Research Paper

Anticoagulant and Fibrinogenolytic Properties of Seed Extract of *Psidium guajava*

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The objective of this study was to identify and evaluate for the first time the anticoagulant and fibrinogenolytic protease from *Psidium guajava* seed extract. The *Psidium guajava* seed extract in sodium dodecyl sulfate polyacrylamide gel electrophoresis showed protein bands in high molecular weight region between 122 kDa and 47 kDa and two distinct bands in the lower molecular weight region at ~16.6 kDa and ~14.3 kDa. The protein content in *Psidium guajava* seed extract was found to be 1 mg/ml with protease specific activity 0.8 U. The proteolytic activity was supported by zymography studies using casein/gelatin as substrates. *Psidium guajava* seed extract hydrolysed all the subunits of fibrinogen and alpha-polymer and alpha-chain subunits of fibrin at a concentration of 20 µg and 120 µg, respectively. *Psidium guajava* seed extract prolonged the clotting time in recalcification time from control 161 s to 487 s, increasing by ~3 folds. This indicates the anticoagulant nature associated with *Psidium guajava* seed extract. Our study provides for the first time, the scientific validation for the presence of a protease with anticoagulant and fibrinogenolytic property from *Psidium guajava* seed extract.

Key words: *Psidium guajava*, fibrinogen, hypertension, diabetes, anticoagulant

Stroke is an immediate pause in the supply of blood to the brain due to the blockage of cerebral artery with thromboembolic occlusion[1]. The causes of thrombosis are multifactorial. The thrombus formation occurs by the activation of blood coagulation factors during vascular injuries or imbalance in endogenous anticoagulants[2–5]. Stroke can be divided into two categories: ischemic and hemorrhagic. Majority of the stroke caused leads to cerebral infarction, venous thrombosis and embolism[6,7]. The most common causes of cerebral infarction are hypertension, diabetes, hyperlipidaemia, smoking and genetic diseases[8].

The possible contributors for these vascular complications during these disease conditions are found to be majorly platelet hypersensitivity, endothelial cell dysfunction and imbalance in blood coagulation mechanisms[9–11]. Blood coagulation is a complex cascade of enzymatic reactions, which arrest the blood from bleeding; while, thrombolysis/ fibrinolysis is the process of dissolution of clot by the degradation of fibrin into soluble fragments[12]. Under normal hemostasis condition, there is balance between blood coagulation and fibrinolysis. But, when there is pathological abnormality under disease condition the imbalance is observed, which may lead to thrombus formation[13].

Antithrombotic drugs such as anticoagulants, antiplatelet and fibrinolytic drugs either target procoagulant factors or dissolve the thrombus formed. These are used as prophylaxis or treatment of arterial and venous thrombosis[14-16]. Currently used anticoagulants are heparin, warfarin and their derivatives. Antiplatelet drugs that are currently used are aspirin, clopidogrel and abciximab[16-19]. Fibrinolytic agents are proteases which have potential to degrade thrombi directly or indirectly. Proteases acting on thrombi directly are plasmin,

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brinase and trypsin; while, protease dissolving thrombi indirectly is Tissue Plasminogen Activator (t-PA)[20–23]. The major concerns against using these thrombolytic drugs are excessive bleeding, anaphylactic reactions, heparin-induced thrombocytopenia and toxicity[24–28]. Hence, currently there is lot of interest in the use of natural products as the new thrombolytic drugs with more potency, safety, chemical stability which is devoid of risk of hemorrhage and toxicity from natural sources.

