



## In Vitro Cardiac Risk Reduction Bioactive Substance from the Seedlings of *Echinochloa frumentacea* and *Panicum sumatrance*

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### Abstract

Drug replacement with appropriate remedies are required to eradicate existing problem in thrombosis. It rectified with partially purified thrombolytic enzyme from the seedling of *Panicum sumatrance* and *Echinochloa frumentacea*. The cell free extract of *P. Sumatrance* and *E. frumentacea* seedling was partially purified by dialysis method and observed 36 and 38 KDa molecular weight protein. The isolated enzyme exhibited optimum blood clotting activity at pH 7 at 30°C. Thrombolytic model used to screen the clot lysis potential of bioactive metabolites and Proteolytic enzyme, extracted seedling. Streptokinase treatment was considered as confirmative and saline water as blank control for this study. The Extracts exhibited a good hemolysis activity and Anticoagulant effect with a maximum activity at 20µl concentration in in-vitro condition.

**Keyword:** *P. sumatrance*, *E. frumentacea*, Antithrombosis, Cardiac risk, Phytoremedy.

### Introduction

Clotting in blood circulation attack the smooth function of heart it leads to death. Blood clotting is caused different kinds of factors (thrombosis), it increase each year for heart problem. Approximately 1,000,000 patients affected by lung blood clot in USA alone. World Health Organization (WHO) report in 2008<sup>[1]</sup>, 17.3 million people death recorded in every year by cardiovascular diseases (CVDs). Thromboses are evolved due to reaction between the platelets and blood vessels, which leads to cause cardiovascular diseases<sup>[2]</sup>. Platelets play major role in blood clot, it reduce the cardiovascular integrity. Some time the platelets are imply on pathological progression of

atherosclerotic lesions and arterial vascular thrombosis<sup>[3]</sup>. Aggregation of platelet is developing the uncontrolled activity in arterial thrombosis; it leads to cause the routine function of heart<sup>[3]</sup>. This kind of diseases is controlled using antiplatelet agents<sup>[4]</sup>. Development of aspirin is valuable recovery mechanism of ischemic cardiovascular disorders, it develop hemorrhagic activity and top of gastrointestinal bleeding as disadvantages<sup>[3]</sup>. From plasminogen, plasmin production initiated by thrombolytic drugs, it defend hemostatic as well as target Thrombo emboli are broken down<sup>[4]</sup>. Plasmin initiated the activity of fibrin, it destruct the blood clot<sup>[5]</sup>. Phytochemical isolated from various plant substances are useful to recovery of coagulant,

platelet and fibrinolytic activity and there is support consuming such kind of foods in suspected heart patient to avoid heart attack<sup>[6]</sup>. Rutin is an one kind of phytochemicals integrated with the foods. Flavanol obtained in Rutin initiates dissociation of formation of thrombus in blood vessels. Rutin was found to be a good anticoagulant<sup>[7]</sup>. Bacterial organism such as *Bacillus natto* secreted the natto enzyme it hydrolyse the thrombi as well as convert plasminogen to plasmin.

*P. sumatrance* is cultivated in tropics, used as constant food in financially poor background people in different part of global. This kind of cereals showed various kinds of phytochemicals during screening it acts as antioxidants, intermediate free radical helps to arrest oxidation of fatty acids and oils<sup>[8]</sup>. *E. frumentacea* (millet) is a species of Echinochola, utilized for traditional and novel methods<sup>[9]</sup>. *E. frumentacea* having phytochemicals are inhibits digestive tract enzymes. From the above analysis, it is clear that formation of blood clot in the blood vessel leads to a major disease known as CVD (Cardiovascular disease) and the blood clots are generally lysed using fibrinolytic drugs. Thrombolytic drugs are produced from various plant sources of which these two millets (*P. sumatrance* and *E. frumentaceae*) are seemed to be much effective since they both have a wide range of protective compounds that reduces the risk of CVD

## Materials and Methods

**Screening of Protein:** From the seedling of chosen plants were extracted protein using filtrate by method of Bradford<sup>[9]</sup>. Culture filtrate (1 ml) with mixed 5 ml of CBB and observed optical density value at 595 nm in a Beckman DU-50 Spectrophotometer. An inoculated microbes free medium used as blank and BSA as standard for this experiment.

