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RESEARCH COMMUNICATIONS

Anticoagulant, fibrinogenolytic and anti-platelet aggregation activities of *Lablab purpureus* (L.) Sweet seed radicle aqueous extract

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ABSTRACT
The current study explores the anticoagulant, fibrinogenolytic and anti-platelet aggregation activities of *Lablab purpureus* (L.) Sweet seed radicle extract (LPRE). It is firstly reported that LPRE has protein at a concentration of 1.5 mg/ml and further evaluated for protein in gel electrophoresis. The proteolytic activity of LPRE was evaluated using casein and gelatin as substrate. LPRE showed a specific activity of 0.35 U. LPRE increased the plasma clotting time significantly showing its anticoagulant property. LPRE hydrolyzed the Aα, Bβ and γ chains of fibrinogen. Furthermore, LPRE significantly inhibited the platelet aggregation induced by agonist adenosine diphosphate (ADP).

Introduction

According to World Health Organization (WHO) cardiovascular disease (CVDs) remains a prominent cause of mortality from myocardial infarctions, strokes from usual blood clots (1, 2). About 31% of the global deaths are from CVDs, and about 85% of them are from stroke and heart attack. Overactivity of the coagulation cascade (hypercoagulation) increases the risk of thrombosis formation leading to thromboembolisms which in turn lead to ischemia by blocking blood flow and damaging the organs (3).

Blood coagulation has an intricate, highly controlled network of reactions involving coagulation factors which gets activated sequentially forming a blood clot. Unregulated blood clot formation leads to formation of thrombus (4). Thrombosis could lead to occlusion of blood vessels and should be treated with anti-coagulant drugs (5).

Thrombosis is treated with anticoagulants such as unfractionated heparin (UFH), coumarins, low molecular weight heparin (LMWH) and pentasaccahride fondaparinux (6). Even though the anticoagulants are efficient, the problem in their administration and the deleterious life-threatening side effects such as bleeding complications limit their usage (7). Since the anticoagulant drugs are expensive and have serious side effects, there is a need to explore alternative anticoagulants with more efficacy and less side effects.

Medicinal plants/plant extracts have been used in traditional Ayurvedic medicinal practitioners to prevent and/or treat various ailments (8). There are several medicinal plants reported to be having anticoagulant property and can serve as an alternative for current anticoagulant agents (9). Several studies have reported that dietary anticoagulants/ phytoconstituents could reduce the risks of thromboembolism (10, 11).

The *Lablab purpureus* (L.) Sweet belongs to the family Fabaceae. Different parts of the plant is used as anti-inflammatory, aphrodisiac, antispasmodic, antidiabetic, febrifuge, bilious, stomachic and phlegmatic disorders (12, 13). In addition, the plant decoction has been used in alcoholic intoxication to treat cholera, diarrhoea, gonorrhoea, leucorrhoea and in globefish poisoning. The seed pods juice was used to treat the digestive problems, expel worms and to treat inflamed throat and ears. The flowers are used to treat uterus inflammation and increase menstrual flow (14, 15).

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In view of finding a natural dietary anticoagulant, this study we have investigated the *in vitro* anticoagulant, fibrinogenolytic and anti-platelet aggregation activities of *Lablab purpureus* (L.) Sweet seed radicle extract (LPRE).

Materials and Methods

Sample collection and aqueous extract preparation

Lablab purpureus (L.) Sweet seeds (round and matured) were procured from merchandise in Tumkur district (13.3379° N, 77.1173° E), Karnataka state, India. *L. purpureus* (L.) Sweet plants were grown in Tumkur at temperature between 28-30 °C in high organic matter soil. The seeds were harvested during September – December months. The plant was identified by Prof. Sharanappa, Professor, Department of Biosciences, Hemagangotri, University of Mysore, Hassan. A herbarium specimen was maintained in Department of Studies and Research in Botany, Tumkur University, Tumkur (TUB-012).

Seeds (500 gm) were soaked in distilled water (1l) for 48 hr. The soaked seeds were kept in a closed container for 48 hr at 37 °C for sprouting. The sprouted seeds were collected. Sprout radicle was separated from cotyledon and dried in an incubator at 40 °C with humidity upto 70% for 48 hr. The dried sprout radicles were finely powdered using electrical grinder and stored at ⁻20 °C freezer (humidity about 95%) until further use. The radicle powder (10 gm) was suspended in distilled water (100 ml) and kept on magnetic stirrer for 24 hr at a speed of 400 rpm to prepare aqueous extract. Later the extract was centrifuged at 10000 rpm (Remi C-24BL centrifuge) and supernatant was collected. The supernatant was concentrated and named as *Lablab purpureus* (L.) Sweet seed radicle extract (LPRE). The LPRE stored at ⁻20 °C (humidity about 95%) freezer until further use.