Medicinal plants are enormous and important source for the indigenous medical systems[29]. *Psidium guajava* (*P. guajava*), belongs to family Myrtaceae, commonly called as 'guava', is a small medicinal tree native to South America but extends throughout the European, African and Asian continents[30]. *P. guajava* is used as both nutraceutical (fruit) and medicinal (leaves, bark, seeds, roots) agents in traditional formulations[31]. *P. guajava* is mostly grown for the food industry to produce candies, juices, jams and frozen pulp[32]. The decoctions or pastes of whole plant or shoots are used to treat skin ailments and as astringents in dysmenorrhea, miscarriages, uterine bleeding and premature labor. The bark is used as an astringent to treat of ulcers wounds and diarrhea. The leaf extracts are used as febrifuge, antispasmodic, to treat viral disease and for rheumatism[33]. The seeds are associated with antimicrobial, gastrointestinal and anticarcinogenic activities. The different extracts of the parts of the tree are known to possess antimicrobial, hypoglycaemic, anti-inflammatory, anti-hypertensive, gastroenteritis, dysentery, conjunctivitis, constipation and antitumour effect[31,34-36]. In addition, it is used to treat hyperlipidemia, diabetes mellitus, cardiovascular diseases, liver disorders and parasitic infection[37]. Previous studies have shown that *P. guajava* inhibits cholesterol levels and incidence of stroke[32]. But the mechanism of inhibition of stroke is not clearly depicted. Different parts of the plants have been used in traditional system of medicines. But there is no report for the presence of protease in the seeds of *P. guajava* fruit. This is the first report on protease associated with anticoagulant and fibrinolytic activity from the seeds of *P. guajava* fruit.

MATERIALS AND METHODS

Materials:

Casein, gelatin and human fibrinogen were purchased from Sigma-Aldrich chemicals, St. Louis, MO, USA. Liquicellin and uniplastin was procured from Tulip Diagnostics Pvt. Ltd, Goa, India. All other reagents were of analytical grade.

Sample collection:

Pink *P. guajava* fruits were purchased from local market. The fruits were cut and the seeds were taken out of the fruit part. The collected seeds were washed with distilled water, wiped with filter paper and then shade dried at room temperature for 3 to 4 d. The dried seeds were then pulverized into fine powder.

Preparation of extract:

The finely powdered seeds were weighed using digital balance and extracted with sodium phosphate buffer (10 mM, pH 7.0) in the ratio 1:25 on a magnetic stirrer at 500 rpm for about 24 h at room temperature. The *P. guajava* Seed Extract (PgSE) was collected and stored at -20° until further use.

Electrophoresis:

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as described by Laemmli[38],. Briefly, PgSE (160 µg) was treated with non-reducing sample buffer and incubated in boiling water bath for 3 min. The sample was then loaded onto 12 % SDS-PAGE along with standard molecular weight markers ranging from 7 to 175 kDa and electrophoresis was performed. The gel was stained with 0.25 % Coomassie brilliant blue R-250 to visualize the protein bands.

Zymography:

Zymography was carried out according to the method of Laemmli[38]. Briefly, the 12 % gel was incorporated with 0.2 % casein and gelatin separately as substrate for the detection of proteolytic activity. The PgSE (80 µg) in 10 mM sodium phosphate buffer (pH 7.0) was incubated with non-reducing sample buffer at 37° for 30 min and electrophoresis was carried out. After electrophoresis, the gels were washed with 2.5 % of Triton X-100 for 1 h to remove SDS. The gels were incubated overnight in incubation buffer containing Tris–HCl (50 mM, pH 7.6, 10 mM calcium chloride ($CaCl_2$) and 150 mM Sodium Chloride (NaCl)). The gels were then stained with 0.25 % Coomassie brilliant blue R-250 to observe the activity bands.

Protease activity:

Protease activity was performed as described by Satake *et al*.[39], using casein (2 % in 200 mM Tris–HCl buffer, pH 7.0) as substrate. Briefly, 0.4 ml of casein was incubated with different concentration of PgSE (0-300 µg) in 10 mM sodium phosphate buffer (pH 7.0)

at 37º for 2.5 h. About 1.5 ml of Trichloroacetic acid (TCA) (0.44 M) was added to terminate the reaction and allowed to stand for 30 min at room temperature. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant (1 ml) was mixed with 0.4 M sodium carbonate (2.5 ml) and 1:2 diluted Folin reagent (0.5 ml). The colour developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase in the absorbance of 0.01 at 660 nm. Protease activity was expressed as units/min/mg.

