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Research Article

FIRST REPORT ON FIBRINOLYTIC AND THROMBOLYTIC ACTIVITY OF *EUTYPHOEUS GAMMIEI* AN EARTHWORM SPECIES COLLECTED FROM TRIPURA, NORTHEAST INDIA

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ABSTRACT

Objective: The present investigation for the first time evaluated the *in vitro* fibrinolytic and thrombolytic activities of crude extracts from *Eutyphoeus gammiei,* native, large size earthworm of Tripura, Northeast, India. The present study was designed to evaluate the therapeutic use of the organism *E. gammiei* as a source of fibrinolytic and thrombolytic agent(s).

Methods: The fibrinolytic activity was studied using by fibrin plate and zymography assays. Thrombolytic assay was carried out according to Prasad *et al.* (2006) using whole blood.

Results: The results obtained clearly indicated *E. gammiei* as a potential source of fibrinolytic and thrombolytic agents. Both in fibrin plate assay and thrombolytic assay with whole blood, *E. gammiei* crude homogenate showed similar and close results in respect to that of streptokinase. Fibrin zymography also showed antifibrinolytic activity with producing clear bands. Dose and time dependency also is evident from the results.

Conclusion: The results of the present study conclude that the studied earthworm species *E. gammiei* possessed profound fibrinolytic and thrombolytic activity on human blood and *E. gammiei* might prove to be useful alternative source for the development of new drugs for treatments involving blood coagulation and fibrinolysis.

Keyword: *Eutyphoeus gammiei,* Earthworm, Fibrinolytic, Thrombolytic activity, North-East India.

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INTRODUCTION

Thrombolytic disorders including cerebral stroke and myocardial infarctions have become a serious concern all over the world. Commonly used fibrinolytic agents that dissolve clots are urokinase, streptokinase (ST), and tissue plasminogen activator. These are not specific for fibrin and have adverse and dangerous side effect including severe bleeding and thereby blood loss which may result in death [1,2]. On the other hand, earthworm fibrinolytic enzyme, also known as Lumbrokinase (LK), is very specific to fibrin and it does not cause excessive bleeding [1,2]. Lumbrokinase has also shown promise for use in lowering whole blood viscosity and reducing platelets aggregation [3,4]. Earthworm fibrinolytic enzymes can be transported into the blood through the intestinal epithelium, and the enzyme works after oral administration [5]. The most common source of earthworm fibrinolytic enzyme is *Lumbricus rubellus*, *Eisenia foetida,* and *Paritima* sp. Isoenzymes constituents of each earthworm may vary depending on the species and living environment which may lead to differences in their activities [6]. Approximately 4400 different species have been identified worldwide. According to Julka *et al.* [7], of the 12 families of earthworms, the subcontinent has representative genera species form 9 families. In the year 2009 Julka *et al.* [8] reported that Indian earthworm fauna comprises about 590 species. Altough there are many reports regarding earthworm fibrinolytic enzymes from China, Japan, and other Far-East Countries, there are very few reports from India and almost no report from North East India. This present study intends to explore for the first time fibrinolytic and thrombolytic activity of *Eutyphoeus gammiei* an earthworm collected from Tripura, Northeast India.

METHODS

Sample collection and identification

Adult earthworm *E. gammiei* was collected by hand sorting and digging method by spade from Agartala, Tripura at early morning. The sample was first identified by Prof. P S Choudhuri, Earthworm Research Laboratory, Department of Zoology, Tripura University. The sample specimen was also submitted to ZSI, Kolkata, for authentication (voucher number- An 5649/1) (Fig. 1).

Collection site

The sample was collected from Agartala (Shibnagar), Tripura West, India. The site was demarcated using global positioning system (Latitude: N=23.82914° and Longitude: E=091.29485°) (Fig. 2).

Preparation of earthworm homogenate

Earthworm *E. gammiei* was first washed with running tap water and then fed with wet blotting paper for 18–20 h to clear their gut. The gut cleared worms were again washed with distilled water. 20% gut cleared earthworm homogenate was prepared in 0.2M phosphate buffered saline (PBS), pH-7.2. Homogenate was filter sterilized before use.

Determination of protein concentration

Protein concentration was determined following Lowry's *et al.* method [9].

