

## Thrombolytic and Cytotoxic Activities of *Terminalia bellerica* Roxb.

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### Abstract

The present study was designed to investigate the thrombolytic activity and cytotoxic potential of the methanolic extract of bark of *Terminalia bellerica* Roxb. The cytotoxicity was assessed with the brine shrimp lethality bioassay and thrombolytic effect with human blood. The brine shrimp lethality bioassay was used to evaluate cytotoxicity ( $LC_{50} = 3.21 \mu\text{g/ml}$ ) compared to vincristine of sulphate ( $LC_{50} = 0.512 \mu\text{g /ml}$ ). It was also evaluated as thrombolytic agent as compared to streptokinase. It has significant thrombolytic activity (32.95%) compared to standard streptokinase (70%).

**Key words:** Thrombolytic, Cytotoxic, Clot lysis, *Terminalia bellerica*.

### Introduction

Thrombosis is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system. When a blood vessel is injured, the body uses platelets (thrombocytes) and fibrin to form a blood clot to prevent blood loss. Even when a blood vessel is not injured, blood clots may form in the body under certain conditions (Furie and Furie, 2008; Handin, 2005). In the patient admitted to hospital, thrombosis is a major cause for complications and occasionally death. In UK, for instance, the Parliamentary Health Select Committee heard in 2005 that the annual rate of death due to thrombosis was 25,000, with at least 50% of these being hospital-acquired (Hunt, 2008).

Streptokinase (SK) belongs to a group of medications known as fibrinolitics, and complexes of streptokinase with human plasminogen can hydrolytically activate other unbound plasminogen by activating through bond cleavage to produce plasmin. SK is used as an effective and inexpensive thrombolysis medication in some cases of myocardial infarction (heart attack) (Sikri and Bardia, 2007) and pulmonary embolism (Meneveau *et al.*, 1997). The plant represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity

has been assayed. Therefore, research activities have been focused to the phytochemical investigation of higher plants which have ethnobotanical information associated with them. Herbal preparations are used as potential source of medicine since ancient times to maintain health and regain healthy state of mind. Herbs showing thrombolytic activity have been studied and some significant observations have been reported (Giuseppina *et al.*, 2004).

Natural products have served as a source for cancer chemotherapy. The brine shrimp lethality bioassay has routinely been used in the primary screening of the crude extracts to assess the toxicity towards brine shrimp. The bioassay has a good correlation with cytotoxic activity in some human solid tumors as well as pesticidal activity (Jerry *et al.*, 1998). A number of novel antitumor and pesticidal natural products have been isolated using this bioassay (Mayer *et al.*, 1982).

*Terminalia bellerica* also known as *Terminalia billirica* (Gaertn.) Roxb. (Family: Combretaceae, Bengali name- Bahera), is a large deciduous tree, 10-20 m high, with tall trunk and long horizontal branches. Leaves are 10-20 cm long, alternate, clustered at the end of branchlets, obovate or broadly elliptic, acute or acuminate, base narrowed. Flowers are greenish-yellow, small in simple axillary spikes, shorter than the leaves. Fruit is a drupe about 2.5 cm long, globose or narrowed at the base,

