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Antithrombotic and Thrombolytic activity of *Terminalia belerica* fruit extracts

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ABSTRACT

Atherothrombotic diseases such as myocardial or cerebral infarction are serious consequences of the thrombus formed in blood vessels. Thrombolytic agents are used to dissolve the already formed clots in the blood vessels. However, these drugs have certain limitations which cause serious and sometimes fatal consequences. Herbal preparations have been used since ancient times for the treatment of several diseases. So, the aim of present work was to investigate whether our selected herbal preparations of *Terminalia belerica* fruits possess thrombolytic and antithrombotic activity or not. An in vitro model was used to check the clot lysis and antithrombotic effect of *Terminalia belerica* fruits along with Streptokinase as a positive control. From this study it was found that after addition of Streptokinase clot formation is delayed upto more than 90 min whereas after addition of test solution it was found that as the concentration of extract was increased the delay in clot formation also increases. At 0.20 mg/dl concentration it showed the maximum delay (more than 90 min.) in clot formation. For thrombolytic activity, at concentration 1.00 mg/dl the clot dissolution time is minimum i.e. 58 and 66 min for aqueous and alcoholic extracts respectively. Through our study it was found that *Terminalia belerica* fruits possess thrombolytic and antithrombotic activity in vitro; however in vivo clot dissolving properties and active component of *Terminalia belerica* for clot lysis are yet to be discovered.

Keywords: *Terminalia belerica* , Antithrombotic, Thrombolytic, Streptokinase

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INTRODUCTION

Atherothrombotic coronary artery disease, associated with deep vein thrombosis, is one of the most common causes of death worldwide [1]. Atherothrombotic diseases such as myocardial or cerebral infarction are serious consequences of the thrombus formed in blood vessels. Thrombolytic agents are used to dissolve the already formed clots in the blood vessels. However, these drugs have certain limitations which cause serious and sometimes fatal consequences [2].

Venous thromboembolism (VTE) is the third most common type of cardiovascular disease. VTE causes over 500000 deaths in Europe every year. An estimated 300000 VTE related deaths occur in the US each year [3]. About 1 in 10 deaths that occur in the hospitals is caused pulmonary embolism. In the UK, PE kills more people than breast cancer, AIDS and traffic accidents combined [4].

A blood clot (thrombus) developed in the circulatory system due to failure of haemostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death. Thrombolytic agents that include tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc. are used all over the world for the treatment of these diseases [5]. In India, though SK and UK are widely used due to lower cost, as compared to other thrombolytic drugs (tPA), their use is associated with hyper risk of hemorrhage severe anaphylactic reaction and lacks specificity. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs. Many antithrombotic drugs may have a deleterious effect on normal haemostasis leading to bleeding complications. Therefore it is necessary to find out a new drug which shows less adverse effect.

Heparin and Aspirin are only moderately efficient for acceleration of lysis and prevention of reocclusion, but are safe. More selective thrombin inhibitors and antiplatelet agents are more potent, but their safety remains to be confirmed. Continued investigation in this area will provide new insights and promote progress toward the development of the ideal thrombolytic therapy, characterized by maximized stable coronary arterial thrombolysis with minimal bleeding [5].

Several third generation thrombolytic agents have been developed. Compared with the second generation agents (altreplase), third generation thrombolytic agents such as monoteplase, tenecteplase, reteplase, lanoteplase, pamiteplase, and staphylokinase result in a greater angiographic potency rate in patients with acute myocardial infarction, although, thus far, mortality rates have been similar for those few drugs that have been studied in large-scale trials. Bleeding risk, however, may be greater. Recently, preventive measures against thrombosis have been tried. Oral administration of the fibrinolytic enzyme nattokinase was one example, which has been reported to enhance fibrinolytic activity in plasma and the production of tPA [6].

Considerable efforts have been directed towards the discovery and development of natural products from various plant and animal sources which have antiplatelet, anticoagulant, antithrombotic, and thrombolytic activity. Epidemiologic studies have provided evidence that foods with experimentally proved antithrombotic effect could reduce risk of thrombosis. Herbs showing thrombolytic activity have been studied and some significant observations have been reported [7].

