COMPARATIVE IN VITRO STUDY OF THROMBOLYTIC EFFECT OF PUNICA GRANATUM AND AZADIRACHTA INDICA

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Abstract: The aim of the study was to investigate the thrombolytic activity of Punica granatum and Azadirachta indica over canine, bovine, caprine and avian blood clot using streptokinase and water as positive and negative control respectively. Using an in vitro thrombolytic model, the clot lysis activity of A. indica was observed as 48.06±0.59%, 50.01±1%, 47.6±0.69% and 28.03±0.35% and of P. granatum as 44.73±0.65%, 48.53±0.56%, 43.83±0.6% and 47.17±0.32% in cattle, dog, goat, poultry respectively. The percentage (%) clot lysis was statistically significant (p<0.05) when compared with vehicle control whereas streptokinase showed clot lysis 43.13±0.9%, 58.4±0.72%, 55.07±0.58% and 44.67±0.65% respectively. We observed that these herbal extracts possess thrombolytic properties that could lyse blood clots in vitro. However, in vivo clot dissolving properties and active component(s) of these extracts for clot lysis are observed to be protein in nature as the protein extract of Azadirachta indica exhibits different protein bands between 16.6 to 90.4 kda where as that of Punica granatum exhibits only 2 bands of 10.9 and 17.3 kDa MW. So the individual components from these bands can be used for further research.

Key words: Punica granatum, Azadirachta indica, Thrombolytic effect

INTRODUCTION

Thrombolysis, essential to prevent fatality, is a defence mechanism, which requires therapeutic intervention in myocardial infarction (heart attack), deep vein thrombosis, thrombo-embolic strokes, and pulmonary embolism to clear a blocked artery and avoid permanent damage to the perfused tissue (e.g. myocardium, brain, leg) [1]. Thrombo-embolic attacks are the main causes of morbidity and mortality in both man and animals throughout the world. Epidemiological findings report the fact that approximately 14 million individuals died of cardiovascular disease in 1990, and this statistics is projected to rise to about 25 million by the year 2020 [2]. When animals are concerned, cattle are more prone to venous thrombi, where as in dogs and cats, arterial thrombi appear to be more clinically important [3,4]. Among all species of animal, canines have the higher incidence of cardiovascular diseases (CVDs) such as cranial venacava thrombo-sis, aortic thromboembolism, portal vein thrombosis [5,6].

Although currently clinically approved thrombolytic therapies such as alteplase (activase), tenecteplase (TNKase), urokinase (abbokinase), staphylokinase, reteplase (retavase), anistreplase (eminase), streptokinase (kabikinase, streptase), lanoteplase, have markedly reduced mortality against acute myocardial infarction [7]. These drugs are gaining increasing acceptance for the treatment of various other thrombo-embolic disorders, but have
significant drawbacks, including the need of large therapeutic doses, short half life, limited fibrin-specificity and significant associated bleeding tendency, allergy and reocclusion. To overcome these problems, many efforts are being made to develop novel recombinant variants of these drugs with improved efficacy and safety profile.

Biotic component of the nature being well diversified, it is assumed a good number of organisms do have the enzyme for their use. Yet there are several other plants which are well known for their medicinal values and locally available are still to be exploited for any thrombolytic effect. In our study we evaluated the thrombolytic effect of *Azadirachta indica* and *Punica granatum*. Medicinal values of these plants have been already established and they are used since ancient times for curing vascular diseases. The thrombolytic effects of *Azadirachta indica* have already been established in human [8]. The extracts of *Punica granatum* have been found to possess antioxidant effect [9,10] and also promote the wound healing [11], thus opening a possibility for its thrombolytic effect. Herbal products are often perceived as safe because they are “natural” and are bestowed with incredible pharmacological activities, economic viability and less side effects in different healthcare management system [7]. Considerable efforts have been directed towards the discovery and development of natural products from various plant and animal sources for cardiovascular diseases which have antiplatelet [12], anticoagulant, antithrombotic and thrombolytic activity [7,13].

The present study aims to isolate and characterize thrombolytic enzymes from natural biotic source for use by greater section of the population. At present, bioactive principles harvested and isolated from a wide variety of plants, fungus, lower vertebrates like earthworm, snail and microbes constitute a rich source of traditional medicine and are exceedingly used in the primary thrombolytic therapy [12]. Nevertheless, there is always a further need to isolate the enzyme from other different sources for the welfare the human community.