**Fibrinolytic Enzyme Analysis:** In this assay 0.5 ml of 1% casein was added to haemoglobin suspension (0.1 M tris-HCl, pH 8.0). Then 0.5 ml of seedling extracts were added separately and incubated at 37 °C for half an hour. At the end of incubation reaction stopped by adding 1.0 ml of 0.15 %

trichloro acetic acid. Tyrosine was observed from the reaction contained and recorded at 570 nm. From this value calculated single fibrinolytic enzyme Unit has release 1 $\mu$  mole tyrosine per minute.

**Separation of protein (SDS-PAGE):** Protein separated from the seedling extracts using SDS-PAGE by the method of Laemmli<sup>[10]</sup>. Weight of protein determined with help of 100kDa ladder.

**Screening of Caseinolytic activity:** Seedling extracts streaked on casein agar medium for screening of casein activity. After streaking Casein plate at 37°C for a day, Opaque zone formation confirmed as positive for casein activity after incubation period.

**Determination of fibrinolytic activity:** Isolated protein stained with silver staining method of Blum. After staining, the gels were stored in 7 % (v/v) acetic acid. After staining protein solution fibrinogenolytic activity was analysed by modified fibrinogenolytic assay. Fibrinogen (200 $\mu$ l) mixed with the protein sample (60 $\mu$ l of 0.2 mol/l) and incubated at 37°C and read value at one hour interval upto 3 hours.<sup>[11]</sup>

**Biochemical analysis** – Biochemical changes were observed at different ph and temperature. The effect of various fibrinolytic enzyme inhibitors, relative protease activity, metal ions (eg, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Al<sup>3+</sup>, Fe<sup>3+</sup>, Na<sup>+</sup>, Zn<sup>2+</sup>, Hg<sup>2+</sup>, and Cu<sup>2+</sup> [5mM]) and RBC haemolysis were prepared according to Chinese Pharmacopoeia, 2010<sup>[16]</sup>. Hemolysis effect was determined by the formula as following:

$$\text{Hemolysis rate (\%)} = (\text{OD}_t - \text{OD}_{nc}) / (\text{OD}_{pc} - \text{OD}_{ncp}) \times 100$$

**Anticoagulant analysis:** Blood sample (1 ml) with different doses of enzyme (25U, 50U, 75U, 100U) was added with 1 mL Tris-HCl and incubated at 37°C for 30 min for observation of anticoagulant effects.

## Results

**Growth studies of *P. sumatrance* and *E. frumentacea* seedlings** - *P. sumatrance* and *E. frumentacea* seeds were grown in petridishes for 4 days. The germinated seeds was harvested at every

24h interval and its fibrinolytic activity was measured (Table 1).

**Table 1** Growth Studies of *P. Sumatrance* and *E. Frumentacea* Seeds

S.No	Selected seeds	Germination of seeds		
		1 day	2 day	3 day
1	<i>P. sumatrance</i>	3mm	12mm	20mm
2	<i>E. frumentacea</i>	2mm	6mm	25mm

**Screening of fibrinolytic activity on selected germinating seeds-** Screening of fibrinolytic activity of *P. sumatrance* and *E. frumentacea* seeds on Skimmed milk in the ratio of 0.1 were performed. The result showed higher hydrolytic activity of 19mm on *E. frumentacea* in casein plate assay. Comparatively 17mm produced highest hydrolytic zone than *P. sumatrance* germinating seeds.

**The Partial characterization of *P. sumatrance* and *E. frumentacea* seedling extract by TLC -** The *P. sumatrance* and *E. frumentacea* seedling extract (5 µl) loaded on thin layer plate and separated with solvents of hexane, ethyl acetate and acetic acid (10:5:0.5 ratio) and observed under UV 240nm and 360nm (Table-2).

**Table 2** The Partial Characterization of *P. Sumatrance* and *E. Frumentacea* Seedling Extraction

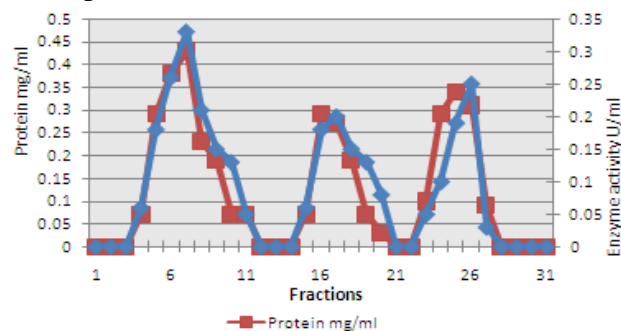
Normal Light	<i>P. sumatrance</i>		<i>E. frumentacea</i>	
	360nm	240nm	360nm	240nm
-	0.28	-	0.21	-
-	-	0.30	0.35	-
-	0.51	0.51	-	0.46
-	0.62	-	+	Seedling extract
-	0.71	-	0.69	0.69

**Purification of fibrinolytic enzyme from *P. sumatrance* and *E. frumentacea* seedling -** The cell free extract of *P. sumatrance* and *E. frumentacea* seedling was extracted with PBS solution and precipitated with ammonium sulfate. The precipitated protein lyophilized and used to screen the activity of fibrinolysis. Observation of fibrinolysis shown in Table-3.