Fibrinogen degradation assay

Fibrinolytic activity of the extract was carried out by the fibrin plate method [10]. Fibrin plate was prepared by mixing 6 ml of 0.6% bovine plasma fibrinogen and 6 ml of 1.5% agarose containing 10 NIH units of bovine plasma thrombin. 0.1 M phosphate buffer, pH 7.4 was used throughout the experiment. The prepared solution was quickly poured into a 90 mm diameter Petri plates and allowed to stand for 1 h at room temperature and labeled as fibrin plate. The earthworm was applied directly into a small pore created in the Petri plate containing artificial fibrin. ST was used as a positive control, and PBS served as negative controls. The Petri plate was incubated at 37°C for various lengths of time. Fibrinolytic activity was determined by examining the formation

of the zone of lysis in the form of a clear hollow in the fibrin plate. Fibrinolytic activity was assessed by measuring the lysis zone. The procedure was repeated 4–5 times.

Sodium dodecyl sulfate-poly acryl amide gel electrophoresis (SDS PAGE)

SDS PAGE was done to determine the purity and molecular weight of the enzyme, as described by Laemmli [11] using a 5% (w/v) stacking and a 10% (w/v) separating gels. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit (Bio-Rad) as a marker.

Fibrin zymography assay

Fibrin zymography was performed as described previously [12] bovine fibrinogen (0.12% w/v, Sigma) dissolved in 20 mM sodium phosphate buffer (pH 7.4) and 100 µl of bovine thrombin (10NIH unit ml Sigma) were copolymerized with 12% (w/v) acrylamide. 0.32% (w/v) bis acrylamide and 375 mM Tris/HCL (pH 8.8) to make the fibrin gel as the running gel. Then 5% w/v0 acrylamide, 0.11% (w/v) bis acrylamide, and 330 nM Tris/HCL (pH 6.8) (no fibrinogen) were used for the stacking gel, which was poured into a mini-gel cast (Bio-Rad). The samples for analysis were prepared by diluting the culture supernatant 5-fold with zymogram sample buffer (0.5M Tris/HCL, pH 6.8, 10% SDS, 20% SDS, 20% glycerol, and 05.% Bromophenol Blue) [13]. After the prepared samples (10 µl) were loaded into the wells, electrophoresis (Laemmli, 1970) was carried out in the cold room (4°C) at a constant 12mA. After electrophoresis, the gel was incubated for 30 min at room temperature on a rotary shaker in 50 nM Tris/Cl (pH 7.4) which contains 2.5% Triton X-100. The gel was washed with distilled water to remove Triton X-100, and then incubated in Zymogram reaction buffer (30 mM Tris/HCL, pH 7.4, 200 mM Nacl, 10 mM $CaCl_2$, and 0.02% Brij-35) at 37°C for 12 h. The gel was stained with Coomassie blue for 1 h and then distained. The digested bands were visualized as the non-stained regions of the fibrin gel.

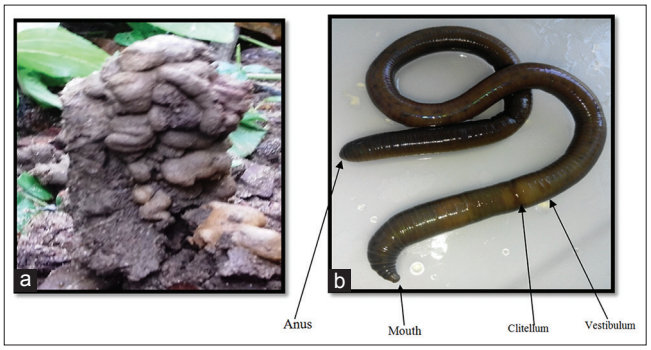

Fig. 1: (a) Earthworm cast of *Eutyphoeus gammiei*, (b) earthworm *E. gammiei*

Thrombolytic assay

Thrombolytic assay was carried out according to Prasad *et al*., [14]. Venous blood was drawn from healthy volunteers after taking informed consent (n=3). 0.5 ml venous blood was put into pre-weighed sterile microcentrifuge tubes and was incubated at 37°C for 60 min. After that, serum was removed from the blood clot. Phosphate buffer (20 mM), as blank, and 100 µl of homogenate (0.5 mg/ml) in Phosphate buffer (20 mM) was added separately to the clot and incubated at 37°C for 2 h, 6 h, 12 h, and 24 h. Fluids were removed from the remaining clots, and the tubes were weighed. Thrombolytic activity was calculated by comparing the initial weight of blood clot to that of lysed blood clot. ST was used as positive control.