Protease activity

Protease activity was analysed (16) using casein (2% in 200 mM Tris-hydrochloric acid (HCl) buffer, pH 8.0) as substrate. LPRE was incubated with 0.4 ml of casein at 37 °C for 180 min. Later, the reaction terminated by adding trichloroacetic acid and the sample centrifuged (3000 rpm for 5 min). After centrifugation 1 ml of supernatant was collected and sodium carbonate and diluted Folin-Ciocalteu reagents were added and kept at 37 °C for 20 min. The intensity of the colour developed was measured using Elico CL63 colorimeter at 660 nm. Enzyme activity was stated as the enzyme required for an incremental increase of absorbance by 0.01 at 660 nm in 1 hr.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to standard procedure (17). LPRE was loaded onto 10% gel and electrophoresis was carried out for 2 hr at 90 Volts. Gel was stained with Coomassie brilliant blue R-250 (CBB) and visualized using Gel.LUMINAX gel documentation system.

Zymography

Casein/gelatin zymogram was carried out (17). The 10% gel incorporated with gelatin (0.2%) as substrate used for zymogram assay. LPRE was incubated with sample buffer and electrophoresis was done. SDS was removed using Triton X-100 and gel was incubated in buffer (COMPOSITION) overnight. The gel was stained with CBB.

Fibrinogenolytic activity

Fibrinogenolytic activity was carried out (18). Human fibrinogen (50 µg) incubated for 2.5 h at 37 °C with different concentrations of LPRE in sodium phosphate buffer. After incubation reducing sample buffer was added and we analyzed the hydrolyzed products in 12% SDS-PAGE.

Coagulation assays

Recalcification time (RT)

The RT was determined following standard procedure (19). Citrated human plasma (100 µl) was incubated with LPRE (3 - 18 µg) at 37 °C for 15 min. After incubation, 100 µl of 25 mM $CaCl_2$ was added and time taken for visible clot formation was recorded.

Prothrombin time (PT)

PT was determined as per standard procedure (20). 100 µl of plasma was incubated with different concentrations of LPRE (6 - 36 µg) at 37 °C for 15 min. After incubation, 0.2 ml of liquid calcified thromboplastin (Uniplastin reagent from Tulip Diagnostics Pvt. Ltd., Goa) was added and time taken for clot formation was recorded.

Activated partial thromboplastin time (aPTT)

APTT kit was procured from Tulip Diagnostics Pvt. Lt. Goa. The assay was performed as described in the manufacturer's protocol. Citrated human plasma (100 µl) was incubated with LPRE (6 - 30 µg) at 37 °C for 5 min. After incubation 100 µl of Liquicelin-E was added and kept for 5 minutes. 100 µl of $CaCl_2$ was added and time for visible clot formation was recorded.

Platelet aggregation

Platelet aggregation was analysed in Chrono-Log Model 700-2D aggregometer (ChronoLog Corp., USA). We collected blood from healthy volunteers. Platelet-rich plasma (PRP) was obtained by centrifuging human blood (1:9 v/v in 3.2% trisodium citrate) at 900 rpm for 15 min; the resultant supernatant was PRP. The blood sample was centrifuged at 3000 rpm for 20 min to obtain Platelet Poor Plasma (PPP) as the supernatant. 250 µl of PRP was taken in siliconized glass cuvette with stir bar stirring at 1200 rpm. The platelet aggregation was initiated by adding ADP (10 µm). Different concentration of LPRE incubated with PRP for 15 min and aggregation were initiated by adding ADP. The aggregation was monitored for 6 minutes by change in turbidity with PRP and PPP representing 0% and 100% transmittance respectively.

Protein estimation

Protein concentration was estimated (21) and BSA is used as standard.

Statistical analysis

Results were represented as mean ± standard error. One-way analysis of variance was used for statistical significance of intergroup differences and the Tukey test was used for comparison of means. The Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA) was used to perform all analysis.

