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Elettaria Cardamomum Seed Extract Exhibits Antioxidant and Procoagulant Activities

Research Article

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Abstract

Elettaria cardamomum seeds stores robust phytochemicals responsible for several therapeutic efficacy, hence it is a rich source of useful components that has medicinal properties and one of the main applications is in controlling bleeding. Thus, current study investigates the antioxidant, procoagulant and clot fibrinolytic efficiency of *Elettaria cardamomum* Seed Aqueous Extract (ECSAE). The antioxidant potential of the extract was assessed by antioxidant activity using 2, 2 - diphenyl, 1- picryl hydrazyl (DPPH) radical scavenging activity. The ECSAE was found to have 63.00% of DPPH scavenging activity with an IC50 value of 45.48µg/ml compare to positive control ascorbic acid. Preliminary phytochemical screening was analyzed by instrumentation method such as RP-HPLC and GC-MS. The protein blue prints of ECSAE revealed similar banding pattern from 15-200kDa on 10% SDS-PAGE suggested that ECSAE reserves only monomeric proteins. ECSAE exhibited proteolytic activity by degrading casein. The specific activity was fond to be 0.27 units/mg/min at 37°C. The proteolytic activity was strongly inhibited by both PMSF and IAA. While, EDTA and 1,10 Phenanthroline did not show inhibition, suggesting the presence serine and cysteine protease in the seeds. ECSAE showcased procoagulant effect as reduced the clotting time of PRP from control 214.2s to 94.2s which promotes blood coagulation. ECSAE found to hydrolyze fibrin clot with specific activity of 1.1 to 4.5units in a dose dependent manner. Furthermore, ECSAE did not hydrolysis the RBC suggested its non-toxic properties.

Keywords: *Elettaria Cardamomum*; Protease; Antioxidant; Procoagulant

Introduction

Haemostasis, coagulation, and fibrinolysis systems are those that are part of the physiological system that maintain the human body functioning normally. By restricting one another, the haemostatic system preserves homeostasis. When deviations in a single system's operation may result in bleeding or thrombosis, which may then lead to a number of illnesses, including vascular disease and haemophilia [1]. A class of inherited hemorrhagic diseases with coagulation issues is known as haemophilia. The dysfunction of active thrombozyme, the extension of the coagulation time, the enduring propensity to bleed following small trauma, and "spontaneous" bleeding in severe individuals who have not experienced evident trauma are the common hallmarks of haemophilia[2]. Haemophilia is mostly treated with local haemostatic therapy and other therapies; nevertheless, there are notable side effects, including an increased risk of hepatitis, haemolysis, and acquired immunodeficiency sickness (AIDS), and a poor cure rate. Vasoconstriction and platelet response, coagulation and anticoagulation systems, and the fibrinolytic system are the three interrelated components of the complicated physiological, biochemical, and pathological process that makes up normal physiological procoagulant and anticoagulant mechanisms [3]. The

body's ability to coagulate normally is mostly based on the whole vascular wall's structure and function, the quantity and quality of functional platelets, and the regular activity of plasma coagulation factors. Anticoagulation and fibrinolytic systems also have an impact on the coagulation and anticoagulation systems [4]. Numerous studies have been conducted on medications that activate clotting factors and promote platelet coagulation blood to successfully halt bleeding but their use is restricted by the side effects[5]. Nowadays, procoagulant-active natural compounds are a major topic in research. Medicinal plants are a massive and vital source of knowledge for indigenous medical systems. All most all part of plants have been using for therapeutic application but seeds from the plants have been least explore. One of such medicinal plant is perennial (*Elettaria cardamomum*). Seeds of *Elettaria cardamomum*are cardamom [5]. It is belonging to the ginger family (*Zingibaraceae)*. Cardamom is a valuable spice originates from the coastal area of India. Occurring in Guatemala, Tanzania, Sri Lanka, El Salvador, Vietnam, Laos and Cambodia [6]. India is the chief exporter of dried cardamom. α-terpineol, myrcene, heptane, subinene, limonene, cineol, menthone, α-pinene, β-pinene, linalol, nerolidol, β-sitostenone, phytol, eugenyl acetate, bisabolene, borneol, citronellol, geraniol, geranyl acetate, stigmasterol and terpinene were found in cardamom [7]. In this connection, the seeds such *as Azadirachta indica, Allum cepa, Elettaria cardamomum, Myrista fragrans, Brassica rapasubsp rapa*seed extracts were screened for various biological activity. However, *Elettaria cardamomum* seed extract exhibited procoagulant activity. Thus, *Elettaria cardamomum* seeds have been selected for further examinations.