**MATERIALS AND METHODS**

**Collection of plant materials and preparation of extracts:** The samples i.e. leaves and fruits of *Azadirachta indica* and *Punica granatum* were collected from Medicinal Nursery and was identified and classified by Department of Botany, OUAT, Bhubaneswar. The crude extract was prepared by cold percolation method. The extract was prepared only once and used in all experimental set up. 20 mg extract was suspended in 100 mL distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a whatman filter No. 1. The protein was precipitated by 50% acetone. Then, the sample was centrifuged at 10000 rpm for 15 min at 4°C. The supernatant was discarded and the precipitated protein was reconstituted using 500 µl PBS [14].

**Estimation of protein:** Protein content of the aqueous extracts of the sample collected was estimated by using the method described by Lowry et al. [15].

**Streptokinase solution:** To the commercially available lyophlized recombinant streptokinase vial (marketed as myokinase, Biocon, India) of 15,00,000 I.U, 5 ml NSS was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for *in vitro* thrombolysis [14].

**Collection of blood and clot lysis:** Equal amount of blood (3 ml) from healthy bovine, canine, caprine and poultry species (n = 6) without a history of anticoagulant therapy was transferred to pre weighed Eppendorf tube (500 µl/tube), incubated at 37°C for 90 minutes and clot formed was weighed. The clots along with 500 µl sample was incubated at 37°C for 150 minutes and weight measured at the end. Difference in weight observed was expressed as percentage of the thrombolytic activity of aqueous extract of above said samples for clot lysis (% clot lysis = (Weight of the lysis clot / Weight of clot before lysis) × 100). Streptokinase and water were used as a positive and negative (non thrombolytic) control respectively.

**Fibrinolytic assay:** Fibrinolytic activity was determined using the method described by Astrup and Mullertz, with minor modifications [16]. The fibrin agarose plate was made of 1 mm thickness and contained 1.2% agarose, 0.4% fibrinogen, and 20 units/ml of thrombin. The clot was allowed to stand for 1 hour at room temperature. The filter paper
discs 6mm in diameter were impregnated with 100 microlitre of sample solution and 30,000 I.U of streptokinase as positive control. The plate was incubated for 24 hour at 37°C and the diameter of the lytic circle was measured. The area of lysis was compared to that of streptokinase by the formula 1mm² = 1 unit. The area was directly proportional to lytic activity.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):** SDS-PAGE in discontinuous buffer system was carried out in order to determine the electrophoresis pattern of proteins present in protein extract of sample [17]. The electrophoresis was run on separating of 7.5% acrylamide gel and staking gel of 4.5% acrylamide concentration using the mini gel electrophoresis apparatus (ATTO, Japan). 20 ìl of each sample was diluted with equal volume of sample buffer. 10 ìl of molecular weight marker ranging from 116 kDa to 14.4 kDa (Fermantas, Life Science) was taken in micro-centrifuge tube. The samples with sample buffer were heated in boiling water bath at 100°C for 5 minutes prior to loading into the gel. 10µl of sample proteins were loaded in the wells after properly washing. 10µl of marker protein was also added for determination of molecular weight.

**Statistical analysis:** The data was subjected to statistical analysis by analysis of variance (ANOVA) and least significant difference (LSD) using Microsoft’s MS 2000 Data Analysis package[18] and also student’s t test as and wherever required.

**RESULT**

The protein content in aqueous extract of the experimented plants was evaluated by Lowry’s method. The protein content of aqueous extract of *Azadirachta indica* and *Punica granatum* were estimated to be 0.69±0.12g/ dl and 0.31±0.03g/ dl respectively.

