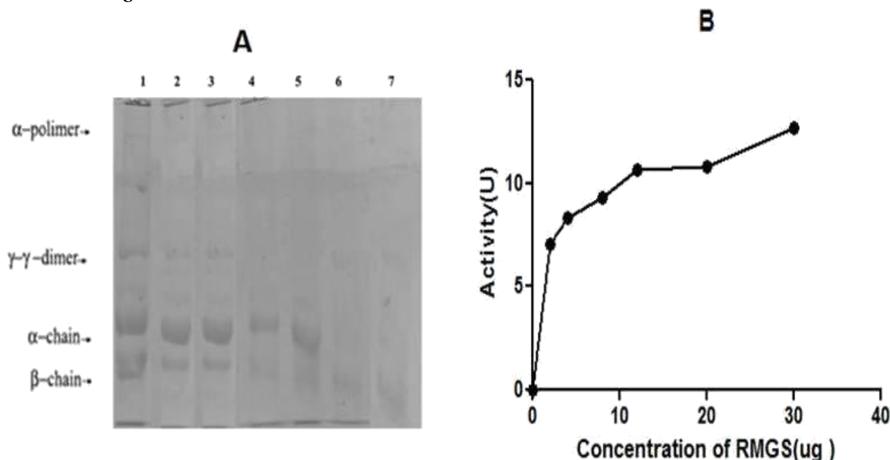

Fig. 4: Fibrinolytic activity

(a) Washed plasma clot was incubated with 0-40µg of RGMS for 2.30h and then the OD was measured at 660 nm. (b) Washed plasma clot was incubated for 6 h and then separated on SDS-PAGE (7.5%) plasma clot alone (1) and plasma clot treated with 4µg (2), 8µg (3), 12µg (4), 16µg (5), 20µg (6), and 40µg (7) of RGMS, respectively.

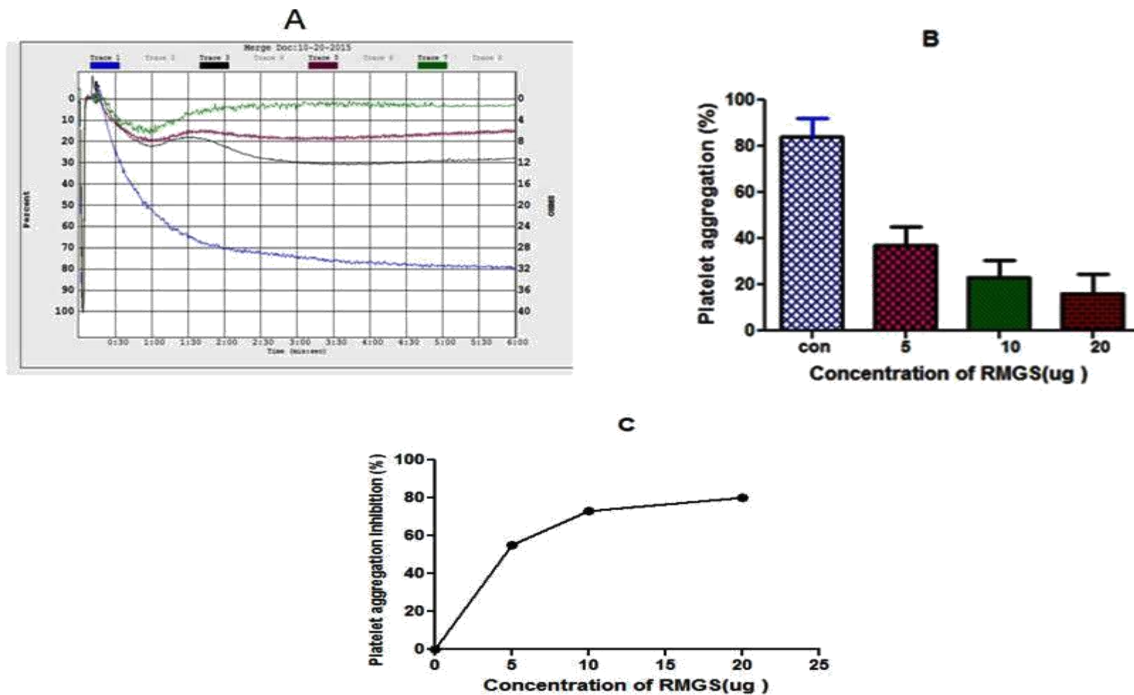

Fig. 5: Platelet aggregation was initiated by adding ADP as an agonist

(A) and (B)Traces of platelet aggregation: Trace 1 (ADP 10μM); Trace 2 (ADP 10μM +5μg of RGMS); Trace 3 (ADP 10μM +10μg of RGMS); Trace 4 (ADP 10μM +20μg of RGMS). The values represent ± SD of three independent experiments and (C) Dose dependent platelet aggregation inhibition %.