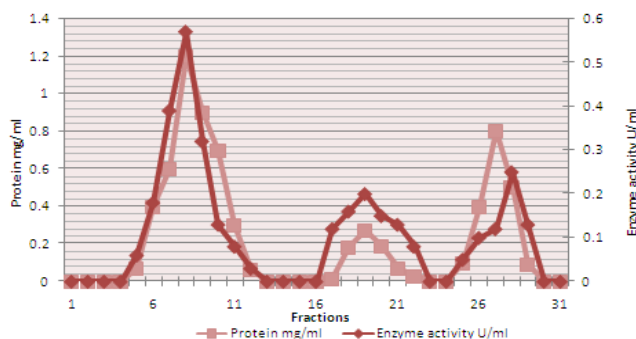
**Table 3** Purification of Fibrinolytic Enzyme from *P. sumatrance* and *E. frumentacea* Seedling

Purification stage	Activity U/ml		Protein mg/ml		Specific activity U/ml		Purification fold		Yield %	
	P.s	E.f	P.s	E.f	P.s	E.f	P.s	E.f	P.s	E.f
Crude sample	25	25	20	22	1.39	1.32	1.0	1.0	100	100
Dialyzed	18	18	13	16	1.51	1.43	1.15	1.04	76	71
Sephadex G-75	12	12	6	8	2.8	2.3	1.42	1.38	68	63

**Purification of fibrinolytic enzyme by Sephadex G-75 column chromatography -** The concentrated protein (25 mg) was loaded on Sephadex G-75 column and fractionated in 3 types with 30 ml each. Fibrin active protein observed in peak I-fractions from 15 to 38& 12 to 33 peak II-fractions from 43 to 59& 45 to 60 and peak III -fractions from 72 to 83. (Graph 1-2).

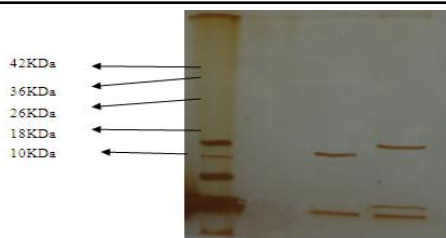


**Graph-1** Separation of fibrinolytic enzyme from *P. sumatrance* by Sephadex G-75 column chromatogram



**Graph-2.** Separation of *fibrinolytic enzyme* from *E. frumentacea* by Sephadex G-75 column chromatogram.

**Molecular mass analysis:** Isolated protein molecular mass of the fibrinolytic enzyme calculated as 36kDa and 38kDa with help of protein ladder (Fig-1).



**Fig-1** SDS-PAGE patterns of the Partial purified Fibrinolytic enzyme from *P. sumatrance* and *E. frumentacea* seedling

**Biochemical characterization of partially purified fibrinolytic enzyme from *Panicum sumatrance* and *E. frumentacea* seedling**

**Effect of pH on the activity and stability:** For the screening of pH optimum was found pH 7.0 as showed effective fibrinolytic activity. The stability of protein is good in broad range of pH 6 to pH 9 (Table-4).

**TABLE 4** Effect of pH on the Activity and Stability of the Fibrinolytic Enzyme from *P. sumatrance* and *E. frumentacea* Seedling

pH	O.D (U/ml)		Temp (°C)	O.D (U/ml)	
	<i>P. sumatrance</i>	<i>E. frumentacea</i>		<i>P. sumatrance</i>	<i>E. frumentacea</i>
pH 4	0.15	0.13	20	0.15	0.13
pH 5	0.19	0.17	30	0.20	0.17
pH 6	0.20	0.19	40	0.18	0.16
pH 7	0.23	0.22	50	0.16	0.15
pH 8	0.18	0.20	60	0.12	0.10
pH 9	0.10	0.11	70	0.10	0.09
pH 10	0.06	0.07	80	0.06	0.05

**Effect of Temperature on the activity and stability-** The activity of protein analyses performed in different temperatures (20°C to 70°C) for 50 minutes. Good responses occur in 20°C and 70°C and 100% stable on 40°C even after 350 minutes of incubation (Table – 4).