Results and Discussion

The *Lablab purpureus* (L.) Sweet seed radicle extract (LPRE) has a protein concentration of 1.5 mg/ml. Analysis of LPRE by SDS-PAGE showed protein bands distributed majorly in medium molecular weight region (Fig. 1). LPRE showed a proteolytic activity of 0.35 U. In zymogram study (using casein and gelatin as substrate) LPRE showed translucent activity bands, confirming protease (Fig. 2). Proteases from seeds have broad specificity. They can hydrolyse casein, gelatin and extracellular matrix (22, 23).

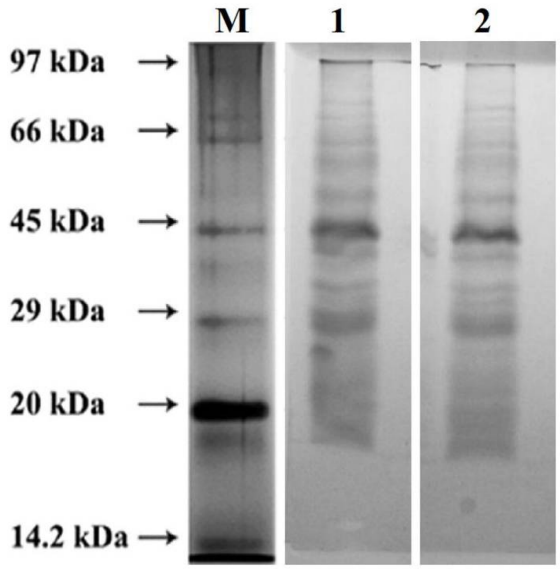


Fig.1. Electrophoresis. LPRE (120 µg) was loaded on to the 10% SDS-PAGE in non-reducing and reduced conditions. Lane M: molecular weight markers, lane 1: LPRE (120 µg) in non-reducing condition, lane 2: LPRE (120 µg) in reducing condition.

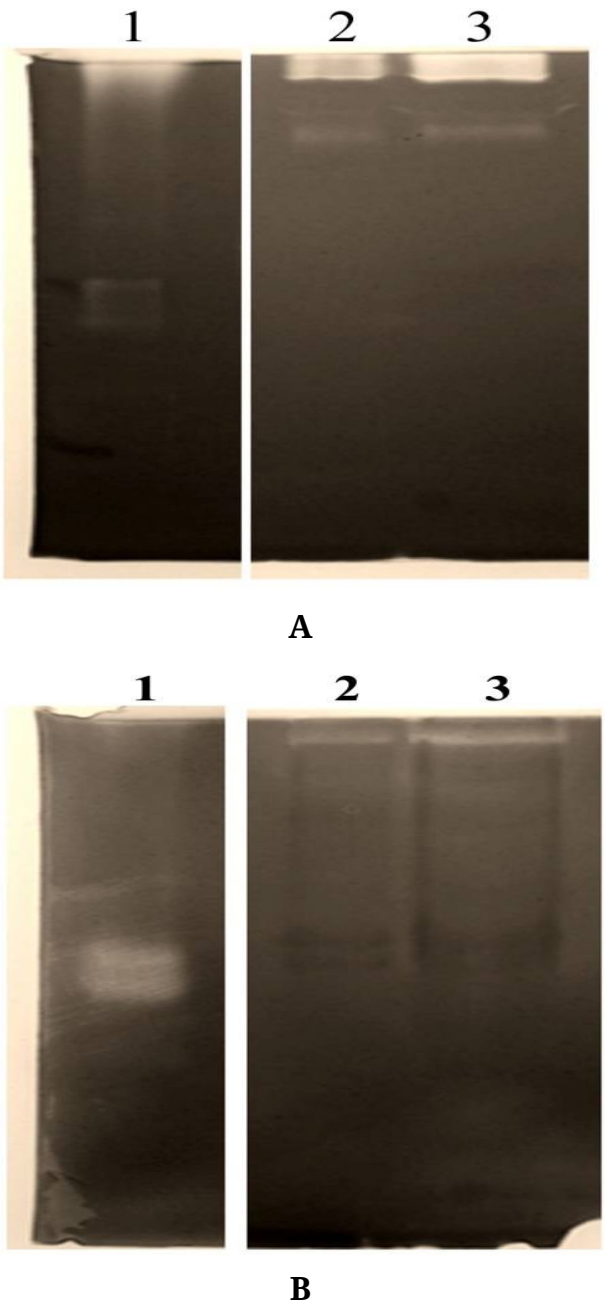


Fig. 2. Zymogram assay. The LPRE was loaded onto SDS-PAGE co-polymerized with gelatin **(A)** or casein **(B)** for the detection of proteolytic activity. After electrophoresis, SDS was removed by using Triton X-100. Later incubated overnight in incubation buffer and stained with CBBR-250. Lane 1: Trypsin (5 µg) 2 and 3: LPRE 50 µg and 100 µg.