silky-brownish-velvety. Fruits are laxative, astringent, anthelmintic and antipyretic; useful in hepatitis, bronchitis, asthma, dyspepsia, piles, menstrual disorder, diarrhoea, coughs, hoarseness of voice, eye diseases and scorpion-sting, and are used as a hair tonic. Decoction of the green fruit is used for cough. Pulp of the fruit is useful in dysenteric-diarrhoea, dropsy, piles and leprosy. Kernel of the fruit is narcotic. Seed oil is used in rheumatism. Gum of the bark is demulcent and purgative. The triterpenoids present in the fruits possess significant antimicrobial activity. Kernel oil has purgative action and its prolonged use was well tolerated in mice ([www.mpbd.info](http://www.mpbd.info)). Fruits contain tannins, which is composed of gallotannic and ellagittanic acids, phyllembin,  $\beta$ -sitosterol, mannitol, sugars, hydrocarbons, triptriacanthone, tetratriacanthane, a hexahydroxy diphenic acid ester, ditriacanthanol, resins and a greenish yellow oil containing palmitic, stearic, oleic and linoleic acids. Presence of three glycosidal compounds, a saponin, gallic acid, ellagic acid, ethyl gallate, chebulagic acid, mannitol, glucose, galactose, fructose, rhamnose, a new cardenolides type cardiac glycoside (bellericanin) have also been reported in fruits. Heartwood and bark contain ellagic acid; bark also contains substantial amount of oxalic acid. Seed coat contains gallic acid. The triterpenoids arjungenin, tomentosic acid, belleric acid, bellericagenin, bellericagenin A, bellericagenin B, bellericoside, bellericaside A, bellericaside B and arjunglucoside I have also been isolated from this plant ([www.mpbd.info](http://www.mpbd.info)).

## Materials and Methods

**Collection and extraction:** The plant of *Terminalia bellerica* was collected at their fully matured form, in October 2012, from local area of Chittagong, Bangladesh and was identified by Dr. Sheikh Bokhtear Uddin, Associate Professor, Chittagong University, Bangladesh. After separating, the bark was taken and air dried for 10 days, and then kept in an oven at 45°C for 72 hours. 500 gm of dried powder was cold extracted with ethanol. Dried powder was soaked in methanol for 15 days, filtered off to get the methanol extract and placed in a beaker on the water bath (at 40°C - 50°C) to evaporate the solvent from the extract to afford crude extract.

**Sample preparation:** The crude extract was suspended in 10 ml distilled water and shaken vigorously on a vortex

mixer. Then the suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a filter paper. The solution was then ready for *in vitro* evaluation of clot lysis activity.

**Streptokinase (SK) solution preparation:** To the commercially available lyophilized SK vial (Polamin Werk GmbH, Herdecke, Germany) of 15,00,000 I.U., 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100  $\mu$ l (30,000 I.U) was used for *in vitro* thrombolysis.

**Specimen:** Whole blood (5 ml) was drawn from healthy human volunteers (n = 10) without a history of oral contraceptive or anticoagulant therapy. 500  $\mu$ l of blood was transferred to each of the ten previously weighed alpine tubes to form clots.

**Thromolytic assay:** Experiments for clot lysis were carried as reported earlier (Sweta et al., 2007). Venous blood drawn from healthy volunteers was transferred in different pre-weighed sterile Eppendorf tube (500  $\mu$ l/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed). Each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Each eppendorf tube containing clot was properly labeled and 100  $\mu$ l of plant extract was added to the tubes. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid obtained was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference in weight taken before and after clot lysis was expressed as percentage of clot lysis. Streptokinase and water were used as positive and negative control, respectively. The experiment was repeated several times with the blood samples of different volunteers.

$$\% \text{ clot lysis} = (\text{Weight of the lysis clot} / \text{Weight of clot before lysis}) \times 100.$$

**Statistical analysis:** The significance between % clot lysis by herbal extract by means of weight difference was tested by the paired t-test analysis. Data are expressed as mean  $\pm$  standard deviation.

**Brine shrimp lethality bioassay:** For the preparation of sea water 38 g of sodium chloride was weighed, dissolved in distilled water to make 1 liter solution and then filtered off to a get clear solution. This simulated sea