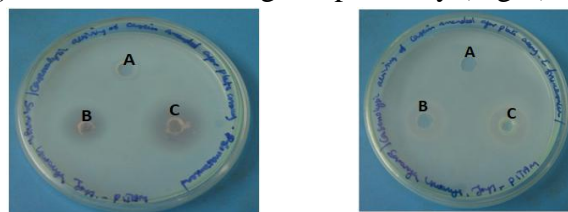
**Effect of Metal Ions and Inhibitors -** Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Fe<sup>2+</sup> metal ions were increased and stabilized with fibrinolytic enzyme. Zn<sup>2+</sup>, Cu<sup>2+</sup>, pb, Na<sup>+</sup>, and Al<sup>3+</sup> did not show any appreciable effect on enzyme activity were observed and recorded (Table-6). EDTA impact was negligible in fibrionlytc activity was observed and recorded (Table-5).

**Table 5** Effect of Various Metal Ions on Fibrinolytic Enzyme Activity

Metal Ions (5mM)	Relative Protease Activity (%)	
	<i>P. sumatrance</i>	<i>E. frumentacea</i>
Control	100	100
Zn <sup>2+</sup>	89	78
Cu <sup>2+</sup>	90	86
Mg <sup>2+</sup>	109	102
Ba <sup>2+</sup>	81	74
Ca <sup>2+</sup>	121	116
Na <sup>+</sup>	93	89
Al <sup>3+</sup>	79	76
Pb	65	60
Fe <sup>3+</sup>	101	98
EDTA	118	110

**Caseinolytic fibrinolytic enzyme purification by *P. sumatrance* and *E. frumentacea* seedling-**

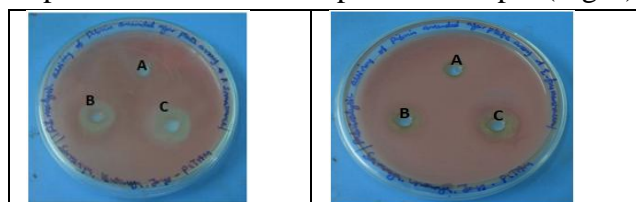
Fibrinolytic enzyme was indeed produced by *P. sumatrance* and *E. frumentacea* seedling. When the dialysis and purified Fibrinolytic enzyme of *P. sumatrance* and *E. frumentacea* seedling hydrolysed casein showed 19 and 17 mm zone and purified sample 23 and 20mm as *P. sumatrance* and *E. frumentacea* seedlings respectively (Fig-2).



A-Control; B-Dialysis sample C-Partial purified enzyme

**Fig-2** Plate assay for caseinolytic activity of processed enzyme

**Fibrinolytic activity of *P. sumatrance* and *E. frumentacea* seedling partially purified sample on fibrin plates** – Isolated protein hydrolyzed clear zone were measured as 14 and 17 mm in dialysed sample and 21 and 24 mm purified sample (Fig-3).



A-Control; B-Dialysis sample C-Partial purified enzyme

**Fig 3** Fibrinolytic activity of *P. sumatrance* and *E. frumentacea* seedling from fibrinolytic enzyme

**Anticoagulant effect:** Effects anticoagulant activity of fibrinolytic enzyme observe in meagre dose (5U) had no notable effect on clot of human blood. When increase the dose above 200U it involved blood clott, it observed *P.sumatrance* anticoagulant more activity than *E. frumentacea* .

**Hemolysis:** Various concentrations of *P. Sumatrance* and *E. frumentacea* seedling fibrinolytic enzyme not have shown any remarkable change in hemolysis and agglutination against human erythrocyte cells. Hemolysis rates of high concentration fibrinolytic enzyme (30U and 40U) were a little more than 3% (3.23% and 3.67%, respectively), but low concentration fibrinolytic enzyme (10U and 20U) did not hemolyze erythrocytes obviously, and their hemolysis rates were less than 5%.

