Fibrinolytic effect of phyto-proteins on animals and poultry blood

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Phyto-proteins of *Mangifera indica* L., *Aegle marmelos* (L.) Corrêa, *Volvariella volvacea* (Paddy straw mushroom), *Carica papaya* L., *Ocimum sanctum* L. and *Aloe vera* (L.) Burm. f. extracted in water and PBS under cold percolation are precipitated in 50% acetone and quantified after reconstitution. PBS suspension with higher protein is assayed for thrombolytic effect on blood clots of Bovine, Caprine, Canine and Avian species and the fibrinolytic effect on fibrin-agarose plate with streptokinase as positive control. Phyto-proteins exhibit clot lysis effect of 49.41-92.04% in poultry, 41.60-93.73% in cattle, 46.67-85.85% in goat and 34.39-77.09% in dog blood as compared to 100 % of streptokinase. In 150 min incubation, significantly higher (p < 0.05) amount of clots are disintegrated by proteins of *M. indica* and *A. marmelos* in dog, *V. volvacea* in poultry and goat, *C. papaya* and *O. sanctum* in goat, dog and cattle and *A. vera* in goat blood as compared to rest species. Phyto-protein and streptokinase impregnated discs depict 74.05-274.49 mm² and 484.48 mm² fibrinolysis zones on fibrin-agarose plate, respectively. Proteins of *M. indica*, *A. vera* and *O. sanctum* exhibits > 50% and those of *A. marmelos*, *V. volvacea* and *C. papaya* exhibits < 50% lysis than streptokinase enzyme. Phyto-proteins pose variable fibrinolytic activity on clots of animals and poultry blood similar to streptokinase.

**Keywords:** Fibrinolysis, Thrombolysis, Phyto-proteins, Fibrin agarose plate, Streptokinase

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Thrombus, an intra-vascular clot is formed during blood coagulation incorporating fat, air, amniotic fluid, plasminogen, tumour or any foreign particle. It travels as an embolus to occlude narrow vessel to cause thrombosis. Thrombotic diseases are responsible for heavy toll in death of 25-30% in most of the industrialised countries worldwide. About 14 million deaths in 1990 and that of forthcoming 25 million are attributed to cardiovascular diseases by 2020¹. It is aptly called as the modern epidemic where surgical embolectomy is considered as the best possible method of treatment. Since, it is associated with a high rate of morbidity / mortality and re-thrombosis and re-perfusion injury, it may not be considered as the sole viable therapy for peripheral thrombo-embolism. Therefore, clinically approved enzymes of Streptokinase and Urokinase are extensively used for treatment of thrombo-embolic disorders. Significant drawbacks of high cost, large therapeutic doses, short half life, fibrin-specificity limit, bleeding tendency, allergy and re-occlusion are evident on use of these drugs. An easily accessible cost effective drug with potent thrombolytic / fibrinolytic effect with no / less adverse reactions can overrule the associated complications.

Around 80 % of the global population still relies on botanical drugs for which several herbal medicines have advanced to clinical use in modern times²³⁴. Bioactive principles of higher plants, fungus, lower vertebrates (Earthworm and snails) and microbes are exceedingly used in the primary thrombolytic therapy⁵. Isolates of *O. sanctum*⁶, enzymes of *Cordyceps militaris* and Chinese traditional medicinal mushroom⁷ are also considered as important sources of thrombolytic agents. This prompts to search for thrombolytic / fibrinolytic properties of traditionally used medicinal herbs. *O. sanctum* L. and *A. marmelos* Correa are holy and medicinal plants in Hindu culture and possess antifertility, anticancer, antidiabetic, antifungal, antimicrobial, antiemetic, antipyretic, antispasmodic, anti-inflammatory, antilipidemic, analgesic, hepatoprotective, cardioprotective, adaptogenic and diaphoretic properties⁸⁻¹³. The fruits of *C. papaya* L. and seed of *M. Indica* L. are the rich