Terminalia belerica is one of the constituents of famous preparation "Triphala" which finds use in wide array of areas ranging from hair care, as laxative, in headache, leucorrhoea, liver diseases to gastro-intestinal complaints and it is the best single herb for controlling Kapha. It is a powerful rejuvenative herb that nourishes the lungs, throat, voice, eyes and hair. It expels stones or other kapha-type accumulations in the digestive, urinary, and respiratory tracts. It is unique in being both laxative and astringent, so it purges the bowels, while simultaneously toning the tissues of the digestive tract. It provides strength to the tissues of the sense organs. The overall tonic effect of this fruit has been known for thousands of years in India and other Asian countries.
It acts as anthelmintic, antiseptic, astringent, expectorant, laxative, lithotriptic, rejuvenative, tonic. It is useful in asthma, biliousness, bronchitis, inflammations, sore throat, and treating the diseases of eyes, nose, heart and bladder [8].

The aim of our work was to investigate whether our selected herbal preparations of *Terminalia belerica* (fruits) possess thrombolytic and antithrombotic activity or not by using an in-vitro procedure taking streptokinase as positive.

MATERIALS AND METHODS

Streptokinase (SK): To the commercially available lyophilized SK vial (**ST-Pase**® Cadila pharma) of 15, 00,000 I.U., mixed in sterile Normal Saline properly. This suspension was used as a stock from which working solution (5,000 I.U) were prepared freshly by normal saline each time.

Specimen: Whole blood was drawn from healthy rabbits. Required volume of blood taken in to each of the micro-centrifuge tubes (MCTs).

Plant material: The fruits of *Terminalia belerica* were purchsed at their fully mature form, from local market and were identified and authenticated from Dr. Aminuddin Scientist E2 at NBRI Lucknow.

Preparation of Extract: The fruits of *Terminalia belerica* were air dried for 5 days, and then kept in an oven at 45°C for 72 hours. The extraction has been done by maceration process. 50 gm of dried powder was taken each time for both alcoholic and aqueous extraction. After completion of the extraction process the extract is collected and concentrated by evaporation method to obtain a semisolid extract.

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Dilution of extracts: Two dilutions (5 times and 10 times) have been made in NS for both extracts i.e. 0.5g of plant extract dissolves in 2.5ml and 5ml of NS respectively.

Clot lysis and antithrombotic activity:

Table 1 Method various Volumes of Solutions Used

Tube no.	Vol. of blood (ml)	Vol. of solutions in ml.					
		For anti- thrombotic activity			For thrombolytic activity		
		S.K.	Test solution	N.S.	S.K.	Test solution	N.S.
1.	0.5	---	---	0.50	---	---	0.5
2.	0.5	0.2	---	0.30	0.5	---	---
3.	0.5	---	0.02	0.48	---	0.1	0.4
4.	0.5	---	0.04	0.46	---	0.2	0.3
5.	0.5	---	0.06	0.44	---	0.3	0.2
6.	0.5	---	0.10	0.40	---	0.5	---

The volume of each tube was made up to 1ml by adding sufficient amount of NS. Same method was applied for both (5X and 10X) dilutions of aqueous and alcoholic extracts

Experiments for clot lysis and antithrombotic activity were carried as reported earlier [9]. Slight modification in procedure was done for antithrombotic activity as well as clot lysis estimation. Different concentrations of both extract (alcoholic and aqueous) were added in previously washed micro centrifuge tubes (MCTs) containing 0.5 ml of blood in each tube. Streptokinase was used as positive control. In first test tube only N.S. was added in place of sample solution and taken as blank. SK was added in tube no. 2. In tube no. 3 to 6 increasing concentration of extract solution was added (as shown in table 1). Meanwhile time was noted before each addition. The volume of each tube was made upto 1ml. For antithrombotic activity reactants (extract solution, SK, NS) was added before clot formation (immediately after taking blood in tubes) and for thrombolytic activity, the reactants were added just after clot formation. All reactions have been maintained at 37°C in water bath. The experiment was repeated 4 times with all 4 dilutions of both the extracts.

