Study of invitro thrombolytic activity of different extracts of *Syzygium cumini*

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Abstract
The present study was carried out to investigate the invitro thrombolytic activity of leaf and seed extracts of *Syzygium cumini*. The thrombolytic activity of different concentration of extracts was compared with the standard drug Streptokinase. The blood collected from human volunteers was taken as test sample for carrying out thrombolytic activity. 800ug/ml leaf extracts of *Syzygium cumini* showed the mean % of clot lysis as 57.07%+/-0.76, 71.42%+/-0.75 & 86.99%+/-1.33 after incubation for 24hrs, 48hrs, and 72hrs respectively at 37^0C. 800ug/ml seed extracts of *Syzygium cumini* showed the mean % of clot lysis as 44.93%+/-2.49, 66.58%+/-1.41, & 83.32%+/-2.27 after incubation for 24hrs, 48hrs, and 72hrs respectively at 37^0C. The mean % clot lysis for the standard drug streptokinase was found to be 75.81% +/-0.71, 88.54%+/-1.02, and 93.04%+/-3.69 after incubation for 24hrs, 48hrs, and 72hrs respectively at 37^0C.

Keywords: Aqueous extract, Syzygium cumini, Control, Streptokinase, In-vitro thrombolytic activity.

Introduction
Thrombosis is defined as the formation of a clot inside a blood vessel, obstructing the flow of blood through the circulatory system. Blood clotting occurs as a result of complex interactions between activation of the coagulation factors, fibrinolytic systems, vascular endothelium, and of the cellular mechanisms, the interaction results in to a clot.[1] Platelets play an important role in the maintenance of cardiovascular integrity, as they are used to control bleeding in the body parts after injury through formation of blood clots.[2] Arterial thrombosis due to uncontrolled platelet aggregation is critical leading to fatal disorders like deep vein thrombosis, pulmonary emboli, strokes and heart attacks.[3] Thrombolytics are agents used to dissolve the fibrin of blood clot and thereby dissolve the formed clot inside blood vessel; therefore, thrombolytic agents are used in the treatment and/or prevention of cardiovascular thrombotic diseases by removing formed clots. Alteplase, antistreplase, streptokinase, urokinase and tissue plasminogen activator (tPA) are the mainly used drugs having thrombolytic activity to dissolve clots.

Some other anticoagulant drugs like heparins, vitamin K antagonists, and their derivatives have used for decades in the clinical treatment of thrombosis. But these drugs have there own demerrits. Though aspirin is one of the antithrombotic agent which still provides an effective secondary prevention of ischemic cardiovascular disorders, this drug can produce hemorrhagic events and upper gastrointestinal bleeding as major drawbacks. During the past years, several researches had been carried out for novel compounds having thrombolytic activity that can be used in treatment of disease.[4] Most of the thrombolytic agents used now a day still have significant drawbacks, like large doses to be maximally effective, limited fibrin specificity, bleeding tendency and high cost. Due to drawbacks of available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs. Therefore plants can be explored as an alternative to the thrombolytic drugs.[5] Therefore the leaves, seeds, twigs, stem, bark and underground parts of plants are most often used in traditional medicines for treatment of different diseases.[6] Presently, in different parts of the world, more and more research has been carried out towards exploitation of higher plant products as novel chemotherapeutants due to their non phytotoxicity, systemicity, easy biodegradability and stimulatory nature. Phytochemicals like alkaloids, tannins, saponins, flavonoids etc are naturally occurring in the medicinal plants that have defense mechanism and protect the plant from various diseases and other environmental factors but recent research demonstrates that phytochemicals can have different pharmacologicl activity and used in treatment of humans against diseases.[7]