RESULTS

Result obtained in the form of clear zone in fibrin plate is presented in Figs. 3 and 5. The diameter of the circles proportionately increased with the increase of incubation time and amount of homogenate (Fig. 5). Clear zone appeared around test and positive control (well no-II, III, IV, V, VI, and VII) whereas, in negative control (well no-I) containing only PBS, no clear zone appeared. Mouth and anus parts of the whole organism showed no activity (Fig. 3.).

When the activity of the homogenate was compared with that of positive control (ST), at the given protein concentration (0.5 mg/ml), the response of the homogenate was found to be very close to that of ST. Results clearly indicated that *E. gammiei* possessed fibrinolytic activity which was very similar to ST activity. In SDS PAGE, prominent bands ranging from 44 KD to 20 KD were observed, Fig. 6. To confirm the fibrinolytic activity, zymography with fibrin was performed. In zymogram two bands, though diffused, were observed, indicating the presence of isozymes (Fig. 7).

Thrombolytic activity

Visual blood clot lysis is shown in Fig. 8. Negative control containing ×1 PBS was mixed to blood clot, no obvious lysis was observed. Both standard ST (positive control) and homogenate increased the percentage of clot lysis in time-dependent manner. Highest clot lysis was observed with the addition of 100 µl of standard ST containing

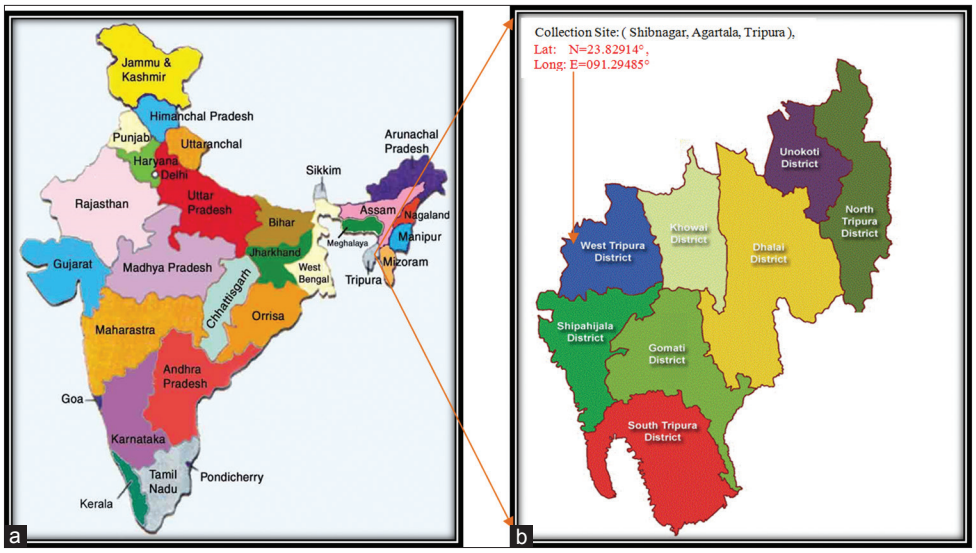

Fig. 2: Site of sample collection ([a] map of India and [b] map of Tripura showing the exact location of the site of collection)