RESULT

A clear visual repetition of clot lysis is shown in fig 1 for clot lysis experiment. When NS was added to the control (tube#1) the clot lysis was negligible. Whereas tube to which SK was added, the clot lysis can be seen in 40-50 minutes and in case of extract solutions, significant clot lysis could be seen as according to concentration. Maximum clot lysis was observed in tube #6 in which maximum concentration of extract was added.

In case of antithrombotic experiment, the clot was formed in normal time or slight delay when NS was added to the control (tube#1). Whereas tube, to which SK was added, the clot was not formed and in case of extract solutions, significant delay in clot formation time is noted as according to concentration. Maximum delay in clot formation time was observed in tube #6 in which maximum concentration of extract was added (as shown in table 2).

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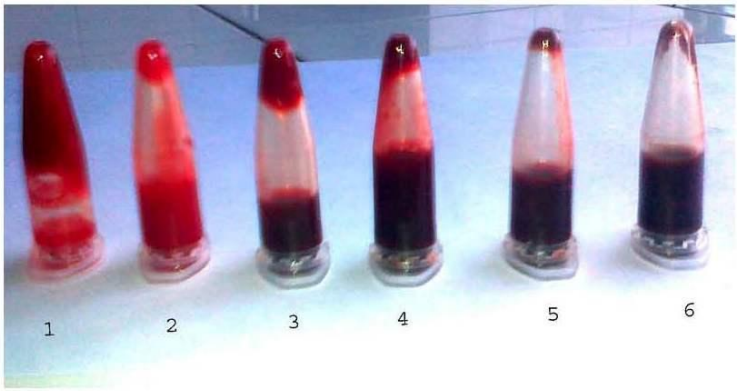
Table 2 Observations of Different Concentrations of both Aqueous and Alcoholic Extracts

S.No.	For anti- thrombotic activity			For thrombolytic activity		
	Conc. Of drug (mg/dl)	Delay in clot formation in minutes		Conc. Of drug (mg/dl)	Clot dissolution time in minutes	
		For Aqu.	For Alc.		For Aqu.	For Alc.
1	0.00 (blank)	04	04	0.00 (blank)	No*	No*
2	0.02	05	04	0.10	75	75
3	0.04	10	07	0.20	75	73
4	0.06	25	16	0.30	70	71
5	0.08	27	18	0.40	65	70
6	0.10	30	24	0.50	63	69
7	0.12	No*	26	0.60	61	67
8	0.20	No*	No*	1.00	58	66
9	SK	No*	No*	SK	45	47

*More than 90 minutes.

Observation for both a anti- thrombotic and thrombolytic activity is summarized here. In case of both activities, monitoring was done for 90 min. but clot formation of positive control (in case of anti-thrombotic activity) and clot dissolution of negative control (in case of thrombotic activity) not occurred hence 90 min. is taken as reference value for graphical expression.



Clot lysis can be clearly observed. Tube no.1 is negative control to which NS was added, no clot lysis was observed in tube no.1. Tube no.2 is positive control to which 0.5ml (5K IU) SK was added. Clear clot lysis can be seen tube no.2. Tube no.3 to 6 have different concentrations of test solutions with increasing order.