Materials and methods

Phenyl Methyl Sulphonyl Fluoride (PMSF), Ethylene Di-amine Tetra-acetic Acid (ED- TA), Iodo-Acetic Acid (IAA) and 1,10-Phenanthroline were purchased from Sigma chemicals company (St. Louis, US- A). Molecular weight markers were from Bangalore Genie Private Limited, India. Fresh human blood was collected from healthy donors for Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP).

Preparation of *Elettaria cardamomum* Seed Aqueous Extract (ECSAE)

Elettaria cardamomum seed were collected from were purchased from local provisions store of Bangalore. Seeds were separated from coat and 20% homogenized in distilled water and centrifuged at 5000rpm for 20min at 15°C. Then, the supernatant was subjected to ammonium sulfate precipitation (30 %) and spun at 1500g for 20min. Later, the pellet was mixed with water and kept for dialysis overnight. The protein sample was stored at -20°C until further use. Protein concentration was determined as described by Lowry's et al. [8]. Using Bovine Serum Albumin (BSA) as standards.

Reverse Phase High Performance Liquid Chromatography analysis

ECSAE was subjected to RP-HPLC using C18 column (150mm×4.60mm, particle size 5µm) with PDA detector in shimadzu LC-20AD prominence. The column was pre-equilibrated with 0.1% Trifluoroacetic acid (TFA) in water and it was eluted at the flow rate of 1ml/min in linear gradient mode.

GC-MS

GC-MS analysis of samples was analysed on quadrupole mass spectrometers in the electron capture negative-ion chemical ionization (ECNICI) mode with capillary column (30X0.25mm IDX1EM df, composed of 100% Dimethyl poly siloxane). Helium (99.9%) gas was used as carrier gas at the flow rate of 1ml/min and the injection volume of 0.5 El (split ratio of 10:1). Temperature program was set as follows, injector temperature 250°C; ion-illuminator temperature 280°C, oven temperature 110°C (isothermal for 3min) with an increase in temperature of 20°C/min to 220°C, thereafter 5°C/min to 300°C. Mass spectrum was taken at 80ev; a scan interval of 0.5s [12].

Determination of the antioxidant activity by the 1,1-diphenyl-2-picrylhydrazyl assay

The DPPH radical scavenging activity was measured according to the method of Okoh et al. [9]. An aliquot of 50–200mg of ECSAE was made up to 600ml of (0.3mM) DPPH, which was dissolved in 95% ethanol. The mixture was shaken and incubated for 30min in a dark at room temperature. Then, the absorbance of the mixture was recorded at 517nm. Ethanol was used as blank and ascorbic acid (1mg/ml) was used as a positive control. The scavenging activity was calculated by the following formula: DPPH scavenging activity (%) = Absorbance of blank-Absorbance of sample X 100 Absorbance of blank. The antioxidant activity of ECSAE was expressed as IC50. The IC50 value was defined as the concentration (mg/ml) of the extract required for inhibiting the formation of DPPH radical by 50%.

Ammonium sulphate precipitation of ECSAE and Protein estimation

The prepared extract was further used for the precipitation of proteins and proteins were precipitated using 30% of ammonium sulphate. Precipitated proteins were again centrifuged at 5000rpm for 2min, pellet was subjected to dialyzed overnight. The protein sample obtained was stored at -20°C in freeze condition until further use. This extracted protein sample was used throughout the study and referred as ECSAE. Protein concentration was determined as described by Lowry's et al. [9]. Using Bovine Serum Albumin (BSA) as standards.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel electrophoresis (SDS-PAGE)

10% SDS-PAGE was assayed based on the method described by Laemmli [10]. ECSAE crude (100µg) was treated in both reduced and non-reduced reagents and electrophoresis was performed by using Tris (25mM), glycine (192mM) and SDS (0.1%) for 2h at room temperature. Upon that gels were stained with 0.1% Coomassie brilliant blue R-250 thereafter de-stained with 40% ethanol in 10% acetic acid and water (40:10:50 v/v) to visualize the protein bands. Molecular weight standards were used from 125kDa to 24kDa.