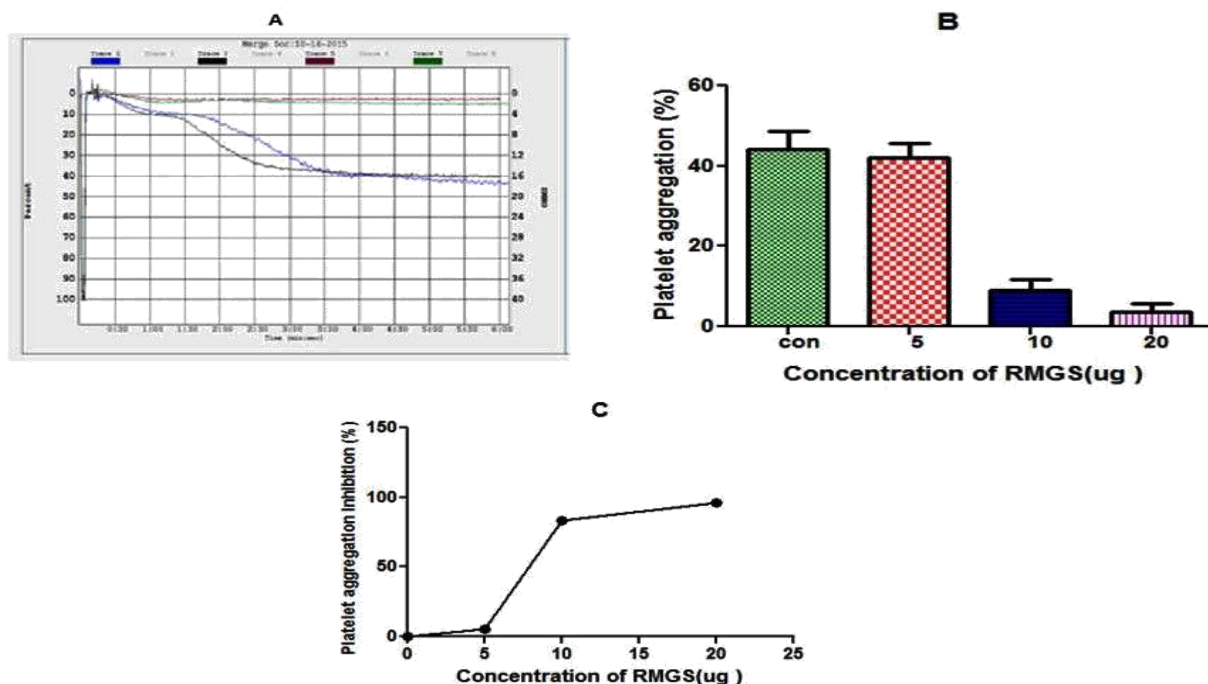

Fig. 6: Platelet aggregation was initiated by adding Epinephrine as an agonist

(A) And (B) Traces of platelet aggregation: Trace 1 (Epinephrine 10 μM); Trace 2 (Epinephrine 10μM +5μg of RGMS); Trace 3 (Epinephrine 10μM +10μg of RGMS Trace 4 (Epinephrine 10μM +20μg of RGMS). The values represent ± SD of three independent experiments and (C) Dose dependent platelet aggregation inhibition %.

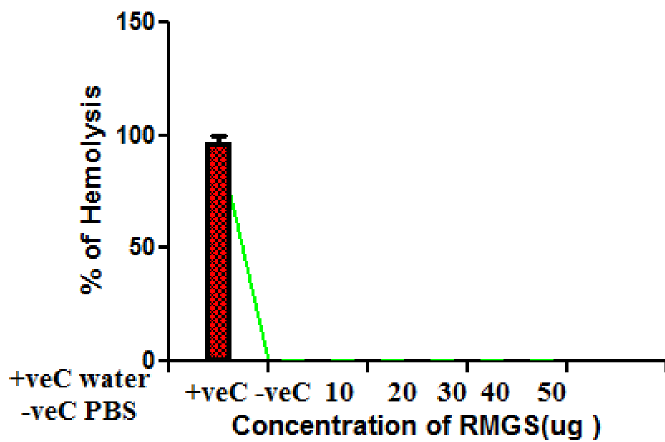

Fig. 7: Direct Hemolytic assay

Packed human erythrocytes and phosphate buffered saline (PBS) incubated with different concentration of RGMS (0-50μg) independently for 1hr at 37°C. +ve control: Water, -ve control: PBS for % of hemolysis. The amount of hemoglobin released in the supernatant was measured at 540 nm.

Table 1: Proteolytic activity with inhibitors

Inhibitors (5Mm each)	% Activity/residual activity
None	100±0.04
EDTA	96.1±0.02
EGTA	80.80±0.04
IAA	65±0.06
PMSF	56±0.03
1,10 Phenonthroline	0.12±0.03

Fibrinogenolytic ability of protease is similar to thrombin which is thrombin like enzymes. They more specifically hydrolyze Aα and Bβ chains of fibrinogen from N-terminal end and generates fibrinopeptide A and B [41]. While, proteases those degrade Aα and Bβ chains of fibrinogen from C-terminal end results in the generation of truncated structure which is short of polymerization potential, leads to the anticoagulation [42]. Fibrinogenolytic enzymes were highly investigated in plants leaf extract, edible fruit pulp latex, seeds of flax, bitter gourd and jackfruit [2, 37, 42].

In other array of RGMS showed strong impact on platelet aggregation function, Platelet activation by several physiological agonists namely, collagen, ADP, thrombin, epinephrine and platelet activating factor play a crucial role in the primary hemostasis. However, like coagulation factors hyper activation of the platelets due to genetic/environmental factors contribute equally for the thrombotic disorders. Eptifibatide, derivative from rattle snake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently using in the treatment of coagulation disorders [29, 43]. Hence, aggregation results shows that RGMS proves to be a valuable candidate in treatment of platelet disorders, in further its exact mechanism of action will be explored. Moreover, RGMS did not cause of RBC lysis, suggesting possibly nontoxic nature. Interestingly, RGMS shows to be a good therapeutic contender in the field of blood clotting/thrombotic disorders prevalence and treatments.

4. CONCLUSION

In summary, in this study an attempt was made to explore the antithrombotic, fibrinogen degradation and fibrin clot hydrolyzing activities along with antiplatelet properties of crude RGMS. Further, purification and characterization of these proteins (metalloprotease)/ Bioactive molecules appears to be interesting.

5. ACKNOWLEDGEMENTS

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Conflicts of interest

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

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