water was used for hatching of brine shrimp. The shrimp were allowed for two days to hatch and mature as nauplii (larvae). In a small beaker, measured amount of the sample was accurately weighed and dissolved in DMSO (Dimethylsulfoxide) to give a final concentration of 10 mg/ml (10 µg/µl). From the test tube containing brine shrimp nauplii, 9 test tubes were taken for the sample where each contained 5ml of seawater and 10 nauplii. These test tubes were marked from 1 to 9 for the sample. To these test tubes different concentrations (160 µg/ml, 120 µg/ml, 80 µg/ml, 60 µg/ml, 40 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml and 2.5 µg/ml) of the sample were added. Then the samples were subjected to brine shrimp lethality bioassay (Meyer et al., 1982). In this case, only 50 µl DMSO was added in 5 ml sea water containing 10 nauplii. No extract was added to prepare control solution. Measured amount of the vincristine sulphate (Techno Drugs Ltd., Bangladesh) was dissolved in DMSO to get an initial concentration of 0.512 µg/µl. 9 test tubes for the standard sample were taken where each contained 5 ml of seawater and 10 nauplii. These test tubes were marked from 1 to 9. In test tube no 1, 160 µl of the vincristine sulphate solution was added to give a concentration of

10µg/ml. In a similar way, 120 µl, 80 µl, 60 µl, 40 µl, 20 µl, 10 µl, 5 µl and 2.5 µl of the sample were added to test tubes 2, 3, 4, 5, 6, 7, 8 & 9 to give the concentration of 160 µg/ml, 120 µg/ml, 80 µg/ml, 60 µg/ml, 40 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml and 2.5 µg/ml of the standard sample solution respectively. After 24 hours, test tubes were observed and the number of dead nauplii were counted and the LC<sub>50</sub> values were calculated.

## Results

*In vitro clot lysis study:* 100 µl SK as a positive control (30,000 I.U.) was added to the clots along with 90 minutes of incubation at 37°C, showed 70% clot lysis. Clots when treated with 100 µl sterile distilled water (negative control) showed only negligible clot lysis (2.8%). The *in vitro* thrombolytic activity study revealed that *Terminalia bellerica* showed 32.95 % clot lysis. The percentage of weight loss of clot after application of extract solution was taken as the functional indication of thrombolytic activity. % Clot lysis obtained after treating clots with different concentration of sample was shown in Table 1 & Figure 1.

**Table 1. Thrombolytic activity of *Terminalia bellerica*.**

No.	Weight of empty tube (A) g	Weight of tube with clot (B) g	Weight of clot (C) (B-A) g	Weight of tube with clot after lysis (D) g	Weight of lysis (E) (B-D) g	% of clot lysis	Average % of clot lysis
01	0.82762	1.1997	0.37208	1.0385	0.1612	43.32402	
02	0.8328	1.18012	0.34732	1.0802	0.09992	28.76886	
03	0.82434	1.13404	0.3097	1.05424	0.0798	25.76687	32.95807
05	0.8264	1.22084	0.39444	1.08372	0.13712	34.76321	
06	0.83294	1.15886	0.32592	1.05402	0.10484	32.1674	

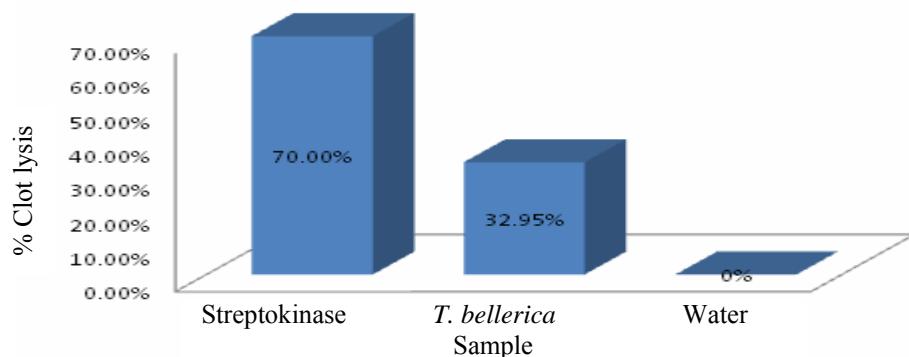


Figure 1. Clot lysis by streptokinase, water and *Terminalia bellerica*.