Proteolytic activity

Analysis of proteolytic activity was assayed based on the method described by Satake et al., [11]. ECSAE crude 80µg was incubated

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with fat-free casein (0.4ml, 2% in 0.2M Tris–HCl buffer, pH 7.6) at 37°C for 2h and 3min. Add 1.5ml of 0.44M Tri-chloro Acetic acid (TCA) in order to precipitate undigested casein and allowed to stand for 30min. Thereafter centrifuged at 2000g for 10min. Upon that add 2.5ml of 0.4M sodium carbonate and 0.5ml of Folin–Ciocalteu's reagent (1:2) to 1ml of the supernatant and the color developed were measured 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 at 37°C. The specific activity was expressed as units/min/mg of protein. The inhibition studies was performed independently by pre-incubating the crude ECSAE (80µg) for 30min with 100mM each of EDTA, 1,10-phenanthroline, PMSF and IAA.

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

PRP and PPP were prepared based on the method described by Ardlie and Han [12]. 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. PRP was prepared using plastic wares or siliconized glass wares.

Plasma re-calcification time

The plasma re-calcification time was assayed based on the method described by Quick et al., [13]. ECSAE crude (5-50µg) was pre-incubated with 0.2ml of citrated human plasma in the presence of 10mM Tris HCl (20µl) buffer pH 7.4 for 1 min at 37°C.

Fibrin clot-hydrolyzing activity by colorimeter

Fibrin clot-hydrolyzing activity was assayed based on the method described by Rajesh et al., [14]. Concisely, 100µl of citrated human plasma was mixed with 20µl of 0.2M $CaCl_2$ and incubated for 2h at 37°C. The clot obtained was washed thoroughly for 5–6 times with PBS and suspended in 400µl of 0.2 M Tris–HCl buffer (pH 8.5). The reaction was initiated by adding varied amounts of ECSAE (20–100µg) in 100µl of saline and incubated for 2h and 30min at 37°C. The undigested clot was precipitated by adding 750µl of 0.44M TCA and allowed to stand for 30min.

Results and Discussion

The present study was designed to investigate the antioxidant, procoagulant and fibrinolytic activities of ECSAE. This study reported to innumerable medicinal application of ECSAE. Recently, considerable attention has been paid on the identification of natural plant-derived bioactive substances (e.g., antioxidants) that may be used in clinical routine. Many natural compounds present in the human diet can lower the risk of developing diseases such as cancer, cardiovascular and neurodegenerative disorders [16]. Living organisms are equipped with enzymatic and chemical antioxidant mechanisms for the control of oxidants. Certain amount of oxidative damage takes place even under the normal conditions; however, increased oxidant and decreased antioxidant levels defeat the ability of the antioxidative mechanisms to prevent oxidative damage [17]. DPPH is stable free radical at room temperature and accepts an electron / hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 5l7nm, induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow [18]. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity. ECSAE exhibited the 76% of DPPH radical scavenging activities at the concentration of 100µg with an IC50 value of 45.38µg/ml compare to positive control ascorbic acid. Ascorbic acid showed 81.34% of DPPH scavenging activity at the concentration of 100µg (Figure 1). In RP-HPLC, assessment of compound was based on the retention time of obtained chromatogram. Five peaks were resolved at the retention time of 16, 17, 19.5, 20 and 20.5min respectively (Figure 2). GC-MS chromatogram indicated the presence of large array of phytochemicals showed in (Figure 3). Interestingly, ECSAE showed the similar protein banding pattern arranged between 24 and 125kDa on 10% SDS-PAGE under reduced and non-reduced conditions (Figure 4). ECSAE exhibited proteolytic activity by degrading casein as a substrate with the specific activity of 0.270 units/mg/min at 37°C. Proteolytic activity of ECSAE was completely neutralized by both PMSF and IAA. While, 1,10, Phenanthroline