Then, protein extract from leaves of *Azadirachta indica* and fruits of *Punica granatum*, were subjected to fibrinolytic assay to study the lytic effect. The streptokinase enzyme as well as the sample extracts formed a clear halo zone on both plate types, indicating that the enzymes were able to degrade the fibrin clot. The area of lysis was directly proportional to lytic activity of the sample as compared to control SK, positive control (30,000 I.U.). The unit is expressed as 1 Unit = 1mm². It was observed that area of fibrinolysis *A. indica* (72.67%) and in *P. granatum* (62.76%) as compared to control (100%) (Table 1).
1. **Azadirachta**

2. **Punica granatum**

3. **Marker protein**

**Fig. 2:** Photograph showing Electrophoretic pattern of proteins from *Azadirachta indica* and *Punica granatum*. 
Protein extract of *Azadirachta indica* was used to see thrombolytic effect on different species and it was observed that dog showed the highest effect (50.01%) with poultry being lowest (28.03%). All groups showed significant (*P<0.05*) lytic effect in comparison to control. No significant difference in thrombolytic effect was observed in between cattle vs dog and cattle vs goat. However significant difference (*P<0.05*) was observed between goat vs cattle and poultry vs dog. There was no significant difference between plant and control and with *Azadirachta indica* (Table 2 and Fig. 1). The *in vitro* clot lysis and fibrinolytic effect of samples on blood of different species was presented in Table 1 and Table 2 respectively.

The protein extracts from *Punica granatum* and *Azadirachta indica* were subjected to SDS PAGE for identifying the bands and determining their molecular weight. The marker proteins were run along with the plant protein to calculate the molecular weights of different component proteins. Molecular weight and Relative mobility (Rm) value of the marker protein was presented in Table 3.

The protein extract of *Azadirachta indica* revealed different protein bands between 15.6 to 90.4 kDa where as that of *Punica granatum* impress only 2 bands of 10.9 and 27.3 kDa MW (Fig. 2). The protein bands of these two plants are distinctly different without similarities in molecular weight where *Azadirachta indica* have higher molecular weight protein bands than that of *Punica granatum*. The study revealed that different types of proteins in different plants source bring the effect of thrombolytic effect.

**DISCUSSION**

Herbal medicines have a long history of use for the prevention and treatment of human diseases. Today, many pharmaceuticals currently approved by the Food and Drug Administration (FDA) have origins to plant sources [7,19]. While a number of studies

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**Table 1:** Invitro clot lysis of samples. *P<0.05*, as compared to the positive control. The data are compared within column.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area of Lysis (1unit=1mm²)</th>
<th>% of lysis in comparisons to control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azadirachta indica</em> (n=6)</td>
<td>352.07±10.18</td>
<td>72.67*</td>
</tr>
<tr>
<td><em>Punica granatum</em> (n=6)</td>
<td>303.8±6.45</td>
<td>62.76*</td>
</tr>
<tr>
<td>Positive control (n=6)</td>
<td>484.48±12.02</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 2:** Fibrinolytic effect of samples. Means bearing different superscripts within a row and within a column differ significantly (p<0.05).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fibrinolytic effect on blood clots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poultry</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> (n=6)</td>
<td>28.03±0.35*</td>
</tr>
<tr>
<td><em>Punica granatum</em> (n=6)</td>
<td>47.17±0.32*</td>
</tr>
<tr>
<td>Positive control (n=6)</td>
<td>44.67±0.65*</td>
</tr>
</tbody>
</table>

**Table 3.** Molecular weight and R_m value of protein marker and screened sample

<table>
<thead>
<tr>
<th>Band</th>
<th>Rm: Marker (M.W)</th>
<th>Rm: <em>Azadirachta indica</em> (M.W)</th>
<th>Rm: <em>Punica granatum</em> (M.W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.156:116</td>
<td>0.248:90.4</td>
<td>0.746:17.3</td>
</tr>
<tr>
<td>2</td>
<td>0.362:66.2</td>
<td>0.447:37.6</td>
<td>0.951:10.9</td>
</tr>
<tr>
<td>3</td>
<td>0.406:45</td>
<td>0.677:20.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.461:35</td>
<td>0.783:16.6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.625:25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.700:18.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.925:14.4</td>
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</table>
have been conducted by various researchers to find out the herbs and natural food sources and their supplements having thrombolytic effect. Side effects include systemic fibrinolysis, anaphylactic reaction and embolism [13]. On the way of quenching safer and more effective thrombolytic drugs, researchers came up with potent bioactive substances as target for development of many cardiovascular drugs both for prophylactic and therapeutic uses. Researchers have paid prime attention on herbal compounds as drug model. Many of the information on medicinal use of plants confined to traditional practitioners of rural India and the modern research is usually ignorant of these ethno-practices, hence the active principle may be further exploited to new drug discovery. This draws our interest to discover new drugs with the active principle of our interest from already established medicinal plant and other sources. While scientists and clinicians are in the search for a better, safer therapeutic agent with fewer side effects, the discovery of the fibrinolytic enzymes from herbal sources may shed the light for the modern thrombolytic therapies.