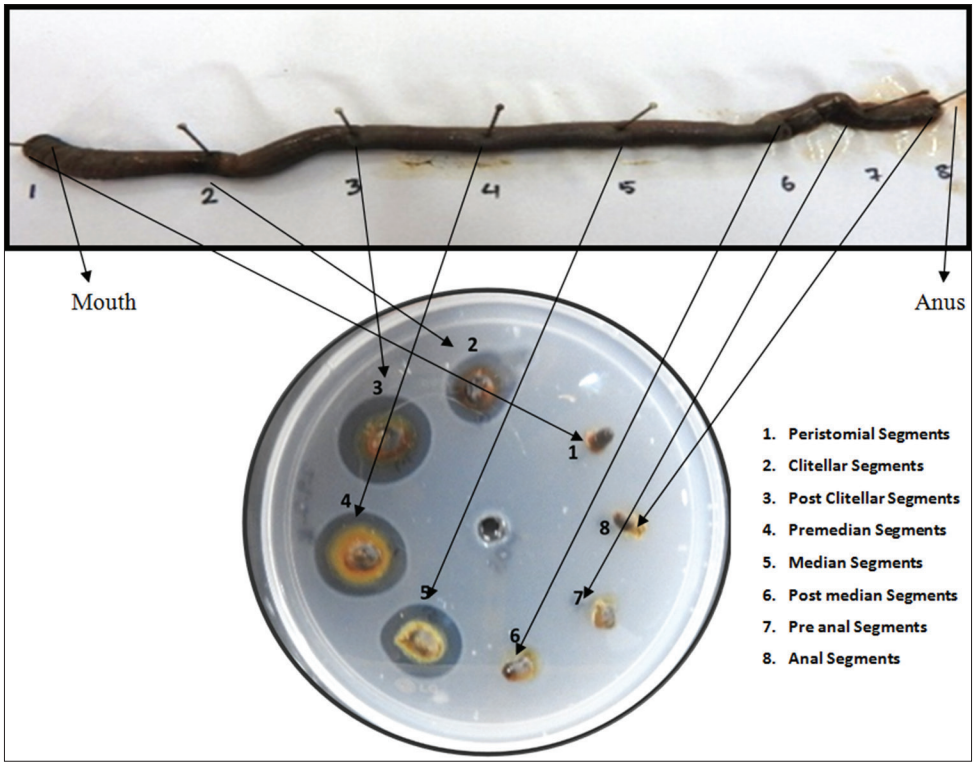

Fig. 3: Different parts of *Eutyphoeus gammiei* exhibit strong fibrinolysis. The sections were applied directly to the Petri dish plate containing artificial fibrin and incubated at 37°C overnight. Phosphate buffered saline was applied in the center well of the plate as negative controls. Bigger lysis halos (2,3,4,5) indicated higher fibrinolytic activity regions

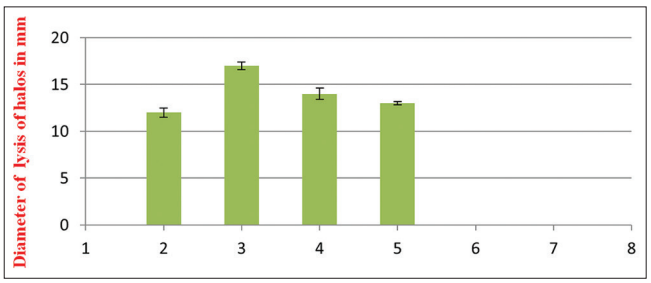

Fig. 4: Graphical representation of the fibrinolytic activity of different sections of *Eutyphoeus gammiei*

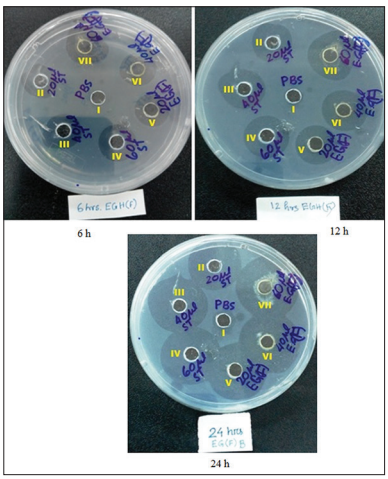

Fig. 5: Effect of homogenate of *Eutyphoeus gammiei* on fibrin plate at different incubation time interval. EGH: *Eutyphoeus gammiei* homogenate, ST: Streptokinase

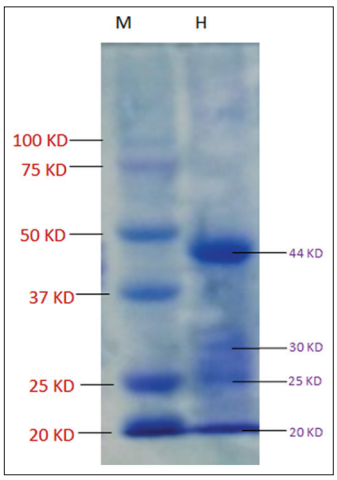

Fig. 6: Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (10%) of homogenate of earthworm *Eutyphoeus gammiei* M: Pre-stained protein marker (Biored), H: Homogenate of *Eutyphoeus gammiei*