Blood coagulation is a complex physiological process that drives immediate response to blood vessel injury. Impairment in coagulation process leads to the hyper-activation of the coagulation factors, which results in thrombotic disorders (4). Thrombosis results in formation of unusual clot in the arteries and veins increasing cerebrovascular/cardiovascular complications (6). Currently available antithrombotic agents have life-threatening side effects. Anticoagulants from the natural sources are preferable for curing thrombotic disorders. Several anticoagulant agents from natural sources of plant/seed extracts have reported and these could be a better therapeutic candidate to treat thrombosis. The seed extract of *Momordica charantia*, *Artocarpus heterophyllus*, *Pisum sativum*, *Macrotyloma uniflorum* and *Linum usitatissimum* seeds found to show anticoagulant and anti-platelet aggregation effects (22, 23, 25).

LPRE showed significant anticoagulant activity. Upon incubation with plasma, LPRE increased the clotting time in RT from control 200 sec to 700 sec with 18 µg (Fig. 3A), in PT from control 16 sec to 700 sec with 36 µg (Fig. 3B) and in APTT from control 36 sec to 665 sec with 30 µg (Fig. 3C) concentration. In RT calcium activates the common pathway of blood coagulation. Calcium along with phospholipid and

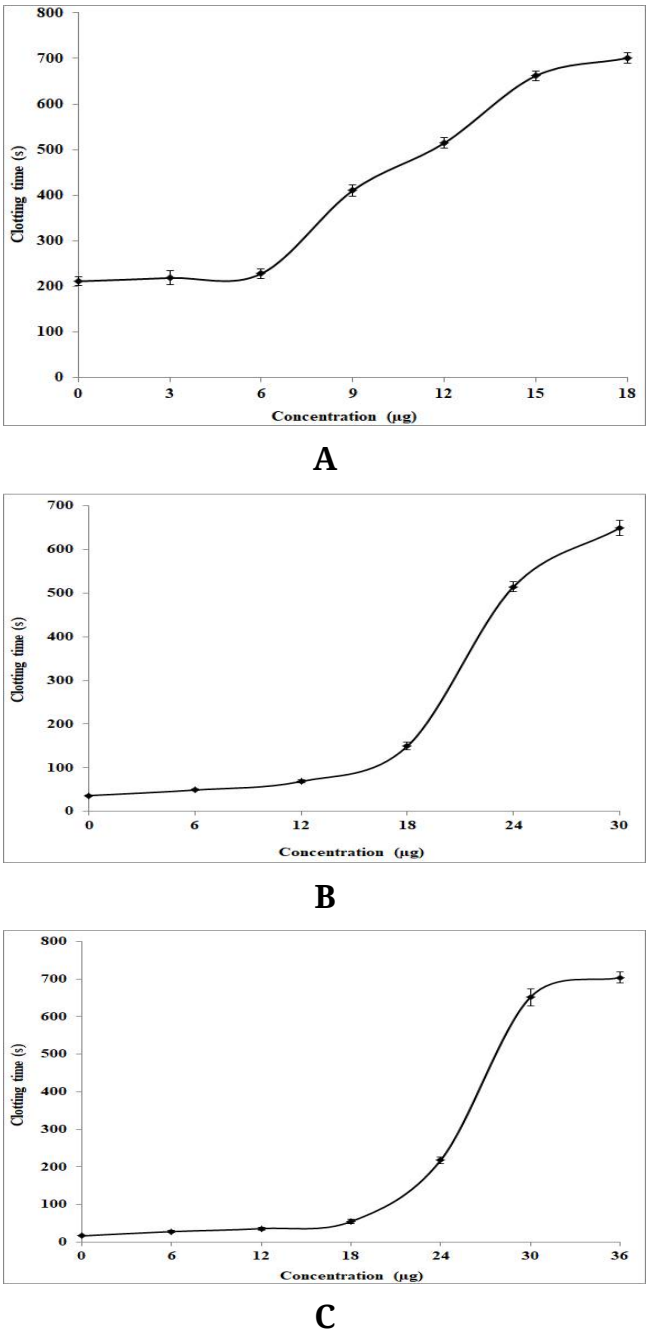


Fig. 3. Coagulation studies. **A.** Recalcification time. Different concentration of LPRE (3 - 18 µg) was incubated with human plasma (100 µl) for 5 min. After incubation, 100 µl of $CaCl_2$ was added and time taken for visible clot formation was recorded. **B.