Syzygium cumini L. belongs to the family of Myrtaceae. It is a large evergreen tree of the Indian Subcontinent localy known as Jamun. It is having presence in Thailand, Philippines, Madagascar, and West Indies, East and West Africa and some subtropical regions including Florida, California, Algeria and Israel. The major phytoconstituents reported to be present in the plant extract contain vitamin C, gallic acid, tannins, anthocyanins, cyanidin, petunidin, malvidinglucoside and other components.[8] The stem bark of *S. cumini* contains butulinic acid, *ß*-sitosterol, friedelin, epi-friedelanol, D-glucoside, kaempterol-3-O-glucoside, quercetin, myricetin, astragalin etc.[9] The seeds of *S. cumini* are used in traditional medicine as astringent, diuretic, hypoglycaemic, anti-inflammatory, antipyretic, psychopharmacological, hypolipidaemic, and antioxidant. Hence, in the present study invitro thrombolytic activity is going to be investigated by using different concentration of leaf and seed extracts of S*yzygium cumini* in search of a better thrombolytic agent.[10]

Materials and Methods
Blood sample
Whole blood (3ml) was drawn from healthy human volunteers (n=10) without a history of oral contraceptive or anticoagulant therapy. From which 500µl (0.5ml) of blood

was transferred to each of the previously weighed micro centrifuge tubes to form clots.

Syzygium cumini

The leaves and seeds of *syzygium cumini* were collected from in and around the villages of Salipur, Cuttack, Odisha during the month of June.

Preparation of aqueous extract of different parts of *syzygium cumini*

Fresh different parts of *Syzygium cumini* plants were collected and washed properly with distilled water. The seed after separation from the berry and leaf sections were shade dried at room temperature for 15 days. Dried parts were uniformly grinded using mechanical grinder. The dried powder of plant material was extracted in distilled water. Ten grams of ground plant material was soaked in 100ml of distilled water in a round bottom flask and loaded in the heating mantle at a temperature of 70°C for 15 minutes for boiling. After15 min temperature was reduced to 40°C and kept in that temperature for 1hour. Then the mixture was filtered using whatmann filter paper number 1. Each grounded plant materials were extracted separately. The dried extracts were weighed and stored in air tight container with necessary markings for identification and kept in refrigerator (0-4°C) for future investigation.[11]

Streptokinase (SK)

Commercially available lyophilized Stukinase (Streptokinase) vial (Samarth pharmaceutical Ltd.) of 15, 00,000 I.U., was collected and 5ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U.) was used for in vitro thrombolysis study.[12]

Procedure for clot lysis

3ml venous blood was drawn from healthy volunteers and distributed in five different labeled, pre weighed sterile micro centrifuge tube (0.5ml/tube) and incubated at 37°C for 45 minutes.

After clot formation, serum was completely removed without disturbing the clot using micro pipette and each tube having clot was again weighed to determine the total clot weight of every individual (clot weight = weight of clot containing tube - weight of empty tube alone).

Various concentrations of leaf and seed crude extract of *Syzygium cumini* i.e. 200µg/ml, 400µg/ml, 600ug/ml and 800µg/ml were tested at various time intervals including; 24hrs, 48hrs and 72hrs duration of incubation at 37^0C for maximum clot lysis.[13] 100 µl of streptokinase was added as a standard (30,000 I.U.) and 100 µl of distilled water was added as a control to the preweighed blood clots along with different time of incubation i.e. 24hrs, 48hrs, and 72hrs at 37°C. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The thrombolytic activities of all extracts were evaluated. The experiment was repeated 10 times with the blood samples of 10 volunteers.

Statistical analysis

To verify the results obtained the statistical analysis of the thrombolytic effects of the plant extracts were carried out by using SPS software and the data obtained is represented as mean ± standard deviation. The statistical analysis used is paired t-test for calculation of significant difference between the control, standard and test results.