3.7 International Unit/µl and homogenate containing 0.5 mg/ml, respectively.

DISCUSSION

Fibrinolytic enzymes dissolve fibrin, the main component of blood clots. Accumulation of fibrin in the blood vessels results in thrombosis, leading to myocardial infarction, and other heart diseases [15,16] which are the leading causes of death throughout the world. Fibrinolytic enzyme has been successfully identified from various sources [17]. Fibrinolytic enzymes and thrombolytic agents currently available for clinical use, such as ST, urokinase, pro-urokinase, reteplase, and alteplase suffer significant unintended physiological

effects such as excessive bleeding, short plasma half-life, limited fibrin specificity, and large therapeutic dose [18]. Therefore, search for less expensive and safer fibrinolytic enzymes and thrombolytic agents from other sources are still an urgent issue [19]. The scientists are always in search of biopotential compounds from natural sources for the treatment of various diseases. More and more researchers have focused on the analysis of bioactive proteins in earthworm coelomic fluid and glycolipoprotein powder [20]. The earthworm protease functions in the fibrinolysis and plasminogen activation have found to be associated with less side effects in comparison to that of urokinase, ST recombinant tissue-type plasminogen activator, etc., [21]. The protease during orally/administered experiments both in animals and clinical shows significant efficacy. The protease has been found to be stable during long-term storage at room temperature [22].

Fig. 7: Fibrin zymography with *Eutyphoeus gammiei homogenate*

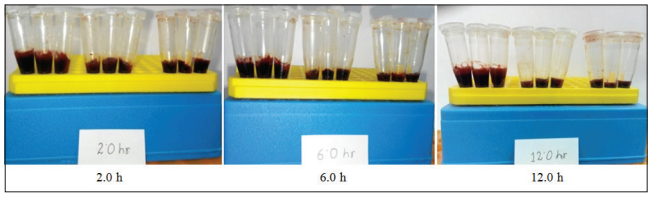

Fig. 8: Thrombolytic activity of homogenate of *Eutyphoeus gammiei* on human blood clot at a different time interval

However, because of the various living environment, different species of earthworms have different resultant isozymes [6]. Not many species of earthworms have been studied for fibrinolytic enzymes across the world.

In this study, *E. gammiei* an earthworm species native to North East India has been explored for fibrinolytic and thrombolytic potentiality. The results obtained (Table 1 and Figs. 4 and 5) clearly indicated *E. gammiei* as a potential source of fibrinolytic and thrombolytic agents. Both in fibrin plate assay and thrombolytic assay with whole blood, *E gammiei* crude homogenate showed similar and close results in respect to that of the standard drug in use ST. Fibrin zymography also showed antifibrinolytic activity with producing clear bands (Figs. 6 and 7). Dose and time dependency also was evident from the results. Further purification and characterization can lead to the clinical use of these enzymes. Therefore, along with other species, so far studied, *E. gammiei* collected from Northeastern part of India can also be a potential new source of fibrinolytic enzyme for clinical use.

CONCLUSION

The study suggested that crude extracts from earthworm of *E. gammiei* might prove to be useful alternative source for the development of new drugs for treatments involving blood clot and fibrinolysis. Further studies can now be designed to characterize and identify the protein of the *E. gammiei* extract involved in the fibrinolytic activity.

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AUTHORS' CONTRIBUTION

Madhusudan Debnath is the main Researcher who had carried out the entire experiment including acquisition of data, analysis, and interpretation of data and drafted the manuscript. Susmita Saha, Research Scholar also helped M.S. Debnath during experiment as labmate. Prof. Samir Kr Sil is the Supervisor (guide) of the research work who had planned, designed, advised, and guided Sri M S Debnath during the implementation of the experiment as well as preparation of the manuscript.

Table 1: Data for comparative fibrinolytic effects of homogenate of *Eutyphoeus gammiei* on fibrin plate at different incubation period

EGH: *Eutyphoeus gammiei* homogenate, ST: Streptokinase	0H	2H	6H	12H	18H	24H
EGH 20 µl (10 µg)	0	11.66±0.33	15±0.57	18.66±0.33	24.66±0.33	25.33±0.33
ST 20 µl	0	12.33±0.33	16.66±0.33	19±0.57	24.66±1.15	27.00±1.00
EGH 40 µl (20 µg)	0	12.66±0.33	16.66±0.66	20.33±0.33	27.00±0	28.66±0.66
ST 40 µl	0	12.66±0.33	18.33±0.88	23.33±0.88	28.00±1.00	28.66±0.66
EGH 60 µl (30 µg)	0	13.66±0.33	19.33±0.67	24.00±0.57	29.33±2.85	29.66±0.66
ST 60 µl	0	14.66±0.33	20.00±1.15	24.66±0.88	29.33±0.66	30.66±0.66

Mean±SD, n=3. EGH: *Eutyphoeus gammiei* homogenate, ST: Streptokinase, SD: Standard deviation

Table 2: Percentage of thrombolytic effect of Homogenate of *Eutyphoeus gammiei*

Control, standard and sample	Thrombolytic effect of *Eutyphoeus gammiei* after 1 h	Thrombolytic effect of *Eutyphoeus gammiei* after 2 h	Thrombolytic effect of *Eutyphoeus gammiei* after 6 h	Thrombolytic effect of *Eutyphoeus gammiei* after 12 h
Control	0%	0%	0%	0%
Streptokinase	6±0.2	41.77±0.02	55.10±0.1	57.14±0.02
Homogenate	2±0.2	40.8±0.15	51.02±0.01	55.10±0.1

CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interests among themselves.