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source of nutrients for human beings and animals where as their leaves pose potent wound healing properties in diabetic rats\textsuperscript{14-16}. Promising property of angiogenesis and better healing of traumatic tissues is claimed by leaf gels of the most common medicinal plant \textit{A. vera}\textsuperscript{17}. Although, many edible mushrooms are considered as the dietary protein supplement, few of them have potent therapeutic value in epilepsy, wounds, skin diseases, heart ailments, rheumatoid arthritis, intermittent fevers, diaphoretic, diarrhoea, dysentery, cold, liver and gall bladder diseases\textsuperscript{18}. Disruption of cell / organelle membrane architect leading to cell lysis is a part of anti-micobial and anti-fungal activities. Also, disintegration of dead tissue / debris and angiogenesis are associated with wound healing. Such medicinal plants can be used for similar / related action during dissolution of intravascular clots by thrombolysis / fibrinolysis. Therefore, the present study includes the medicinal plants of \textit{A. vera} (Leaf), \textit{O. sanctum} (Leaf), \textit{A. marmelos} (Leaf), \textit{M. indica} (Seed), \textit{C. papaya} (Fruit), and \textit{V. volvacea} (Thallus) with an objective to evaluate the fibrinolytic effect and to provide a platform for development of safe and natural thrombolytic / fibrinolytic drugs as per the need of the hour.

Methodology

The study has been conducted in the department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology (OUAT). Plant samples after being collected locally from in and around Bhubaneswar has been deposited in the Department of Botany, College of Basic Science, OUAT and identified by the Professor and Head with reference to the description of Regional Research Laboratory and Orissa Forest Development Corporation Ltd., Bhubaneswar as mentioned in the Flora of Orissa, Volume-II\textsuperscript{19}. Phyto-proteins are exposed to blood clots of animals and poultry birds for thrombolytic effect and phyto-protein impregnated discs are incubated on fibrin-agarose plate for fibrinolytic effect study keeping streptokinase as positive control using chemicals of HIMEDIA make.

Streptokinase (SK) solution

Commercially available lyophilized recombinant Streptokinase (SK) vials (Myokinase, Biocon, India) of 15, 00,000 I.U is reconstituted with 5 mL NSS (Normal Salt Saline) and volumes of 100 µL (30,000 I.U) are used for \textit{in vitro} thrombolytic effect study.

Blood clots

Five hundred (500) µL of non-haemolysed whole venous blood of healthy bovine, canine, caprine and avian (poultry) species are kept in separately labelled and previously weighed eppendroff tubes without treatment of anti-coagulant at 37 °C for 90 min for complete clot formation. The weight of the tube and clot was recorded after aspiration of serum. The clot weight was calculated by subtracting weight of the tube from weight of tube with clot.

Phyto-protein estimation

The fresh leaves (\textit{O. sanctum}, \textit{A. marmelos} and \textit{A. vera}), green fruits (\textit{C. papaya}), seeds (\textit{M. indica}) and thallus (\textit{V. volvacea}) of the experimental plants are pulverized into fine powder after cleaning by water and shade drying. The extracts of the powders are prepared by cold percolation in water and Phosphate Buffer Saline (PBS) @ 20 g / 100 mL with the help of a magnetic stirrer. After 24 h of soaking at room temperature, the contents are filtered and phyto-proteins are recovered by precipitation with 50 % acetone followed by centrifugation at 10,000 rpm for 15 min at 4 °C. The precipitated protein is reconstituted with 500 µL PBS\textsuperscript{20} and quantified in aqueous and PBS extracts separately\textsuperscript{21}.

Thrombolytic assay

The labelled tubes containing blood clots are added each with 500 µL of reconstituted phyto-protein and incubated at 37 °C for 150 min. The incubation is also conducted with streptokinase and water as positive (thrombolytic) and negative (non-thrombolytic) controls respectively. The weights of the tubes are recorded before and after incubation and the percent of weight loss is calculated to express the effect.

Fibrinolytic assay

Fibrin-agarose plate of 1 mm thick is prepared with agarose, fibrinogen and thrombin @ 1.2 %, 0.4 % and 20 units / mL, respectively. The plate is allowed to age for 1 h at room temperature. The filter paper discs of 6 mm diameter impregnated with 100µL of phyto-protein suspensions and 30,000 IU of streptokinase are prepared as tests as positive control, respectively. These discs are embedded on fibrin-agarose plate and incubated for 24 h at 37 °C to measure the diameter of fibrinolysis circle\textsuperscript{22}. One mm$^2$ area of lysis zone is taken as 1 unit and the units of test samples are compared with streptokinase.
Statistical analysis

The data is statistically processed through Analysis of Variance (ANOVA) and Least Significant Difference (LSD) by using Microsoft’s MS 2000 Data Analysis pack and student’s t test\(^\text{23}\). The resulted data are considered significant at \(p < 0.05\) level.

