



Thermo sensitive proteins from *W. coagulans* Dunal fruits exhibit anti-coagulant, anti-thrombin and anti-trypsin activities

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Abstract

Anti-coagulants are used for the treatment and prophylaxis of thrombotic disorders. The applications of traditional anti-coagulants like warfarin and heparin produce severe complications with bleeding. In present study anti-coagulant, anti-thrombin and anti-trypsin activities were studied from crude aqueous extract (CAE) of *Withania coagulans* Dunal fruits. Anti-coagulant and trypsin activities of CAE against human plasma were detected by coagulation tests (aPTT, PT and TT) and dot blot assay on X-ray film. Inhibitor assays of CAE against thrombin and trypsin were carried by using synthetic substrate. Prominent delay in clotting time in coagulation tests suggested CAE exhibits an excellent anticoagulant activity. Six trypsin inhibitors (TI) isoforms from CAE were found on X-ray film. Active proteins from CAE of *W. coagulans* were responsible for activities. CAE showed more anti-trypsin activity (IC₅₀ 30.20µg/ml) than anti-thrombin activity (IC₅₀ 48.56 µg/ml). Various activities of CAE suggested multifunctional proteins are available in *W. coagulans*. The proteins of CAE were found to be thermo labial and showed more activity at 40°C temperature. The proteins showed activity at wide range of pH and optimum pH was found between 7 and 8. The active proteins from *W. coagulans* could be applied an alternative anticoagulants for treatment of thrombotic disorders.

Keywords: anticoagulant activity, *W. coagulans*, thermo labial, trypsin inhibitors active proteins

Introduction

Thrombosis is a state of blood occurs in blood vessel that reduces blood flow as a result of clot formation ^[1] in which the complex interactions of vascular endothelium, platelets and clotting factors ultimately establishes as venous thromboembolism ^[2]. The activation of coagulation factors is increased in disorders like atherosclerosis, diabetes, disseminated intravascular coagulation (DIC) and deep vein thrombosis (DVT) that resulting in thrombin formation ^[3, 4, 5].

Such processes lead to formation of occluding blood vessels, intravascular thrombosis and resulting death. Intravascular thrombosis is the major harmful process in cardiovascular diseases which are the foremost problem of morbidity and mortality occurs in worldwide ^[6]. According to ISCFWT ^[7] thrombosis is a major devastating global disease. The blood anti-coagulants are known to be used as drugs for the treatment and prophylaxis of thrombotic disorders. Some anticoagulants like heparin and warfarin have been used as drugs to control thrombosis ^[8].

However, the use of such anticoagulants associated with some complications like recurrent stenosis, increased bleeding diathesis and thrombocytopenia ^[9]. Anticoagulants from natural sources are safe and an alternative remedies that can be used for treatment of diseases. Many non-proteinaceous anticoagulants have been studied from different natural sources but they can be exerting non-specific function and some side effects. Active proteins from natural sources have been studied as safe for consumption and have a specific activity with minute concentration. Very few active proteins from natural sources have been studied yet as anticoagulants therefore, there is a scarcity of proteins as anticoagulants in available literature.

W. coagulans belongs to *Solanaceae* family and is commonly found throughout Pakistan, North-West India and Afghanistan. The *W. coagulans* fruit possesses milk coagulating property due to presence of milk coagulating proteases hence, it is utilized for 'cheese' and 'paneer' preparation in North-West India and adjoining countries ^[10]. Crude extract (CE) of this plant exhibit anti-microbial, anti-inflammatory, anti-tumor, anti-carcinogenic, anti-hyperglycemic, free radical scavenging, immunosuppressive and central nervous system depressant activities ^[11].

The aqueous extract of *W. coagulans* fruits exhibit anti-angiogenic ^[12] and antidiabetic ^[13] activities. Anti-coagulant activity of proteins from *W. coagulans* has not been studied yet. The present study aimed the evaluation of anti-coagulant and protease inhibitor activities of proteins from *W. coagulans* fruits.