Fibrinolytic properties

Fibrin plate method was used to determine the fibrinolytic activity by using plasminogen free fibrin plate method.[14] The media for fibrinolytic activity was prepared by adding fibrinogen solution made using 2.5 ml of 1.2% (w/v) human fibrinogen in 0.1 M sodium phosphate buffer (pH 7.4) with 10 U of thrombin solution, and 1% agarose. The fibrin plates were heated at 80°C for 30 min to eliminate other fibrinolytic factors. Paper disks of 6 mm diameter were prepared by Whatsman Filter paper number 1. The fibrinolytic activity was studied by dipping the paper discs in respective sample extract and carefully placed them on the plate and incubated at 35°C for 24 h. Streptokinase (30,000 IU), phosphate buffer (20 mM) were used as positive control and blank respectively. The fibrinolytic activity was determined by measuring the clear zone diameter formed around the paper disc at six different positions.

Results

Thrombolytic activity of streptokinase

100 µl of streptokinase the standard was added as a positive control (30,000 I.U.) and 100 µl of distilled water was added as a control to the preweighed blood clots along with different time of incubation i.e. 24hrs, 48hrs, and 72hrs at 37°C showed mean clot lysis of 75.81%+/-0.71, 88.54%+/-1.02 and 93.04%+/-3.69 respectively (Table 1). Similarly the control distilled water was added to blood clot and after incubation i.e. 24hrs, 48hrs, and 72hrs at 37°C showed mean clot lysis of 5.25%+/-0.49, 11.70%+/-0.69 and 19.47%+/-2.32 espectively.

Table 1: In vitro-thrombolytic activity of control (water) and standard (Streptokinase):

Concentrations of crude leaf extract	Incubation Time	Clot Lysis % (Mean+/-SD)
Standard	24hrs	75.81*+/-0.71
	48hrs	88.54*+/-1.02
	72hrs	93.04*+/-3.69
Control	24hrs	5.25+/-0.49
	48hrs	11.70+/-0.69
	72hrs	19.47+/-2.32

* The result is significant at $p < 0.01$

3.2 Thrombolytic activity of Syzygium cumini

When 100µl of different concentration of S*yzygium cumini* aqueous preparation of leaf and seed extract was added to different blood clots, it showed a good thrombolytic activity as shown in Tale 1& 2 comparable to the standard drug streptokinase. The aqueous preparation of syzygium cumini

leaf extract showed more thrombolytic activity as compared to seed extract.

The 200ug/ml leaf extract showed mean % clot lysis of 13.4 +/- 1.35, 20.7 +/-1.76 & 35.6+/-1.50 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. The 400ug/ml leaf extract showed mean % clot lysis of 26.42+/-1.25, 42.91+/-1.07 & 57.26+/-2.00 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. The 600ug/ml leaf extract showed mean % clot lysis of 42.08+/-0.42, 53.77+/-1.61 & 69.06+/-0.76 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. The 800ug/ml leaf extract showed mean % clot lysis of 57.07+/-0.76, 71.42+/-0.75 & 86.99+/-1.33 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. Similarly 200ug/ml seed extract showed mean % clot lysis of 11.08+/-0.58, 20.97+/-0.59& 31.50+/-1.43 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. The 400ug/ml seed extract showed mean % clot lysis of 26.42+/-1.25, 42.91+/-1.07& 57.26+/-2.00 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. The 600ug/ml seed extract showed mean % clot lysis of 35.40+/-0.71, 53.25+/-0.91& 63.74+/-2.23 at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C. The 800ug/ml seed extract showed mean % clot lysis of 57.07+/-0.76, 71.42+/-0.75 & 86.99+/-1.33at incubation time of 24hrs, 48hrs, and 72hrs resp at 37°C.

Table 2: In vitro-thrombolytic activity of aqueous Leaf extract of *Syzygium cumini*

Concentrations of crude leaf extract	Incubation time	Clot Lysis % (Mean+/-SD)
200ug/ml	24hrs	13.4 +/- 1.35
	48hrs	20.7 +/-1.76
	72hrs	35.6+/-1.50
400ug/ml	24hrs	26.42+/-1.25
	48hrs	42.91+/-1.07
	72hrs	57.26+/-2.00
600ug/ml	24hrs	42.08*+/-0.42
	48hrs	53.77*+/-1.61
	72hrs	69.06*+/-0.76
800ug/ml	24hrs	57.07*+/-0.76
	48hrs	71.42*+/-0.75
	72hrs	86.99*+/-1.33