In our study we evaluated the thrombolytic effect of Azadiracta indica and Punica granatum. Medicinal values of these plants have been already established and they are used since ancient times for curing vascular diseases. The thrombolytic effect of Azadiracta indica has already been established in human blood [8]. The extracts of Punica granatum have been found to possess antioxidant effect [9,10] and also promote the wound healing [11], thus opening a possibility for its thrombolytic effect. In this study, streptokinase, a known thrombolytic drug is used as a positive control, whereas distilled water was selected as a negative control. The comparison of positive control with negative control clearly demonstrated that clot dissolution does not occur when water was added to the clot. By comparing with this positive control, a significant (*P<0.05) thrombolytic activity was observed after treating the clots with Azadiracta indica and Punica granatum extracts. The aqueous extracts of Azadiracta indica showed highest thrombolytic activity in dog, followed by cattle, goat and poultry with 50.01%, 48.06%, 47.6% and 28.03% lysis respectively. But, it showed 27.47 % lysis in human clot [8], which may be due to species variation and difference in erythrocytes membrane structure.

The C-terminal domain of streptokinase contains Asp41–His48 as well as residues 48–59 which has plasminogen substrate recognition and activation property [20]. The streptokinase h domain is involved in forming the streptokinase–plasminogen complex that is responsible for activating the plasminogen through a lysine residue [21]. These amino acid sequences are responsible for fibrinolytic effect of streptokinase. These plants may contain the same domain as that of streptokinase in their protein or enzyme. The amino acid sequence of proteins may vary from region to region, soil type, topography, plant or animal species, where their quality and number of repetitive domains are not constant. This may be reason of variable thrombolytic effect between the experimental samples.

Our studies were initially conducted to aid in development of a large and small animal model of thrombosed arterio-venous graft occlusion. Interestingly, out of four species (canine, caprine, bovine, avian), maximum lysis was observed in canine blood clots. The underlying cause may be due to species difference attributed to fibrin differences, thrombin activated fibrinolysis inhibitor [22], activated thrombin (FXIIia) concentration [23]. In addition, differences in each species’ thrombin (FXIII) level or activity, von Willbrand factor activity, or platelet activation may also be participating in events controlling clot morphology and/or strength [23,24]. Factors that influence the rate of thrombin generation may have a significant impact on the clot structure [24]. Bovine erythrocytes contains cholesterol, cholesteryl esters, triglycerides, and certain unidentified hydrocarbons which constitute about 80%, 4%, 10% and 6% respectively of the neutral [25]. The RBC cell membrane consists of lipid bilayer composed of cholesterol and phospholipids in the ratio of 0.92, 0.96, and 0.98 in case of bovine, canine and caprine species respectively [26]. It has been shown that peroxidation of the lipids in the erythrocyte causes hemolysis [27] which may result from disruption of the cholesterol polar lipid complex in the RBC membrane. The difference in clot lysis as per species variation may be attributed to varying cholesterol and protein content.

Clinical application of several plasminogen activators results in activation of circulating plasminogen, which successfully digest abnormal blood clot occluded in arteries or vein; however, risk accompanied with this treatment option may include a life-threatening hemorrhage caused by the systemic
activation of fibrinolytic mechanism. While scientists and clinicians are in the search for a better, safer therapeutic agent with fewer side effects, the discovery of the fibrinolytic enzymes from herbal sources may shed the light for the modern thrombolytic therapies. This preliminary study needs further investigation to exploit medicinal and pharmaceutical potentialities of these plants and further investigations are in progress.

**CONCLUSION**

From this experiment, it can be concluded that the aqueous extracts of *Azadirachta indica* and *Punica granatum* showed significant clot lysis activity as compared to positive control in different animal specifically in canines. So these herbal preparations may be used in formulation of newer and improved drug for thromboembolic disease. While scientists and clinicians are in the search for a better, safer therapeutic agent with fewer side effects, the discovery of the fibrinolytic enzymes from herbal sources may shed the light for the modern thrombolytic therapies.

**REFERENCES**