Material and Method

Chemicals and Reagents

Lyophilized plasma, Liquicelin-E reagent, Uniplastin reagent and FIBROSCREEN reagent were purchased from Tulip Diagnostics Pvt. Ltd. Polyvinylpyrrolidone (PVP), Sodium phosphate, Phosphoric acid, Acetone, Hexane, Calcium chloride, Hydrochloric acid, Sodium acetate, Sodium phosphate and Calcium carbonate were obtained from RANKEM Pvt. Ltd. Bovine serum albumin (BSA fraction V) and Trypsin were obtained from Sisco Research Laboratories (SRL), Pvt. Ltd. N- α -benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-*p*-tosyl-gly-pro-arg-*p*-nitroanilide acetate salt were purchased from Sigma-Aldrich. All chemicals used in this study were of analytical grade.

Preparation of *W. coagulans* Crude Aqueous Extract (CAE)

Whole fruits of *W. coagulans* were purchased from local market of Ayurveda medicine in Aurangabad (Maharashtra) India, and authenticated by taxonomist Department of Botany Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (Maharashtra) India. Fruits were sorted and kept in oven at 37°C until completely dried. Dry fruits were ground to fine powder by grinder and mixer. 100 g of fine powder was washed thoroughly with 300 ml of chilled acetone (four to five times) and then with 200 ml of chilled hexane (two times) to make fat free. The solvents were filtered off and the powder was kept at room temperature for air drying. Dried defatted powder was suspended in phosphate buffer (0.05 M sodium phosphate, pH 7.2 containing 1% polyvinylpyrrolidone) as 1: 6 proportion (w/v) and kept in refrigerator at 37°C for overnight extraction of proteins. Polyvinylpyrrolidone was added during the preparation of buffer to remove and separate the polyphenols from proteins present in powder. Suspension was centrifuged at 10,000 rpm at 4 °C for 15 min and the resulting supernatant was considered as crude protein extract and it was preserved at -20°C temperature.

Protein Estimation

The protein concentration in CAE was estimated by Folin-Ciocalteu assay using bovine serum albumin as a standard protein [14].

Preparation of Blood Plasma For anticoagulant activity

Lyophilized plasma was mixed with distilled water as 1: 0.5 (v/v) proportions and this solution was used for the coagulation tests.

Activated Partial Thromboplastin Time Test (aPTT)

In a coagulation test tube, CAE (50 μ l) was mixed with 50 μ l of plasma and 100 μ l of Liquicelin-E reagent and incubated at 37°C for 5 minutes followed by addition of 50 μ l of calcium chloride. The clotting time in second of plasma was recorded on coagulometer. Simultaneously, an aliquot was prepared without crude extract by the mixing of 50 μ l of phosphate buffer, 50 μ l of plasma, 100 μ l of Liquicelin-E reagent and 50 μ l of calcium chloride as control. The increase in clotting time of test sample as compared to control was assumed the extract have anticoagulant activity. The difference in clotting time was calculated by subtraction of control clotting time from test clotting time.

Prothrombin Time Test (PT)

Prothrombin time test was performed similar to aPTT, in a coagulation test tube containing 50 μ l of CAE and 50 μ l of plasma mixed together and incubated at 37°C for 5 minutes followed by the addition of 200 μ l of Uniplastin. The control tube was prepared by the addition of phosphate buffer instead of plant extract. The clotting time of plasma was recorded on coagulometer. The difference in clotting time was calculated by subtraction of control clotting time from test clotting time.

Thrombin Time Test (TT)

In a coagulation test tube, 100 μ l of CAE was mixed with 100 μ l of human plasma and incubated at 37°C for 5 minutes followed by the addition of 200 μ l of FIBROSCREEN reagent. The control tube was prepared by the addition of 100 μ l of phosphate buffer instead of plant extract. The clotting time of plasma was recorded on coagulometer. The difference in clotting time was calculated by subtraction of control clotting time from test clotting time and percentage delay in clotting time was calculated.