Fibrinogenolytic activity:

Fibrinogenolytic activity was performed as described by Gubbiveeranna *et al*.[13]. Briefly, human fibrinogen (50 µg) was treated with different concentration of PgSE (0.5–20 µg) in 25 µl of 10 mM sodium phosphate buffer (pH 7.0) and incubated at 37° for 2.5 h. Reducing sample buffer (10 µl) containing 1M urea, 4 % SDS and 4 % beta (β)-mercaptoethanol was added to terminate the reaction and kept in boiling water bath for 3 min. The hydrolyzed products were analyzed in 12 % SDS-PAGE and visualized by staining with Coomassie brilliant blue R-250.

Fibrinolytic activity:

Fibrinolytic activity was carried out according to the method of Shivaiah and Kempaiah[40]. Briefly, trisodium citrate (3.2 %) treated blood in the ratio 1:9 was centrifuged at 3000 rpm for 5-10 min. The supernatant obtained was separated and used as Platelet Poor Plasma (PPP). Equal volume of PPP (100 µl) and 25 mM $CaCl_2$ (100 µl) was incubated at 37º to get fibrin clot. The clot formed was thoroughly washed with 10 mM sodium phosphate buffer (pH 7.0) for 5-6 times. The washed fibrin clot was incubated with different concentration of PgSE (15 to 120 µg) in a total reaction volume of 40 µl of 10 mM sodium phosphate buffer (pH 7.0) at 37º for 2.5 h. Reducing sample buffer (20 µl) containing 1M urea, 4 % SDS and 4 % β-mercaptoethanol was added to terminate the reaction and kept in boiling water bath for 3 min. An aliquot (20 µl) of the supernatant was subjected to 10 % SDS-PAGE to analyze fibrin hydrolyzing pattern.

Recalcification time (RT):

Plasma recalcification time was determined according to the method described by Gubbiveeranna *et al*.[13]. Briefly, PPP (100 µl) was pre-warmed to 37º before use and incubated with different concentration of PgSE (0-80 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37° for 5 min. Later, 100 µl of 25 mM $CaCl_2$ was added and the clotting time was recorded. The 10 mM sodium phosphate buffer (pH 7.0) alone without PgSE was considered as negative control.

Protein estimation:

The protein concentration was estimated as described by Lowry *et al*.[41]. Briefly, bovine serum albumin (BSA) was used as standard and the protein concentration of PgSE was determined by comparing with known concentration of BSA.

Statistical analysis:

The experiments were performed in triplicates and the data obtained from the experiments were expressed as mean±standard error of mean (SEM). The results were statistically analysed using one–way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. The data were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

The PgSE (160 µg) was treated with non-reducing sample buffer and incubated in boiling water bath for 3 min. The sample was then loaded onto 12 % SDS-PAGE under non-reducing condition. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 to visualise the protein bands. PgSE exhibited dense bands in the high molecular weight region between 122 kDa and 47 kDa. Two distinct bands were seen in the lower molecular weight region at ~16.6 kDa and ~14.3 kDa (fig. 1).

PgSE was evaluated for proteolytic activity using 2 % casein as substrate. It showed a specific activity of

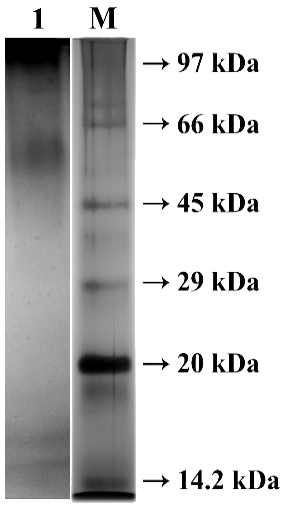

Fig. 1: Protein banding profile of PgSE, (lane 1) PgSE (160 µg); (M) molecular weight markers