REFERENCES

1. Vernooij MW, Haag MD, van der Lugt A, Hofman A, Krestin GP, Stricker BH, *et al.* Use of antithrombotic drugs and the presence of cerebral microbleeds: The Rotterdam scan study. Arch Neurol 2009;66:714-20.
2. Delaney JA, Opatrny L, Brophy JM, Suissa S. Drug drug interactions between antithrombotic medications and the risk of gastrointestinal bleeding. CMAJ 2007;177:347-51.
3. Nakajima N, Mihara H, Sumi H. Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus*. Biosci Biotechnol Biochem 1993;57:1726-30.
4. Ji H, Wang L, Bi H, Sun L, Cai B, Wang Y, *et al.* Mechanisms of lumbrokinase in protection of cerebral ischemia. Eur J Pharmacol 2008;590:281-9.
5. Fan Q, Wu C, Li L, Fan R, Wu C, Hou Q, *et al.* Some features of intestinal absorption of intact fibrinolytic enzyme III-I from *Lumbricus rubellus*. Biochim Biophys Acta 2001;3:286-92.
6. Rong P, Zhang ZJ, He RQ. Earthworm protease. Appl Environ Soc Sci 2010;2010:Article ID: 294258, 13 Pages.
7. Julka JM, Paliwal R, Kathireswari P. Biodiversity of Indian earthworms-an overview. In: Edwards CA, Jayaraaj R, Jayraaj IA, editors. Proceedings of Indo-U S Workshop. Coimbatore, India: Vermitechnology in Human Welfare; 2009. p. 36-56.
8. Julka JM, Paliwal R, Kathireswari P. Biodiversity of Indian earthworms-an overview. In: Edwards CA, Jayaraaj R, Jayraaj IA, editors. Proceedings of Indo-U S Workshop. Coimbatore, India: Vermitechnology in Human Welfare; 2009. p. 36-56.
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin-phenol reagent. J Boil Chem 1951;193:265-75.
10. Astrup T, Mullertz S. The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys 1952;40:346-51.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
12. Kim SH, Choi NS, Lee WY. Fibrin zymography: A direct analysis of fibrinolytic enzymes on gels. Anal Biochem 1998;263:115-6.
13. Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: Detection of picogram quantities of gelatinases. Anal Biochem 1994;218:325-9.
14. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF, *et al.* Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. Thromb J 2006;4:14.
15. Voet D and Voet JG. Biochemistry. New York, USA: John Wiley and Sons Press; 1990.
16. Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y, *et al.* Purification and characterization of a fibrinolytic enzyme produced from bacillus sp. Strain CK 11-4 screened from chungkook-jang. Appl Environ Microbiol 1996;62:2482-8.
17. Takeno T, Okamura T, Sera M, Takana M, Fukuda S, Ohsugi M. Screening of fibrinolytic enzyme of microorganisms. Bull Mukogawa's Univ Nat Sci 1999;47:67-72.
18. Mackman N. Triggers targets and treatments for thrombosis. Nature 2008;451:914-18.
19. Rashad MM, Mahmoud AE, AI-Kashef AS, Mohamed UN. Purification and characterization of a novel fibrinolytic enzyme by *Candida guilliermondii* grown on sunflower oil cake. J Appl Sci Res 2012;8:635- 45.
20. Anjana JC, Sruthy P, Rathinamala J, Jayashree S. *In vitro* evaluation of glycolipoprotein powder from earthworm *Eudrilus eugeniae*. Int J Pharm Pharm Sci 2015;7:377-9.
21. Kim JS, Kang JK, Chang HC, Lee M, Kim GS, Lee DK, *et al.* The thrombolytic effect of lumbrokinase is not as potent as urokinase in a rabbit cerebral embolism model. J Korean Med Sci 1993;8:117-20.
22. Nakajima N, Sugimoto M, Ishihara K. Stable earth-worm serine proteases, application of the protease function and usefulness of the earthworm autolysate. J Biosci Bioeng 2000;90:174-9.