Anti-thrombin activity of CAE

Inhibitor activity of CAE against thrombin was carried out by using a chromogenic substrate (N-*p*-tosyl-gly-pro-arg-*p*-nitroanilide acetate salt) on 96-well microtiter plate reader. As per instruction mentioned in assay procedure the thrombin from bovine plasma was dissolved in buffer (0.05 M Tris-HCl, 0.227 M NaCl, 0.1% BSA, pH 8.3) at a concentration of 0.8 U/ml and the synthetic substrate was dissolved in distilled H₂O at a concentration of 0.18 mM. Briefly, 10 μ l of thrombin was added in microtiter plate reader along with various concentrations of protein of CE in 80 μ l assay buffer (50, 100,.....250 μ g/ml) in total reaction mixture of 100 μ l. For control activity 10 μ l of thrombin was mixed with 80 μ l assay buffer. All mixtures were incubated at room temperature for 10 minutes and enzymatic reaction was initiated with the addition of 10 μ l substrate. Absorbance was recorded on Thermo micro plate reader at 405 nm at an interval of 3 minute up to 30 minutes. The percent

inhibition of thrombin with respect to increasing inhibitor concentration was calculated by considering control as 100% enzymatic activity.

Detection of anti-trypsin activity of crude extract (Dlot assay/spot test)

The detection of anti-trypsin activity in CAE was carried out by using dot blot assay/spot test on X-ray film using the method of Pichare and Kachole^[15]. The principle of this method is based on the digestion of gelatin coated on X-ray film by proteases. An aliquot containing 10 µl trypsin (0.5 mg/ml) and 10 µl of crude extract was incubated at 37°C for 10 min. Another aliquot was prepared without crude extract for control activity. After incubation samples were loaded on X-ray film and X-ray film was incubated at 37°C for 15 min. Then X-ray film was washed under tap/warm water and wetted X-ray film was dried at room temperature. Inhibitory activity was checked by comparing with control spot. Spot appeared as unhydrolyzed gelatin revealed the presence of inhibitory activity in extract. Film was visually assessed and scanned on EPSON scanner at 300 dpi.

Visualization of trypsin inhibitors by Gel-X-ray Film Contact Print Method (GXCP)

For the visualization of trypsin inhibitor, CAE (10µl) was mixed with sample buffer and loaded on 10% SDS-PAGE gel electrophoresis. After electrophoresis gel was removed, washed with distilled water and cut into strips. The gel strips were incubated for 1 hr at room temperature in 100 ml of renaturing buffer (2% Triton X-100) on a rotary shaker. Thereafter the gel was processed for trypsin inhibitory activity by the gel-X-ray film contact print method^[15].

The gel was washed in 0.1 M Tris-HCl buffer (pH 7.8) for 15 min, followed by incubation in 0.1% trypsin solution for 15 min at room temperature. The gel was then briefly rinsed with buffer to remove the excess trypsin. Immediately the wet gel was overlaid on X-ray film in a tray and incubated at 37 °C. The hydrolysis of the gelatin on X-ray film was monitored visually and after extent hydrolysis of gelatin, the gel was removed from X-ray film. The X-ray film was washed with warm water and kept at room temperature for drying. Protease inhibitor bands appeared as unhydrolyzed gelatin against the background of hydrolyzed gelatin. The inhibitory bands were observed by visually and image of X-ray film was taken on scanner.

Anti-trypsin activity of crude extract

The trypsin inhibitors activity in CAE was measured by trypsin assay using N- α -benzoyl-DL-arginine-p-nitroanilide (BAPNA) as synthetic substrate, as described by Erlanger^[16]. In reaction mixtures containing 10µl trypsin (1 mg/ml; 1000U/mg), various concentrations (50, 100.....250µg/ml) of protein of CE and volume of reactions was adjusted with the addition of 0.1 M Tris-HCL buffers (pH 7.8) up to 200 µl. Control tube was prepared without extract by addition of 10µl trypsin and 200 µl assay buffer. All reaction mixtures were incubated at 37°C for 10 min.