Results

Protein content

Phyto-protein contents in aqueous and PBS extracts of the plant parts vary from 0.21 to 1.15 mg/g and 0.46 to 1.35 mg/g, respectively (Table 1). \(M.\) indica extracts in both aqueous and PBS medium estimates the maximum (1.15±0.24 and 1.35±0.21) and those of \(A.\) vera the minimum (0.21±0.01 and 0.46±0.09) mg/g of phyto-proteins, respectively. Significantly higher (\(p < 0.05\)) phyto-proteins are observed in PBS extracts of \(V.\) volvacea, \(O.\) sanctum and \(A.\) vera than those in aqueous extracts.

Thrombolytic effect

The lysis of blood clots (weight loss) caused by streptokinase ranges from 43.13 g (cattle) and 44.67 g (poultry) to 55.07 (goat) and 58.40 (dog). The phyto-proteins depict clot lysis effect of 49.41-92.04 % in poultry, 41.60-93.73 % in cattle, 46.76-86.85 % in goat and 34.39-77.09 % in dog blood as compared to 100 % effect of streptokinase. Clots of dog and goat blood dissolve significantly higher (\(p < 0.05\)) amount of clot than cattle and poultry (Table 2).

Phyto-proteins of \(M.\) indica and \(A.\) marmelos disintegrate significantly higher (\(p < 0.05\)) amount of clots in dog, poultry and goat blood as compared to blood clots of the rest of species, respectively (Fig. 1).

Fibrinolytic effect

As compared to streptokinase, on fibrin agarose plate more than 50 % fibrinolysis is observed by the filter paper discs impregnated with protein suspensions of \(M.\) indica (56.66), \(A.\) vera (55.60) and \(O.\) sanctum (53.66) whereas those of \(A.\) marmelos (37.40), \(V.\) volvacea (28.27) and \(C.\) papaya (15.28) depicts less than 50 % effect (Table 3). The observed zones of fibrinolysis caused by phyto-protein suspensions and streptokinase are 74.05-274.49 mm\(^2\) and 484.48 mm\(^2\), respectively. Phyto-proteins with > 50 % effect exhibit no significant variations between the source plants (Fig. 2). The study concludes that phyto-proteins of different plants at similar concentrations pose significant and variable thrombolytic and fibrinolytic effect on blood clots of animals and poultry birds similar to streptokinase.

Discussion

During blood coagulation and thrombosis fibrin clot is formed by disintegration of fibrinogen. Fibrinolysis is the process of disintegration of fibrin clot by plasmin at specific locations into smaller water soluble products at solid-liquid interface on the outer surface of thrombi. In order to initiate fibrinolysis, inactive plasminogen of thrombi, needs to be activated by plasminogen activators. Tissue type plasminogen activator (tPA) freely diffuses through the pores of the fibrin network basing on size\(^\text{24}\). This process is opposed by the great affinity of the enzyme to bind with fibrin. The blood tPA binds on the surface of fibrin in a thin interface layer to convert inactive plasminogen to plasmin. Plasmin, the proteolytic enzyme breaks cross-links between fibrin molecules to disrupt the structural integrity of blood clots\(^\text{25}\). It digests fibrin and exposes new binding sites for subsequent plasminogen molecules on the surface of the clot. Since, plasmin cleavage is in transversal direction and fibrinolysis occurs in the outer thin layer of the fibrin, the dissolution follows a layer-by-layer process.
Bacillus subtilis similar action to lumbrokinase substrate recognition and activation property. The Asp41-His48 and others at 48-59 with plasminogen enzyme protein contains amino acid residues at fibrinolytic drugs. The C-terminal domain of the plasmin by activating plasminogen like other dissolves fibrin network of blood clots through Streptokinase, the control enzyme of the study lysis of fibrin network. Nattokinase (27.3 kDa to 35 kDa) isolated from fermenting Bacillus natto or Bacillus subtilis bacteria is a serine protease with chain of fibrinogen and fibrin. α-plasminogen activation property to initiate fibrinolysis.