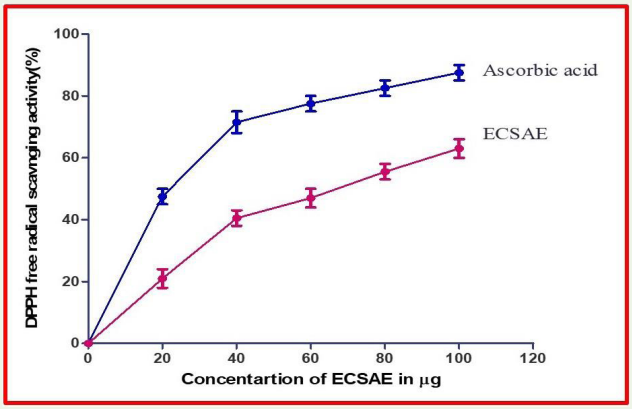

Figure 1: DPPH Free radical scavenging activity. Antioxidant activity of ECSAE, measured by DPPH method. Each value is presented as mean ± standard error.

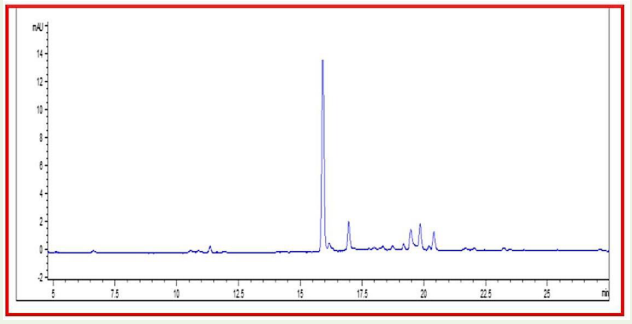

Figure 2: HPLC Chromatogram. ECSAE (20µL) was injected to C18 Column (5mm, 0.21X25cm) which was pre-equilibrated with 0.1% Trifluoro Acetic Acid (TFA) in water and sample was eluted in gradient mode by increasing the concentration (0- 100%) of 0.1% TFA in acetonitrile for 20min at the flow rate of 1ml/min and monitored at 280nm.

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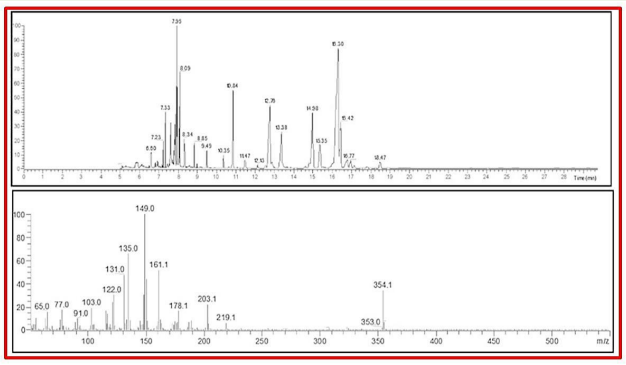

Figure 3: GC-MS Chromatogram of ECSAE

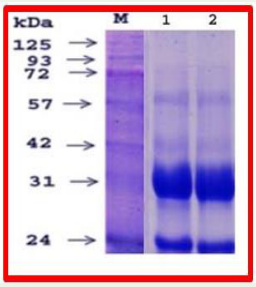

Figure 4: SDS-PAGE 10%: ECSAE as shown in SDS-PAGE (10%): ECSAE (100µg) under non-reduced (a1) and reduced conditions (a2).

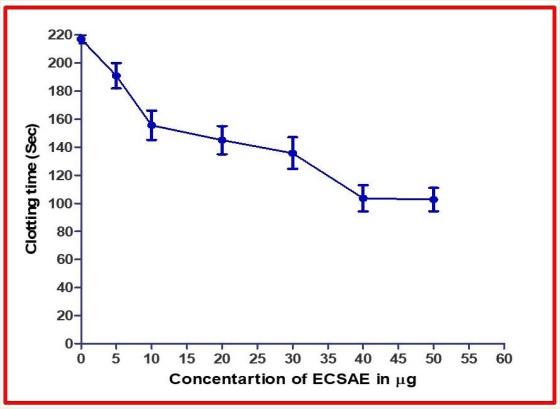

Figure 5: Plasma re-calcification time. ECSAE (5–50µg) was pre-incubated with 0.2ml of citrated human plasma PRP/PPP in the presence of 20 µl 10mM Tris–HCl buffer (pH 7.4) for 1 min at 37°C. 20µl of 0.25M CaCl2 was added to the preincubated mixture and clotting time was recorded.