After incubation, 300µl substrate (1 mM) was added in each reaction and incubated for 30 min at 37°C. The enzymatic activity was terminated by the addition of 30% acetic acid (300 µl) and optical density of each solution was recorded at 410 nm. The percent inhibition of trypsin with respect to increasing inhibitor concentration was calculated by considering control as 100% enzymatic activity.

Determination of Optimum Temperature

The effect of different temperature on CAE for anti-trypsin and anti-thrombin activities was carried out by the incubation of reaction mixtures (as mentioned in assay method section) at temperature ranging from 20 to 100°C (at 10°C intervals). The percent inhibitions of trypsin and thrombin at each temperature were calculated with considering control as 100% enzyme activity. A graph of percent inhibitions verses temperature was plotted for determination of optimum temperature.

Determination of optimum pH

The optimum pH of CAE for anti-trypsin and anti-thrombin activities was determined by incubation of reaction mixtures (as mentioned in assay method section) in different pH solutions ranging from 3.0 to 11.0 at constant temperature.

Different pH buffers were prepared as 100mM sodium acetate buffer (pH 3.0-5.0), 100mM phosphate buffer (pH 6.0-7.0), 100mM Tris-HCl buffer pH (7.0-9.0), and 100mM carbonate buffer (pH 10.0-11.0). The percent inhibitions of trypsin and thrombin at each pH were calculated with considering control as 100% enzyme activity. A graph of percent inhibitions verses different pH value was plotted for determination of optimum pH value.

Results

Anti-coagulant and anti-trypsin activities in *W. coagulans* fruit CAE

The aqueous extract was prepared from whole fruits of *W. coagulans* for isolation of proteins. The extracted protein in CAE was estimated about 4.982 mg/ml. For detection of anticoagulant activity, aPTT, PT and TT tests were performed using human blood plasma. Clotting time of plasma in all tests was prominently increased by the addition of CAE during coagulation tests as shown in Table 1. These test revealed that CAE exhibited anticoagulant activity.

As per the % delay in clotting time (second), CAE showed prominent activity in aPTT test as compare to other tests. The activity of extract also checked against trypsin by dot blot assay on X-ray film. The trypsin was unable to digest gelatin coated on X-film in presence of CAE, this indicates that CAE exhibits anti-trypsin activity (Fig 1).

Table 1: Anticoagulant activity in CAE *W. coagulans* fruit by activated partial thromboplastic time (aPTT), prothrombin time (PT) and thrombin time (TT) assay.

Type of test	Control clotting Time (Second)	Test clotting Time (Second)	Difference (Second)	% delay in clotting Time (Second)
aPTT	36.1	655.0	618.9	1714.40
PT	14.4	86.8	72.4	502.77
TT	8.1	14.1	6.0	74.07

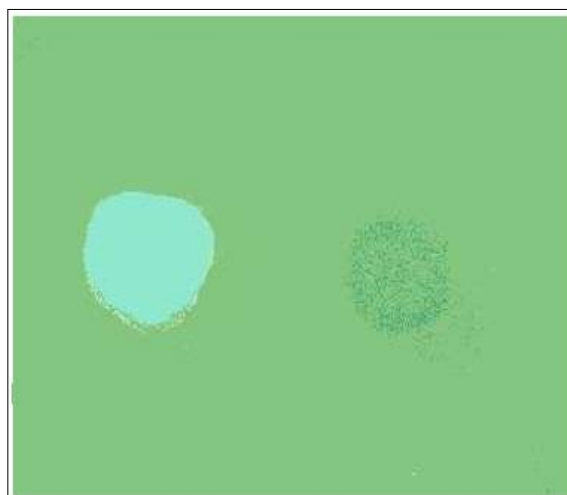


Fig 1: Detection of trypsin inhibitor (TI) activity in CAE of *W. coagulans* fruit by dot blot assay on X-ray film.