** Activated partial thromboplastin time (aPTT). Different concentration of the LPRE (6 - 30 µg) was incubated with 100 µl of plasma for 10 min and after incubation 100 µl of liquicelin-E was added and again incubated for 5 min. To this mixture 100 µl of $CaCl_2$ was added and time taken for visible clot formation was recorded. **C.** Prothrombin time (PT). Different concentration of LPRE (6 - 36 µg) was incubated with 100 µl of plasma for 10 min and after incubation 200 µl of uniplastin was added, the clotting time was recorded. All the values are presented as mean ± standard error of the mean (n=5).

factor V activates factor II. PT was evaluated using uniplastin reagent containing ready to use liquid calcified thromboplastin reagent. Thromplastin in presence of calcium activates the extrinsic pathway of human blood coagulation. While APTT was evaluated using liquicelin-E reagent containing phospholipid with ellagic acid as an activator. Cephaloplastin activates the coagulation factors of intrinsic pathway of coagulation mechanism in the presence of calcium. This suggests that LPRE interfere in intrinsic, extrinsic and in common pathway of coagulation cascade. The protease(s) present in the LPRE sample might be responsible for the anticoagulant property exhibited (22).

We evaluated the fibrinogenolytic activity for LPRE. The LPRE hydrolysed all the chains of fibrinogen (Aα, Bβ and γ) at a concentration of 3 µg in a dose dependent manner (Fig. 4). Generally, proteases which degrade fibrinogen from its C-terminal end generate truncated fibrinopeptides

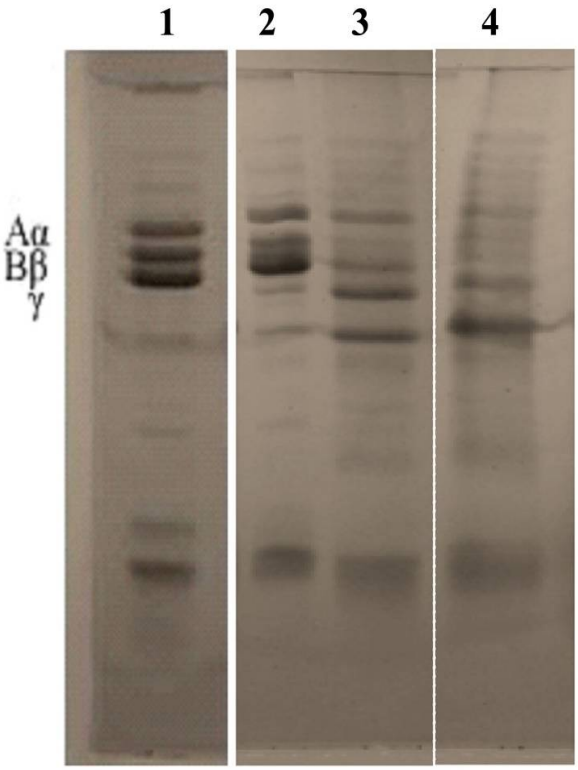
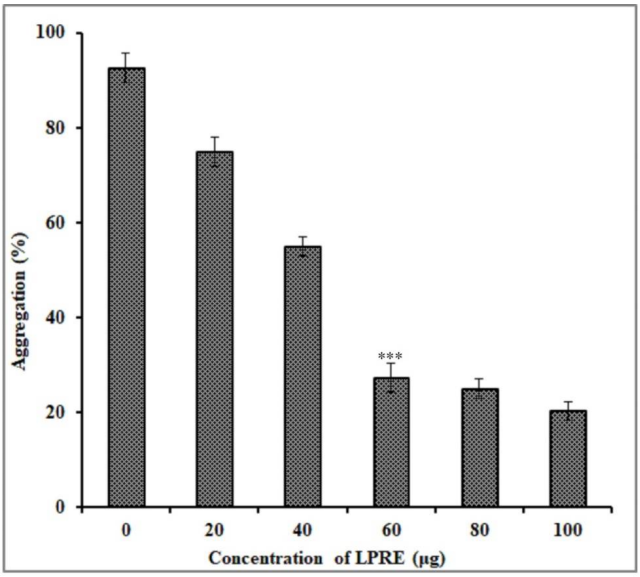