Inhibitors (100mM each)	Activity/Residual activity (%)
None	100
PMSF	10
EDTA	100
1,10,Phenanthroline	54
IAA	20

Table 1: Effect of protease inhibitors on the proteolytic activity of ECSAE

and EDTA, have not inhibited the proteolytic activity of the extract. This suggested that ECSAE comes under serine and cysteine protease family (Table 1). When ECSAE was analyzed for its procoagulant effect it decreased the clotting time of citrated human PRP from control 214.2s to 94.2s and promotes blood coagulation, at the maximum concentration of 50µg after this dose it reached saturation (Figure 5). ECSAE could significantly reduce the plasma recalcification time of citrated human plasma suggested the procoagulant effect of ECSAE. Clotting is the body's normal response to prevent a person from bleeding to death. However, blood clot formation can be dangerous if it occurs within healthy blood vessels or if not degraded after due time. Many diseases like heart attack, stroke and pulmonary embolism are associated with inappropriate blood clot formation [19]. Although procoagulant medicines made entirely of plants. Since proteases have not yet been created, many plant extracts that contain them are currently used extensively in traditional medicine to stop bleeding and for wound healing. Several protein fractions and proteases from plant latex have procoagulant properties that speed up clotting. Serine and cysteine proteases from plant source exhibit procoagulant action irrespective of the plant species and family. But the mechanism involved in procoagulant action differs with the type of proteases [20]. In contrast, cysteine proteases exhibit specific effects on coagulation factors. For example, ficin derived from *Ficus carica* shown to activate

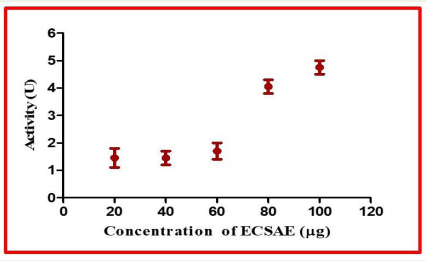

Figure 6: Fibrinolytic activity: Colorimetric assay. Washed plasma clot was incubated with 20–100 µg of ECSAE for 2.30hr and then the OD was measured at 660 nm.

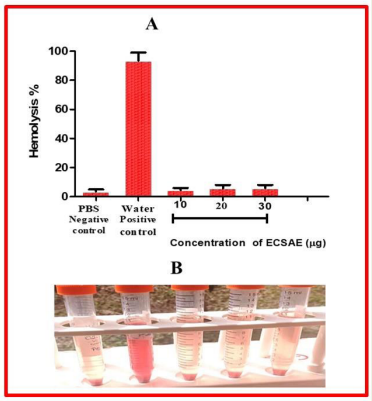

Figure 7: Direct Hemolytic assay. The different concentration of ECSAE (10-30µg) was incubated independently for 1hr at 37° C with the 1 ml of suspension made with packed human erythrocytes and phosphate buffered saline (PBS) 1:9 v/v. The reaction was stopped by adding 9 ml of ice-cold PBS and centrifuged at 1000g for 10min at 37°C. The amount of hemoglobin released in the supernatant was measured at 540nm.

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coagulation factor X, whereas Cysteine proteases from *Asclepiadaceae* exhibit thrombin-like activity [21]. The fibrin clot dissolving ability of ECSAE was carried out by colorimetric method. ECSAE hydrolyzed the fibrin clot in a dose dependent manner and the specific activity was found to be 1.1 to 4.5 units (Figure 6). A Fibrinolytic agent induces enzymatic activation of plasminogen to plasmin which cleaves the fibrin molecules [22]. Consistent with the fibrinolytic effect of ECSAE on fibrin, this supporting the procoagulant activity. ECSAE did not hydrolyze RBC up to the concentration of 200 µg when compare to the water positive control and PBS taken as a negative (Figure 7).

Conclusion

In conclusion, this study for the first time explores the antioxidant, procoagulant, and fibrinolytic activities of protease(s) present in the ECSAE. Thus, it could be better contender in the management of thrombotic disorders.

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Author and co-author contribution

CD, PH and SH together planned and designed the research work. YR,KP and RR assisted in the laboratory work. CSK and HM reviewed the article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that we do not have a conflict of interest.

Ethical clearance

The research work does not involve any animals.

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