The therapeutic properties of the medicinal plant enzymes are distributed both in animal and plant kingdom. The enzyme lumbrokinase isolated from artificial breeding earthworm dissolves fibrin directly and activates plasminogen to cause lysis of fibrin network. Nattokinase (27.3 kDa to 35 kDa) isolated from fermenting Bacillus natto or Bacillus subtilis bacteria is a serine protease with similar action to lumbrokinase. But, the serine protease purified from culture supernatant of the fungus Cordyceps sinensis is a plasmin-like enzyme which cleaves α chain of fibrinogen and fibrin. Although, fibrinolytic activity on fibrin plate method is variably exhibited by the plant materials of Pueraria lobata Maxim., Lonicera japonica Thunb. and Desmodium styracifolium (Osbeck) Merr. but the activity of D. styracifolium resembles to that of urokinase enzyme. Streptokinase, the control enzyme of the study dissolves fibrin network of blood clots through plasmin by activating plasminogen like other fibrinolytic drugs. The C-terminal domain of the enzyme protein contains amino acid residues at Asp41-His48 and others at 48-59 with plasminogen substrate recognition and activation property. The hour domain of streptokinase is involved in streptokinase–plasminogen complex formation through a lysine residue to activate plasminogen and initiate fibrinolysis.

The therapeutic properties of the medicinal plant samples used in the present study are mostly exhibited by the derivatives of lipids and polysaccharides. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent of O. sanctum leaf has been found to be largely responsible for its potent therapeutic role in cancer, diabetes, hepatic and cardiac disorder and in fungal and bacterial infections. The active principles of leaves of A. marmelos are observed as effective as a biosorbent of metal ions. The mucilage of A. vera contains β-sitosterol as one of the potent constituent for promoting angiogenesis during debris removal and wound healing. The wound healing properties of C. papaya fruit and M. indica seed are also observed due to similar constituents. Mushrooms in general are rich source of multiple and diverged proteins which claim for various biological activities.

Proteins are placed in the integral part of the plant composition along with carbohydrates and lipids. Irrespective of source, these are least soluble in aqueous medium and can be easily precipitated in buffer at their iso-electric pH. Therefore, higher amount of phyto-proteins are recovered in PBS than in aqueous extract for all the plant samples under study. Further, the plant samples may contain variable amount of compositional proteins for which the precipitated protein contents vary between the samples.

In the present study, streptokinase exhibit 43.13-58.40 % weight loss of blood clots of animals and poultry birds. The phyto-proteins of C. papaya, V. volvacea, O. sanctum, A. marmelos, M. indica and A. vera depict variable thrombolytic activities of 67.27-93.73, 34.39-92.04, 49.41-88.55, 52.59-85.85, 61.10-81.17 and 46.32-77.66 % with respect to 100 % effect by streptokinase, respectively. The thrombolytic effect of phyto-proteins may be attributed to the presence of structural domain of amino acid residues at 41-59 position with plasminogen activation property to initiate fibrinolysis.

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**Table 2 — Thrombolytic effect of plant protein suspensions on blood clots of animals and birds (Mean Weight loss % ± SE)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cattle</th>
<th>Dog</th>
<th>Goat</th>
<th>Poultry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptokinase</td>
<td>43.13±0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.40±0.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.07±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.67±0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mangifera indica</td>
<td>27.24±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.73±0.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.60±0.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.97±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aegle marmelos</td>
<td>22.67±0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.08±0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.17±0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.10±1.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Volvariella volvacea</td>
<td>17.93±1.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.11±1.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.97±1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.09±1.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carica papaya</td>
<td>40.40±1.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.53±1.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.23±1.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.03±1.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ocimum sanctum</td>
<td>38.17±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.93±1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.13±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.06±0.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aloe vera</td>
<td>30.90±1.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.09±1.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.67±1.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.86±1.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means bearing different superscripts in a row between the columns differ significantly (p < 0.05).
Figures in “( )” denotes percent of clot lysis with reference to streptokinase.
and thrombolysis similar to streptokinase / urokinase activity. The variation of thrombolytic property between the plant samples may be due to variation in the manner and number of repetition of such domain in the phyto-protein structure.