### Discussion

Fibrinolytic therapy is modern method used to safe and effective methods from food-grade microorganisms<sup>[12]</sup>. It is better than plant isolated protein and alternate method for eliminate the risks of thromboembolic diseases<sup>[13]</sup>. In this study, *P.sumatrance* and *E. frumentacea* plants can inhibit the extrinsic coagulation system, which may be the key to antithrombotic and thrombolytic activity. Low dose group (40 U), showed less impact on bleeding and does not affect the body's normal clotting mechanism. Therefore, low dose *P.sumatrance* and *E. frumentacea* plants are recommended for the long term use to prevent thrombosis.

Antithrombotic activity of fruits and vegetables is scarce, few reports available to inhibit platelet aggregation by induce synthesis of ADP and arachidonic acid<sup>[11]</sup>. Fibrinolytic enzyme from *P.sumatrance* and *E. frumentacea* seedling calculated as 34.0 kDa by gel filtration, which was close to the value SDS-PAGE (34kDa). The optimum temperature and pH were 70°C and 10.5, respectively, and the molecular weight was 28,200 as calculated using SDS method. Usama and Ibrahim<sup>[12]</sup> reported gel filtration chromatography showed *Rhizomucormiehei*, Fraction I was weakly

proteolytic, fraction II showed proteolytic and fibrinolytic activity. The *P. sumatrance* and *E. frumentacea* seedling of fibrinolytic enzyme showed broad pH at 7.0 optimal temperature of the enzyme was 30°C. It clarifies the availability of hydrosulfuryl and metal near the active center of the enzyme<sup>[14]</sup>.

### Conclusion

The present findings concluded that *P.sumatrance* and *E. frumentacea* plants seedling and bioactive metabolites has effective fibrinolytic activity. *P. sumatrance* and *E. frumentacea* seedling showed lyse globulin, relieve thrombus symptom on healthy human blood, elongate bleeding and clotting time, and also had an anticoagulant. *P. sumatrance* and *E. frumentacea* seedling has been used to prepare the drug to control thrombosis relevant diseases.

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### Reference

1. WHO, "Report of the WHO Technical Reference Group", WHO Headquarters, Geneva, Switzerland, 2008.
2. V.N. Gordon, "A Developmental Perspective", The Personnel and Guidance Journal, 1981, Vol: 59: Pp: 433–439.
3. S. G. Davies, Thomas, S. E, and P.F. Gordon, General and Synthetic Methods, 1986, 8, 312.
4. S. Johnson, "Known knowns and known unknowns: risks associated with combination antithrombotic therapy," Thrombosis Research, 2008, vol. 123 (1), Pp. S7–S11.
5. J.L. Zehnder, "Drugs used in disorders of coagulation. In: *Basic and Clinical Pharm*", Tata-McGraw-Hill publishers, 2009, 11th ed., Pp.597.

6. PN. Bennett and ML. Brown, “*Clinical Pharmacology*”, Churchill Livingstone Publishers, New York, 2003, 9th ed., Pp.28.
7. V.Kuntić, I. Filipović, Z. Vujić, “Effects of Rutin and Hesperidin and Their Al(III) and Cu(II) Complexes on *in Vitro* Plasma Coagulation Assays”, *Molecules* 2011, 16, 1378-1388.
8. J. Vanithasri, S. Kanchana, G. Hemalatha, C. Vanniarajan, and M. SahulHameed, “Role of millets and its importance in new mellinium”, *Int.J. Food Sci. Technol.* 2012, Vol- 2(1):35-47.
9. MM. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry* [01 May 1976, 72:248-254.
10. U.K. Lammeli, “Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature*, 1970, vol: 227, Pp: 680–685.
11. M. Fujita, K. Hong and Y. Ito, “Transport of nattokinase across the rat intestinal tract” *Biol Pharm Bull* , 1995, 18(9):Pp.1194–1196.
12. HA. Guglielmone, AM. Agnese, SC. Nunez Montoya, and JL Cabrera, “Anticoagulant effect and action mechanism of sulphated flavonoids from *Flaveriabidentis*”, *Thromb Res*, 2002, Vol: 105, Pp.183-188.
13. K. Matsubara, H. Sumi, K. Hori, and K. Miyazawa, “Purification and characterization of two fibrinolytic enzymes from a marine green alga, *Codiumintricatum*”. *Comp Biochem Physiol Biochem MolBio*, 1998, Vol (1119):Pp.177–181.
14. F. Usama, Ali and Z.M. Ibrahim, “Production and some properties of Fibrinolytic enzyme from *Rhizomucormiehei* (Cooney & Emerson) Schipper”, *Journal of applied science Research*, 2008, 4(7): Pp.892-899.