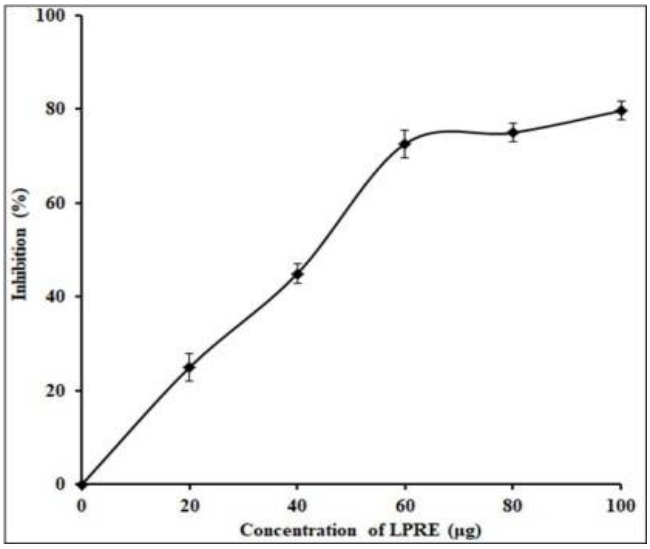
Fig. 4. Fibrinogenolytic activity. Human fibrinogen (50 µg) was incubated with LPRE (0.06 µg, 0.3 µg and 3 µg) at 37 ° C for 2.5 h. SDS-PAGE performed under reducing condition. Lane 1: fibrinogen (50 µg), lane 2 - 4: fibrinogen incubated with 0.06 µg, 0.3 µg and 3 µg of LPRE respectively.

exhibiting anti-coagulant activity. Similar fibrinogenolytic activity has been reported from *Tridax procumbens*, *Jatropha curcas* and *Plumeria alba* (23 - 25).

Platelets are anuclear discoid-shaped cells originating from stem cells of bone marrow megakaryocytes which play an important role in blood coagulation (26). The platelets aggregate to form platelet plug at the site of injury, along with fibrin clot to form blood clot. During thrombotic disorder, platelets also contribute for the formation of an unusual clot in blood vessels. LPRE significantly inhibited the ADP-induced platelet aggregation to an extent of 80% at the concentration of 100 µg (Fig. 5A and B). The factors that act as antiplatelet aggregating agents play a key role in inhibiting unusual blood clots. Several antiplatelet agents have been reported from snake, microbes, plants and animal sources (24, 27).



A



B

Fig. 5. Platelet-aggregation. Aggregation of platelet was monitored in a Chrono-Log Model 700 aggregometer. Platelet-rich plasma (0.25 ml) was incubated with LPRE for 15 min and later aggregation was initiated by adding ADP (10 µm). The reaction mixtures were monitored for platelet aggregation. All the values are presented as mean ± standard error of the mean (n=5). (A) The percentage of platelet aggregation with LPRE; (B) The percentage of inhibition of platelet aggregation by LPRE. All the values are presented as mean ± standard error of the mean (n=5). Statistically significant results are indicated by asteriks *** –p<0.001.

Conclusion

The present study for the first time demonstrates that *Lablab purpureus* (L.) Sweet seed radicle extract (LPRE) has proteolytic activity associated with fibrinogenolytic activity which exhibit anticoagulant and anti-platelet aggregation effect. The findings give an opportunity for isolation of bioactive molecule from LPRE with potent anticoagulant property which can be further used in clinical applications.

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Authors' contributions

TRS collected the specimen, prepared the LPR extract and conducted the experiments. TRS and PD contributed in the preparing and editing the complete manuscript. Both the authors have read and approved the manuscript.

Conflict of interests

The authors declare that there is no competing interest.

Ethical issues

The present study was according to Ethical guidelines approved through the Ethical Committee of Faculty of Pharmacy, Sree Siddaganga College of Pharmacy (Reference Number: SSCPT. Clear/152/2016-17).

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