The per cent of thrombolysis exhibited by phyto-proteins on blood clots of cattle, dog, goat and poultry as compared to streptokinase are observed as 41.60–93.73, 34.39–77.09, 46.76–85.85 and 49.41–92.04 %, respectively. Blood, the liquid connective tissue of animals and birds consists of multiple numbers of nutrients, metabolites and carrier molecules in the fluid part. The cellular part is mainly comprised of Red Blood Cells (RBC), White Blood Cells (WBC) and Platelets. The phospho-lipid bi-layer architecture of RBC membrane includes neutral lipids of cholesterol, cholesteryl esters, mono-, di-, and triglycerides and unidentified hydrocarbons at variable amounts between the species of animals. Such variation influences thrombin generation, fibrin content, factor XIIIa concentration, and morphology and or strength of the clot. Further, the manner of disruption of membrane structure of RBC facilitates hemolysis, fibrinolysis and thrombolysis. Since, the specific domain of phyto-proteins acts on cholesterol, lipo-protein and protein, the difference in membrane lipid content of blood cells regulates the extent and rate of such lysis effect. The species difference in thrombolytic activity may be attributed to the variation in structural composition of blood cell membrane, genesis and morphology of clots basing on the source.

The phyto-proteins of C. papaya, V. volvacea, A. marmelos, O. sanctum, A. vera and M. indica at the same experimental concentration cause 15.28, 28.27, 37.40, 53.66, 55.60 and 56.66 % fibrinolysis on fibrin-agarose plate, respectively. Even though, all proteins are constituted by domain and non-domain amino acid residues, but the number and manner of organization of domain of a particular protein varies between the source species. The variable fibrinolytic effect between the plant samples may be attributed to the difference in the manner of distribution of proteolytic domain and the sequence of amino acid residues at 41-59 position in the phyto-proteins.

**Table 3 — Fibrinolysis Area (1 unit=1mm²) by phyto-proteins with reference to streptokinase (Mean ± SE)**

<table>
<thead>
<tr>
<th>Phyto-protein</th>
<th>Area of lysis</th>
<th>% of lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptokinase</td>
<td>484.48±12.02</td>
<td>100</td>
</tr>
<tr>
<td>Mangifera indica</td>
<td>274.49±9.46</td>
<td>56.66</td>
</tr>
<tr>
<td>Aegle marmelos</td>
<td>181.2±9.46</td>
<td>37.40</td>
</tr>
<tr>
<td>Volvariella volvacea</td>
<td>136.98±10.07</td>
<td>28.27</td>
</tr>
<tr>
<td>Carica papaya</td>
<td>74.05±6.26</td>
<td>15.28</td>
</tr>
<tr>
<td>Mangifera indica</td>
<td>259.97±14.81</td>
<td>53.66</td>
</tr>
<tr>
<td>Volvariella volvacea</td>
<td>269.39±12.44</td>
<td>55.60</td>
</tr>
</tbody>
</table>

Means bearing different superscripts in a row between columns differ significantly (p<0.05)

**Fig.1 — Effect of phyto-proteins on lysis of blood clots of animals and birds (Mean±SE)**

**Traditional significance of the study to the society / researchers**

The traditional knowledge of using plants in folk medicine is a valuable source of information to the
researchers of the scientific world for development of new safe drugs of natural origin. Few rural natives (Village Quacks) of Orissa gain the knowledge of using plants in folk medicine through generation and are practicing confidentially. Use of green papaya fruits and its latex in softening of meat during cooking, extracts of Tulsi and Bail leaf and paste of mango kernel in healing of traumatic wound and mushroom paste in acute inflammation are common practices by rural tribes of the state. The way of using these plants indicates proteolytic, thrombolytic and wound healing property of phyto-proteins. Such information is considered to be validated scientifically and documented for the greater interest of the entire population through development of natural thrombolytic agents by the researchers.

Conclusion
Phyto-proteins of medicinal plants used traditionally in wound healing and inflammation exhibit potent thrombolytic and fibrinolytic activities similar to streptokinase enzyme of bacterial origin. Natural therapeutics of plant origin has less/ no adverse effects for which these are safer than synthetic chemo-therapeutics. Such properties of phyto-proteins may add/ replace the use of chemo-therapeutics / costly enzymes used for dissolution of intra-vascular clots. As required during the process of wound healing, phyto-proteins may also be used to remove blood / tissue debris during treatment of traumatic and post surgical wounds. Isolation and characterization of specific domain of these phyto-proteins and the effect study in large sample size including more subject species and human beings is needed on the platform of drug development.

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