Gel electrophoresis (SDS-PAGE) and visualization of trypsin inhibitors (TIs) of CAE

Separation of protein from CAE was carried out on native-PAGE and after staining distinct/clear bands were not observed on gel (Fig 2 lane A) this may be due association of other molecules with proteins therefore for the visualization of isoform of TIs, SDS-PAGE was carried with using non-reducing sample buffer. GXCP revealed the six distinct isoforms of TIs from CAE of *W. coagulans* fruits with 50ug protein concentration. The detected isoforms of TIs were designated as TI1, TI2, TI3, TI4, TI5 and TI6 as mentioned in fig 2 lane B.

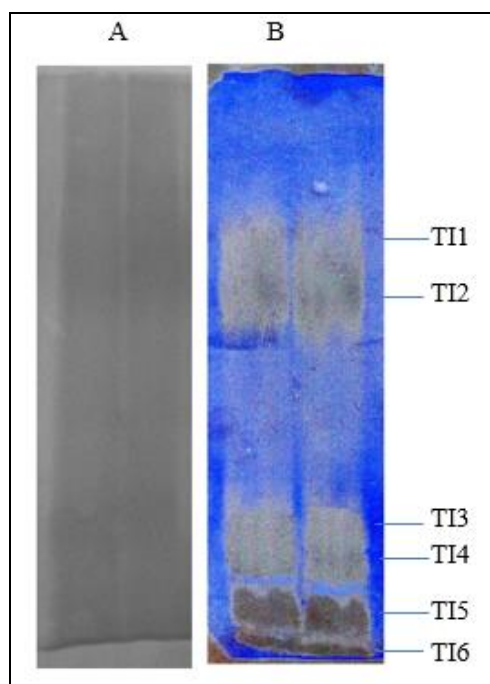


Fig 2: Gel electrophoresis of CAE. Lane A: Separation of proteins from CAE on native-PAGE and Lane B: Visualization of trypsin inhibitors (TIs) on GXCP separated by SDS-PAGE.

Inhibitor assay of CAE against thrombin and trypsin

Inhibitor assay of CAE protein against thrombin and trypsin were performed by using synthetic substrate as dose dependent manner. Fig 3 shows that percent inhibitions of thrombin and trypsin increased with increasing protein concentrations. CAE exhibited more inhibitory activity against trypsin (IC_{50} 30.20 μ g/ml) than thrombin (IC_{50} 48.56 μ g/ml). In both cases, more than 50 μ g/ml protein from CAE could be required for complete inhibition of 0.008U (thrombin) and 10U (trypsin) respectively.