Figure1: Showing dissolved clots

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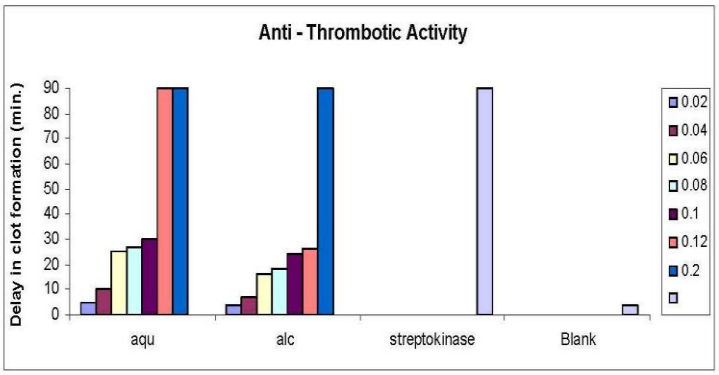
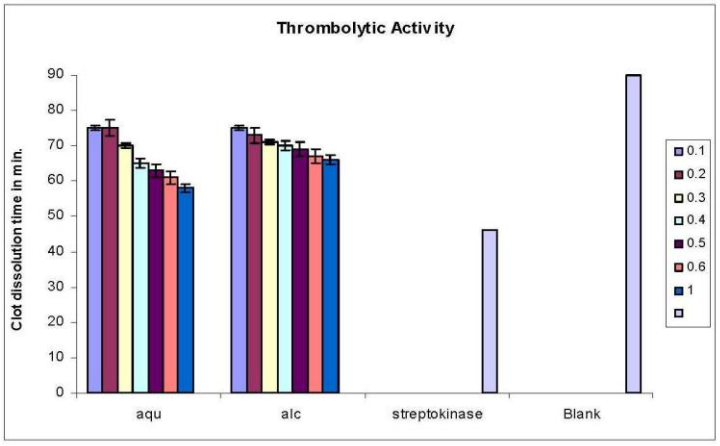


Normally clot formation within four minutes as in case of blank whereas addition of SK delays clot formation more than 90 min. Hence monitoring was done for 90 mins as reported earlier[9], this value have been taken as maximum (and it is believed that clot formation will not occur after such a long time in normal conditions). Activity of aqueous and alcoholic extracts can be seen increasing as increase of drug concentration.

Figure2: Anti-Thrombotic activity



Clot lysis activity expressed here. Clot was not dissolved till 90 mins. In case of blank to which NS was added and in case of positive control to which SK was added clot was dissolved significantly earlier. It can be seen that aqueous and alcoholic extracts of drug are decreasing clot lysis time as concentration increa

Figure3: Thrombolytic activity

DISCUSSION

Herbal preparations are used since ancient times to maintain health and regain healthy state of mind. Advances in phytochemistry and identification of plant compounds, which are effective in curing certain diseases have renewed the interest in herbal medicines. About 30% of the pharmaceuticals are prepared from plants worldwide [10]. A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is

evidence that consuming such food leads to prevention of coronary events and stroke [2]. There are several thrombolytic drugs obtained from various sources. Some are modified further with the use of recombinant technology [11] in order to make these thrombolytic drugs more site specific and effective. Side effects related to these drugs have been reported that lead to further complications [2]. Sometimes the patients die due to bleeding and embolism [2]. Herbal preparations, if taken in appropriate dose, can lead to a better option for curing various ailments.

In our study of anti thrombotic and thrombolytic activity we have taken *Terminalia belerica* which is used since ancient times.

SK, a known thrombolytic drug [12] is used as a positive control. NS, on the other hand, was selected as a negative control. The comparison of positive control with negative control clearly demonstrated that clot dissolution does not occur when NS was added to the clot neither it delays clot formation significantly. Encouraged by the results obtained through the clot lysis activity by SK and with NS as a negative control, we tried extracts of *Terminalia belerica* in the same manner.

When compared with the anti thrombotic and thrombolytic activity obtained through NS (negative control), a significant activity was observed with *Terminalia belerica* extracts.

CONCLUSION

In conclusion, on the basis of beneficial effect of *Terminalia belerica* in the literature and our own results of the experiments in the extract of same herb, *Terminalia Belerica* shows anti thrombotic and thrombolytic activity in vitro; however, in vivo clot dissolving anti thrombotic property and active component(s) of *Terminalia Belerica* for these properties are yet to be found out. Once found *Terminalia Belerica* may be incorporated as a thrombolytic and anti thrombotic agent for the improvement of the patients suffering from Atherothrombotic diseases.

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