0.8 U/mg/ml. Casein (0.2 %) and gelatin (0.2 %) were copolymerized with the polyacrylamide gel separately for the detection of proteolytic activity. PgSE (80 µg) was incubated with non-reducing sample buffer at 37º for 1.5 h and loaded onto 12 % SDS-PAGE under non-reducing condition. After electrophoresis, gels were washed with 2.5 % of Triton X-100 for 1 h to remove SDS. The gels were incubated overnight in incubation buffer containing Tris–HCl (50 mM, pH 7.6, 10 mM $CaCl_2$ and 150 mM NaCl). Gels were then stained with 0.25 % Coomassie brilliant blue R-250 to visualize the activity bands. The bands were observed at molecular weight region ~97 kDa and between 34 kDa and 18.2 kDa for caseinolytic activity. Similarly, translucent activity bands in the molecular weight region ~96.7 kDa and between 52.3 kDa and 24.9 kDa were seen for the gelatinolytic activity (fig. 2).

PgSE was studied for fibrinogenolytic activity using human fibrinogen, which is a 340 kDa soluble plasma glycoprotein, composed of three subunits (Aα, Bβ and γ). Fibrinogen plays a crucial role in arresting blood during vascular injury by getting converted to fibrin upon action of thrombin. The fibrin is subsequently converted to fibrin-based blood clot. PgSE hydrolysed all the chains (Aα, Bβ and λ) of fibrinogen in a dose dependent manner. The fibrinogen (50 µg) was incubated with different concentration of PgSE (0.5 µg to 20 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37° for 2.5 h. The reaction was terminated by adding denaturing sample buffer containing 1 M urea, 4 % SDS and 4 % β-mercaptoethanol and kept in boiling water bath for 3 min. SDS-PAGE (12 %) was performed in reducing condition to visualize degradation pattern. After electrophoresis, the gel was stained with 0.25 % Coomassie brilliant blue R-250 (fig. 3).

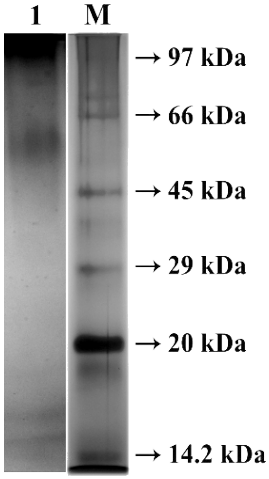

Fig. 2: Zymographic studies of PgSE, (lane 1) PgSE (160 µg); (lane 2) caseinolytic zymogram; (lane 3) gelatinolytic zymogram; (M) molecular weight markers

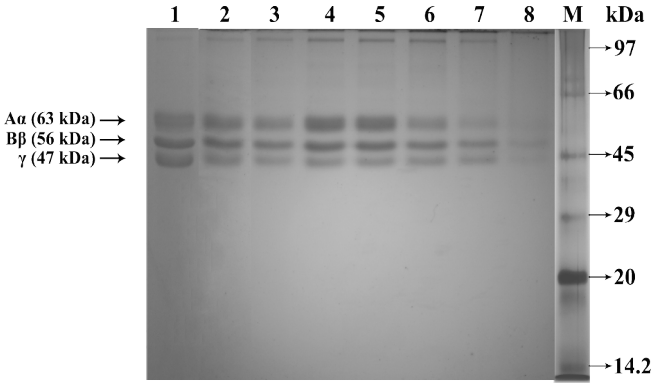

Fig. 3: Concentration dependent fibrinogenolytic activity by PgSE, (lane 1) fibrinogen (50 µg); (lane 2-7) fibrinogen incubated with 0.5, 1, 2, 4, 6, 8, 12, 16 and 20 µg of PgSE, respectively; (lane M) standard molecular weight markers

The fibrinolytic activity of PgSE was studied using human fibrin. PgSE hydrolyzed fibrin clot in a dose dependent manner. α-polymer and α-chain subunits of fibrin were hydrolysed at 90 µg followed by partial degradation of λ-dimer and β-chain subunits at a concentration of 120 µg. Equal volumes of PPP (100 µl) and 25 mM $CaCl_2$ (100 µl) were mixed and incubated to get fibrin clot. The fibrin clot was washed in 10 mM sodium phosphate buffer (pH 7.0) and incubated with different concentration of PgSE (15 µg to 120 µg) in 10 mM sodium phosphate buffer (pH 7.0) at 37° for 2.5 h. The reaction was terminated by adding denaturing sample buffer containing 1 M urea, 4 % SDS and 4 % β-mercaptoethanol and kept in boiling water bath for 3 min. The sample was loaded onto 10 % SDS-PAGE in reducing condition. After electrophoresis, the gel was stained with Coomassie Brilliant blue R-250 to visualize the bands (fig. 4).