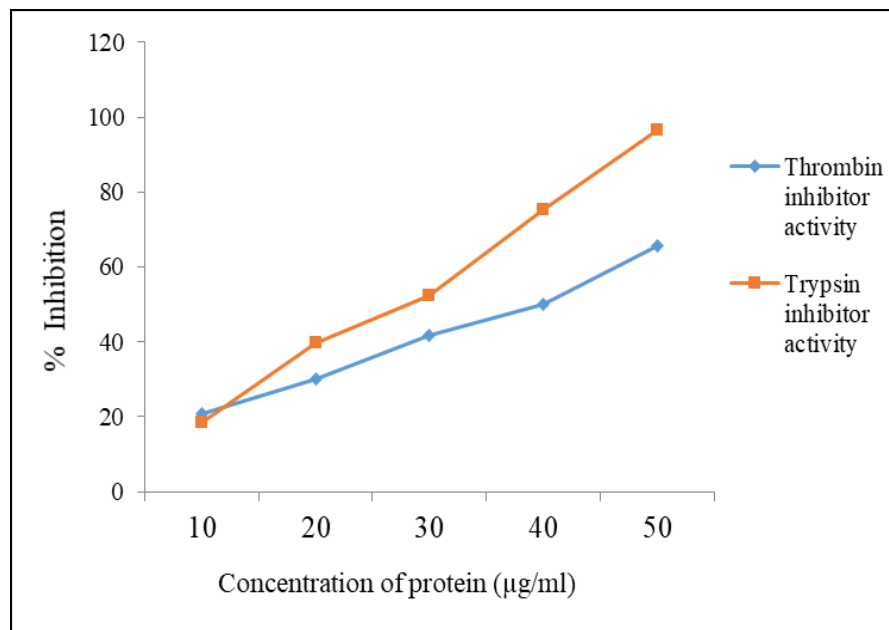


Fig 3: Inhibitory effect of different protein concentration in CAE against trypsin and thrombin activities.

Effect of temperature on CAE for anti-thrombin and anti-trypsin activity

The standard assay system of thrombin and trypsin was used for assessment of temperature effect on activity of CAE. Fig 4 clearly revealed that active proteins present in CAE of *W. coagulans* fruits are thermo sensitive/thermo labial. The CAE showed maximum activity at 40 $^{\circ}$ C (Optimum temperature) against both standard enzymes. The inhibitor activity of CAE slightly increased at 20 $^{\circ}$ C and moderate activity was observed at 30 $^{\circ}$ C and 50 $^{\circ}$ C against both enzymes. The inhibitor activity totally lost at 70 $^{\circ}$ C, indicating the denaturation of active proteins at this temperature.

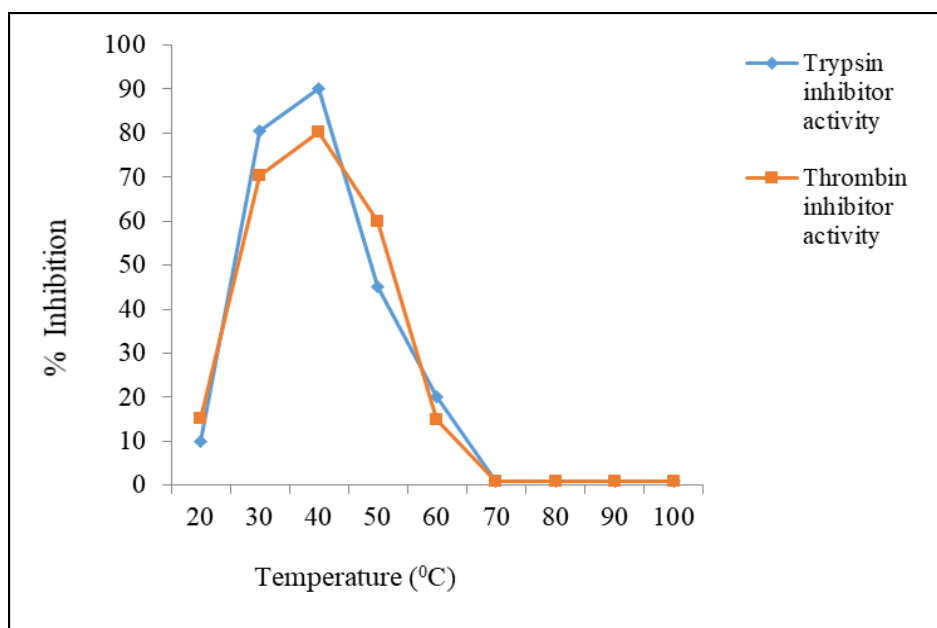


Fig 4: Effect of different temperature on CAE for anti-thrombin and anti-trypsin activities.

Effect of pH on CAE for anti-thrombin and anti-trypsin activity

Different pH buffers were used to assess their effect on active proteins present in CAE. Fig 5 shows that CAE exhibited inhibitor activity against thrombin and trypsin at wide range of pH indicating active proteins present in

CAE of *W. coagulans* fruits are stable at different pH. The optimum pH of CAE was observed between 7 and 8 against both enzymes. Inhibitor activity slightly increased from pH 4 up to optimum pH, decreased step by step with increasing pH value and minimum activity was observed at pH 11.