**Brine shrimp lethality bioassay:** In brine shrimp lethality bioassay, the methanolic extract of *T. bellerica* bark showed positive result in comparison with the positive control vincristine sulphate. By plotting the log of concentration (log C) versus percent (%) of mortality for all test samples showed an approximate linear correlation. From the graph, the median lethal concentration ( $LC_{50}$ )

was determined to check the toxic level of the extract. The crude extract of *T. bellerica* bark showed significant cytotoxic activity against brine shrimp nauplii and  $LC_{50}$  value was 3.21  $\mu\text{g/ml}$  (Table 2 & Figure 2). DMSO was used as negative control to validate the test method.

**Table 2. Cytotoxicity of methanol extract of *Terminalia bellerica*.**

Concentration $\mu\text{g/ml}$	Log C	No. of nauplii taken	No. of nauplii dead	%Mortality	$LC_{50}$ $\mu\text{g/ml}$
2.5	0.39794	10	5	50	
5	0.69897	10	6	60	
10	1	10	6	60	
20	1.30103	10	7	70	3.21
40	1.60206	10	7	70	
60	1.778151	10	8	80	
80	1.90309	10	9	90	
120	2.079181	10	10	100	
160	2.20412	10	10	100	

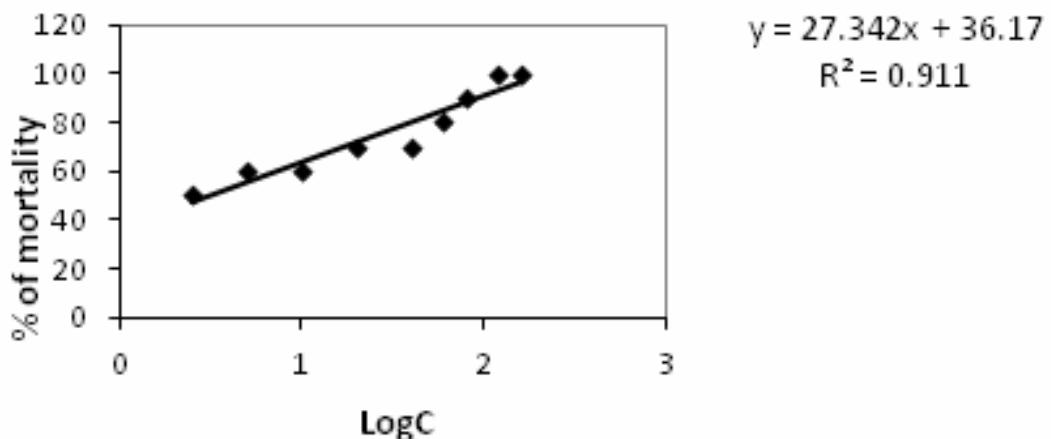


Figure 2. Determination of  $LC_{50}$  value for extract of *T. bellerica* from linear correlation between log C versus % of mortality.

## Discussion

Several thrombolytic drugs obtained from various sources are used for the treatment of thrombosis. Thrombolytic agents are used to disrupt already formed blood clots in clinical settings where ischemia may be fatal (acute myocardial infarction, pulmonary embolism, ischemic stroke, and arterial thrombosis). Thrombolytic drugs dissolve blood clots by activating plasminogen, which forms a cleaved product called plasmin. Plasmin

is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots. Because of these actions, thrombolytic drugs are also called “plasminogen activators” and “fibrinolytic drugs.” There are three major classes of fibrinolytic drugs: tissue plasminogen activator (tPA), streptokinase (SK) and urokinase (UK). While drugs in these three classes all have the ability to effectively dissolve blood clots, they

differ in their detailed mechanisms in ways that alter their selectivity for fibrin clots. Some are modified further with the use of recombinant technology in order to make these thrombolytic drugs more site specific and effective.

Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antineoplastic properties for future work (Cardellina *et al.*, 1999). Brine shrimp lethality test is carried out in order to reveal new anticancer compounds.

### Conclusion

It can be concluded that *T. bellerica* has got the potential as a candidate for future thrombolytic agent. It can also be investigated as a possible source of antitumour drugs. This is only a preliminary study and to make final comment the extract should thoroughly investigated be phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentials.

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