***** The result is significant at $p < 0.01$

Table 3: In vitro-thrombolytic activity of aqueous seed extract of *Syzygium cumini*

Concentrations of crude seed extract	Incubation time	Clot lysis % (Mean+/-SD)
200ug/ml	24hrs	11.08+/-0.58
	48hrs	20.97+/-0.59
	72hrs	31.50+/-1.43
400ug/ml	24hrs	21.45+/-0.57
	48hrs	43.43+/-0.93
	72hrs	54.63+/-2.37
600ug/ml	24hrs	35.40#+/-0.71
	48hrs	53.25#+/-0.91
	72hrs	63.74#+/-2.23
800ug/ml	24hrs	44.93#+/-2.49
	48hrs	66.58#+/-1.41
	72hrs	83.32#+/-2.27

The result is significant at $p < 0.05$

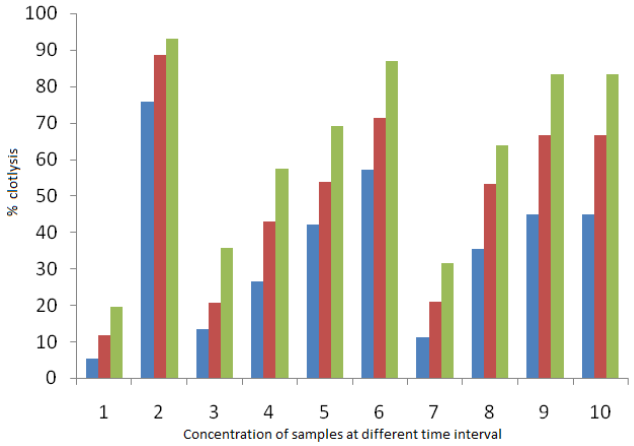

Fig.1: Bar diagram of in vitro-thrombolytic activity

1. Control at 24hrs, 48hrs, and 72hrs of incubation
2. Standard at 24hrs, 48hrs, and 72hrs of incubation
3. 200ug/ml leaf extract at 24hrs, 48hrs, and 72hrs of incubation
4. 400ug/ml leaf extract at 24hrs, 48hrs, and 72hrs of incubation
5. 600ug/ml leaf extract at 24hrs, 48hrs, and 72hrs of incubation
6. 800ug/ml leaf extract at 24hrs, 48hrs, and 72hrs of incubation
7. 200ug/ml seed extract at 24hrs, 48hrs, and 72hrs of incubation
8. 400ug/ml seed extract at 24hrs, 48hrs, and 72hrs of incubation
9. 600ug/ml seed extract at 24hrs, 48hrs, and 72hrs of incubation
10. 800ug/ml seed extract at 24hrs, 48hrs, and 72hrs of incubation

Fibrinolytic properties

The diameter of clear zone measuring 6.4+/-0.7 mm and 6.3 +/- 0.7 mm were formed around the disc loaded with leaf and seed extract respectively. Whereas in the positive control (disc having streptokinase) a zone measuring about 6.86 +/- 0.05 mm diameter was observed. There was no zone formation in the blank. This indicates the specificity of the plant extract towards fibrinogen.

Conclusion

The different concentration of leaf and seed extracts of *Syzygium cumini* can be used to design anti thrombotic agent due to its moderate thrombolytic activity as compared to the standard drug streptokinase. Further research work is needed to isolate the bioactive components or secondary metabolites present in the extract that will provide more precise and accurate thrombolytic activity. The present in vitro thrombolytic study demonstrated that information of

using plants in folk medicine can be as effective as modern medicine without the side effects of synthesised chemical medicine. The herbal preparation containing leaf extract of plant *Syzygium cumini* can be used effectively as a thrombolytic agent instead of using chemically prepared thrombolytic drugs to avoid threatened side effects.

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Conflict of interest
None.

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