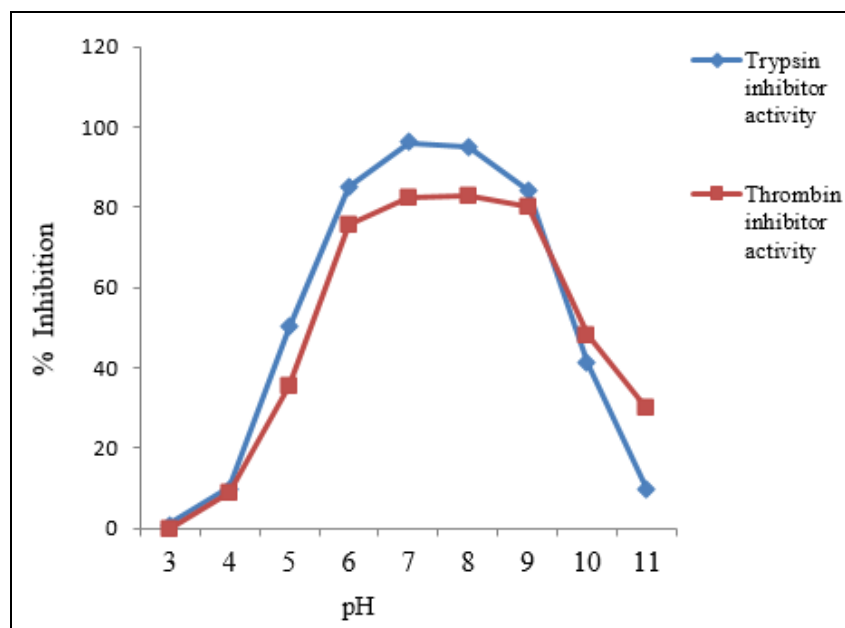


Fig 5: Effect of different pH on CAE for anti-thrombin and anti-trypsin activities.

Discussion

To search blood anticoagulant activity from plant resources we obtained ten different medicinally important tissues of plants from local market of Ayurveda medicine and they were authenticated by taxonomists. Tissues were properly dried, pulverized into fine powders and aqueous extracts (AE) were prepared for extraction of proteins. During preparation of AE we attempted separation of polyphenol compounds from protein molecules by the addition of 1% PVP in extraction buffer. PVP binds and precipitates to polyphenolic compounds and therefore it is widely used in extraction buffer for isolation of proteins from biological samples [17]. AE of all plants were allowed to screen as blood anticoagulant activity with human lyophilized plasma by using different coagulations tests (aPTT, PT and TT). Out of all samples we found that CAE of *W. coagulans* fruits has excellent activity against all coagulation tests (Table 1). In parallel with blood anticoagulant activity, CAE exhibited prominent anti-trypsin activity on X-ray film (Fig 1). Results of aPTT and PT tests suggest that proteins from CAE of *W. coagulans* fruits have efficacy to restrict both intrinsic as well as extrinsic pathway of blood clotting. The both aPTT and PT test are conducted to discriminate the effects of test candidate between the intrinsic and extrinsic pathway. The anti-thrombin activity signifies that, the proteins from *W. coagulans* fruits able to arrest the formation of fibrin clots and the conversion of fibrinogen into fibrin and inhibit thrombin mediated formation of platelet aggregation. From inhibitor assay against thrombin (Fig 3) it has been confirmed that proteins from *W. coagulans* fruits inhibit thrombin in dose-dependent manner with minimum concentration. Few results are available in literature that similar to our study. A novel anticoagulant protein (E-II-1) purified in earlier study from *Hypomesus olidus*, a unique freshwater fish in northern China has significant potential for dose-dependent inhibition of thrombin [18]. Chien [19] studied a recombinant nematode anticoagulant protein c2 (rNAPc2) isolated from hookworm *Ancylostoma caninum* exhibit anticoagulant activity by fX-dependent inhibition of the TF/FVIIa complex. Novel anti-coagulant proteins *W. coagulans* fruits can become an alternative anticoagulant drug suitable for utilization at commercial level as compared to already available anticoagulant drugs which exert some sort of side effect. Standard thrombin is an expensive; therefore a serine protease like trypsin was used for visualization of thrombin inhibitor isoforms from CAE by GXCP method [15]. Six different trypsin inhibitor isoforms (TI1, TI2, TI3, TI4, TI5 and TI6) were observed on X-ray films (Fig 2). This number of isoforms of TIs may have increased, if electrophoresis gel length would have increased during experiment. We have hypothesized that those TI isoforms also acts as thrombin inhibitors and it was quit proved by experiments of temperature and pH effects on CAE. Therefore it was concluded that multifunctional proteins are available in CAE of *W. coagulans* fruits. We have not found clear and distinct separation of proteins on native PAGE, so that SDS-PAGE has been used to achieve proper separation of different proteins. The separation of distinct proteins has not been satisfactorily observed on SDS-PAGE this may be due to association of these proteins with other non-proteinaceous molecules (Fig 2). Various phytochemicals such as tannins, phenolic compounds, steroids, anthraquinone and triterpenoids have been reported in *W. coagulans* [20]. The pattern of trypsin inhibitors from *W. coagulans* fruits detected on X-ray film was quite similar to isoform of trypsin inhibitors present in *Pigeon pea* seeds [15]. Trypsin inhibitors assay showed that a strong inhibitor exist in *W. coagulans* fruits. 50µg/ml protein was near about sufficient for inhibition of 10U trypsin. Trypsin inhibitors from