PgSE which was found to contain protease associated with fibrinogenolytic and fibrinolytic activity showed anticoagulant activity upon incubation with PPP. PgSE increased the recalcification time in a dose dependent manner indicating its anticoagulant property. PgSE increased the clotting time from control 161 s to 487 s at a concentration of 80 µg. The PgSE increased the clotting time by 3.03-folds (fig. 5).

Pathophysiological conditions associated with hypertension, diabetes, inflammation, hypercholesterolemia, genetic diseases and metabolic syndrome reported to disturb the hemostatic and fibrinolytic mechanisms by abruptly activating blood coagulation, hypofibrinolysis and platelet hyperaggregation[42]. With the new research area focusing on identifying new anticoagulants, much attention has been focused on potent anticoagulants from the natural source particularly from medicinal

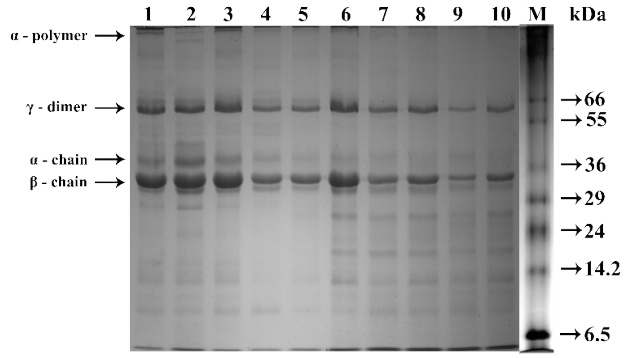

Fig. 4: Concentration dependent fibrinolytic activity by PgSE, (lane 1) control (fibrin clot); lane (2-9) fibrin clot incubated with 15, 30, 45, 60, 75, 90, 105 and 120 µg of PgSE, respectively; (lane M) standard molecular weight markers

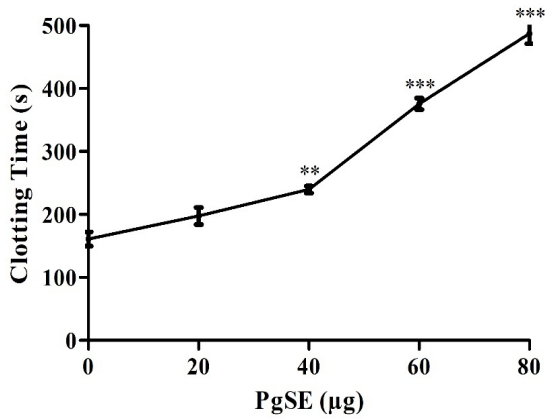

Fig. 5: RT of PgSE, PPP (100 µl) was incubated with different concentration of PgSE (20, 40, 60 and 80 µg) in a total volume of 20 µl of sodium phosphate buffer (10 mM, pH 7.0) at 37° for 5 min. Then, 100 µl of 25 mM $CaCl_2$ was added to initiate clotting and the clotting time was recorded. The 10 mM sodium phosphate buffer (pH 7.0) alone without PgSE was taken as control. Values are presented as means±SEM. **p<0.01, ***p<0.001, analysed by one-way ANOVA, followed by Tukey multiple comparison testing

plant extracts with more efficiency, safety, chemical stability and devoid of side effects.