CAE of this plant have more efficacy than trypsin inhibitor purified from *Phaseolus vulgaris* cv. "gold bean" that shows dose-dependent inhibition of trypsin with an IC_{50} value of $0.4 \mu M$ [21]. The effects of temperature and pH on trypsin and thrombin inhibitors suggested that same isoform of proteins are involved in inhibition of both enzymes. The result of temperature effect showed that thermo labile/ thermo sensitive active proteins from CAE are responsible for activities but, from the result of pH effect it was quite cleared that proteins are active wide pH level. The optimum temperature and pH of CAE were near about equal to physiological temperature and pH ($37^{\circ}C$ and 7.4) therefore CAE of *W. coagulans* fruits somewhat suitable for involvement in anti-coagulant drug preparation. Based on different activities it was confirmed that multifunctional proteins are available in *W. coagulans* fruits.

Conclusion

Based on in-vitro results, we concluded that CAE of *W. coagulans* fruits exhibit anti-coagulant, anti-thrombin and anti-trypsin activities. Inhibitory effects are due to the presence of active proteins in *W. coagulans* fruits. Inhibitory assay confirmed that active proteins have an excellent potential for inhibition of thrombin and trypsin activities. From our visual observation on X-ray film revealed that six or more than that active isoforms of proteins exist in *W. coagulans* fruits. The optimum pH and temperature of active proteins are near about equal to physiological pH and temperature that indicating the proteins from *W. coagulans* fruits are suitable for preparation of anti-coagulant drugs. Based on our observations the active proteins are thermo labial and have multifunctional properties. These findings have considerable significance, if future studies establish the purification and characterization of active proteins showing anti-coagulant activity at lower concentration and able to reduce harmful incidence in thrombotic disorders.

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Conflict of interest

The authors declare that there is no conflict of interest.

Abbreviations

CAE: Crude aqueous extract; aPTT: Activated Partial Thromboplastin Time; PT: Prothrombin Time; TT: Thrombin Time Test; GXCP: Gel X-ray film contact print method; TI: Trypsin Inhibitor; TIs: Trypsin inhibitors; AE: Aqueous extract.

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