Several medicinal plant extracts have been reported to exhibit anticoagulant activity[10]. Compounds such as chitosan, glucan, polysaccharides, proteins, sulfated flavonoids, tannins, tea catechins and triterpenes have been reported to show anticoagulant activity and/or antithrombotic effects[43]. *P. guajava* is traditionally used for various therapeutic purposes. Studies have reported that *P. guajava* leaf extract showed significant anticoagulant activity associated with protective effect on antithrombin III activity[44]. The leaf extract has been shown to contain three phenolic compounds gallic acid, ferulic acid and quercetin. The anticoagulant nature of the leaf extract has been attributed to these phenolic compounds[45]. Hydroalcoholic leaves extract of *P. guajava* and its bioactive fractions showed antithrombocytopenic activity. The analysis showed the presence of phenolic and flavonoid compounds such as quercetin, ellagic acid and kaempferol[37].

In this study, we have analysed PgSE for the presence of protease and its effect on fibrinogen, fibrin and blood coagulation cascade to evaluate its anticoagulant property. PgSE showed protein bands in high molecular weight region between 122 kDa and 47 kDa and two distinct bands in the lower molecular weight region at ~16.6 kDa and ~14.3 kDa as analysed by SDS-PAGE. The protein concentration in PgSE was found to be 1 mg/ml. PgSE showed a specific activity of 0.8 U/mg/ml using casein as substrate. The proteolytic activity was supported by zymography studies using casein and gelatin as substrates. In zymography studies, PgSE hydrolyzed casein at molecular weight region ~97 kDa and between 34 kDa and 18.2 kDa. The degradation of gelatin was observed in the molecular weight region ~96.7 kDa and between 52.3 kDa and 24.9 kDa.

Plant proteases are involved in variety of physiological processes in plant life such as regulated cell death, photosynthesis, immune response, embryogenesis, remodelling of extracellular matrix and regulation of signal transduction[46-48]. Plant proteases have been extracted in aqueous solution from different parts of the plants such as flowers, seeds, roots and leaves[5]. Plant proteases have high stability, unique substrate specificity and wide pH range for enzyme activity[49-51]. Hence, proteases isolated from plants have been used to treat diverse diseases. Some of them exhibit antitumor, antimicrobial and antifungal activities[50]. The plant proteases have been widely used in wound healing, digestion disorders, burn debridement and blood coagulation, thrombolysis process and oral healthcare[52].

PgSE showed fibrinogenolytic activity and degraded the subunits of fibrinogen and fibrin. The degradation is dose dependent manner. PgSE hydrolyzed Aα, Bβ and γ chains of fibrinogen at a concentration of 20 µg while the fibrin subunits (α-polymer and α-chain) were degraded at a comparatively high concentration of 120 µg. γ-dimer and β-chains of fibrin were resistant for hydrolysis. The α-polymer and α-chain are more susceptible for degradation compared to the γ-dimer and β-chains of fibrin. PgSE hydrolyzed the cross-linked fibrin subunits. Fibrin together with platelet aggregate stabilizes the platelet plug, which stops bleeding from vascular injuries. Anticoagulant plant proteases have been studied which increases the plasma coagulation

time. These proteases digest fibrinogen in different pattern; some hydrolyse fibrinogen completely while others show partial hydrolysis. Most of the proteases also showed fibrinolytic activity and ability to lyse blood clots. Some of the proteases are Kitamase from *Aster yomena*, protease from *P. japonicas*, Hirtin from *Euphornia hirta*, Codiase from *Codium fragile* and protease from *Costaria costata*[52].

PgSE interfered in the blood coagulation cascade as analysed by RT. PgSE, at a concentration of 80 µg, prolonged the clotting time in RT from control 161 s to 487 s, increasing the RT by ~3.03 folds. This clearly indicates the anticoagulant nature associated with PgSE. The increase in clotting time of plasma in RT method indicates the interference of PgSE in common pathway of blood coagulation.

In conclusion, PgSE exhibited proteolytic activity degrades fibrinogen, fibrin and inhibited blood coagulation cascade indicating its interference in thrombus formation. Our study provides for the first time, the scientific validation for the presence of a protease with anticoagulant property from PgSE. This study provides an opportunity to identify, isolate and characterize the active principle from PgSE having clinical significance and decipher its molecular mechanism.

Conflicts of interest:

There is no conflict of interest to disclose.

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