IN VITRO THROMBOLYTIC ACTIVITY OF EUDRILUS EUGENIAE.

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Abstract
Atherothrombosis is the main cause of cardiovascular diseases. Cardiovascular diseases (CVDs) take the lives of 17.7 million people every year, 31% of all global deaths due to the Heart attacks and strokes caused by a vessel blockage by thrombus. Thrombolytic drugs are available in market, however, they cause some side effects and expensive. Therefore, present research work carried out on, In vitro thrombolytic activity of earthworm: Eudrilus eugeniae extract (EEE) using plasma clot method. Clot lysis is check at various concentrations of extract against the artificially prepared plasma clot. Streptokinase used as standard and sterilized distilled water used as control. The percentage of clot lysis was analysed by computing the difference between weights of clot. The earthworm: Eudrilus eugeniae extract (EEE) at various concentrations exhibit significant activity i.e. 29.95 ± 1.96 and 31.70 ± 0.57% at the concentration 0.050 and 0.1 mg/ml respectively. The thrombolytic activity of EEE concentration at 0.050 and 0.1mg/ml was parallel to the standard drug 31.80 ± 0.20 %. Hence, it may conclude that, the earthworm extract have the potential to dissolve clot, therefore, it is urgent need to isolate the active molecule which is therapeutically beneficial in future use.

Key words: Thrombosis, Eudrilus eugeniae, Earthworm extract, Thrombolytic activity.

I. INTRODUCTION
Atherothrombosis is the main cause of cardiovascular diseases. Thrombosis made impairment in blood flow, however, one of the important substance plasmin present in blood in inactive form which dissolve clot intravenously. Plasmin is the trypsin like enzyme which catalyzed the fibrin and converts the clot in to soluble substance; hence, the restoration of the blood flow takes place. Plasmin is the key substance in fibrinolysis system; it is formed by the activation of plasminogen. Plasmin, formed under the influence of plasminogen activators (Rozhchenko, 2003). Plasminogen is activated by Tissue-type plasminogen activator (t-PA) and Urokinase and Streptokinase -type plasminogen activator (u-PA and s-PA respectively) (Adanna et al., 2013). Moreover, there are various plasminogen activators available in market which broadly categorized into tissue plasminogen activator and other plasminogen activator of which some are popular existing antithrombolytic drugs used by medical personnel’s. These are Streptokinase, Anistreplase, Urokinase however, they have some side effects like Hemorrhage, purpuric rashes, thrombocytopenia and Contradiction with severe hypertension, peptic ulcer disease, ulcerative colitis, pancreatitis, sub acute bacterial endocarditis, coagulation defects due to liver or kidney disease, recent surgery, childbirth, hypersensitivity, increased risk of cerebral bleeding, trauma, pregnancy, active internal bleeding, bleeding GI lesions, besides this they are extremely expensive (Asiful et al., 2016). To overcome the said problem, some researcher works on exploration of plasminogen activator substance from plant and animal origin, which dissolve clot without any side effects. Jen et al., in 1989 studied and screened 111 extracts prepared from 37 kind of 16 invertebrate and 21 vertebrate animals and reported that the animal origin extracts exhibits good fibrinolytic activity than plant origin extracts. The plant originated active compounds or groups i.e. Protease enzyme named Kitamase, from Aster yomena Honda (Choi et al., 2014), Tannins, saponins, terpenes and flavonoids from Campanum Africanus xanthocarpa (Jonatas et al., 2012), Bromelain (combination of proteolytic enzymes) from Ananas comosus (Glaser et al., 2006), Polyphenols and citrate from Mulberry (Yamamato et al., 2006), from Animal products i.e. Flavonoids and Polyphenols from...
Honey (Manukumar and Shruti 2014), Fungi origin i.e. Serine protease from Aspergillus oryzae (Norifumi et al., 2012), Serine metalloprotease from Candida guilliermondii (Mona et al., 2012). Protease enzyme from Fusarium sp. (Ueda et al., 2007), are well documented in literature. Among these, on the basis of update literature, the earthworm has a long association with medicine, since its use in various remedies date back to 1340 A.D (Reynolds and Wilma 1972). For instance, doctors practicing folk medicine in Burma and India; uses earthworms in the treatment of various diseases. The fibrin clot dissolving enzyme extract potency of earthworm was first time reported by Dr. Hisashi Mihara and his colleagues in 1983 (http://earthwormvietnam.com/useful-earthworm-powder-human-care.html). They purified and characterized various fractions from earthworm Lumbricus rubellus. One of them was labelled as lumbrokinase and reported its fibrinolytic activity in fibrin plate model (Mihara et al., 1991). The exhaustive and comprehensive review on lumbrokinase ignite an idea to find out such type of molecule from the native earthworm species, hence, in present study an attempt has been made to evaluate Thrombolytic activity of the extract of Eudrilus eugeniae (Kinberg, 1867).

II. MATERIALS AND METHODS

Collection of Animal

Earthworms were purchased from Sanjeevanee Gandul Khat Prakalp, Waghoda (BK), Tal. Raver, Dist. Jalgaon of North Maharashtra Region, after the permission granted by the Maharashtra State Biodiversity Board. The earthworm Eudrilus eugeniae was identified and authenticated by Zoological Survey of India, Kolkata. The earthworm acclimatized and cultured in the laboratory in vermicomposting unit and use for further study.

![Fig.1 Eudrilus eugeniae Kin.](image)

Extraction of thrombolytic enzyme

The extract used in present study was achieved by following the extraction method of Hwan et al., (2004). Earthworms were washed with tap water, then after kept and allow in annelid saline for 2- 3 hrs to removing the cast from alimentary canal of earthworm. Weight the cleaned earthworm, and homogenate in 20 mM phosphate buffer; pH 7.4. Homogenate kept for 4 hrs at 45°C, after that, homogenate set aside in 0.025% sodium azide for 15 days for autolysis. After autolysis of homogenate, centrifuge it and filter by using celite and 0.45µm membrane through vacuumed filter twice. Obtained filtrate evaporates on water bath at 60°C. The selected concentration of Crude extract suspended into 0.1 M Phosphate buffer; pH 7.4 and labeled as earthworm: Eudrilus eugeniae extract (EEE) and used for further study.

Blood collection

Volunteer of either sex used to draw the 3.0 ml blood; collected from median cubital vein, immediately place it in to the tri-sodium citrated bulb and centrifuge to obtain citrated plasma.

Preparation of Plasma clot

The method for plasma clot was adapted as mentioned by Purnima et al., (2015). Then blood was mix immediately with 3.8% tri- sodium citrate in volume ratio of 9:1 and centrifuge at 3000 rpm for 15 min. to obtain citrated plasma. 400 µl citrated plasma was added to pre- weighed eppendorf tube and add to in it 100 µl 0.2 M CaCl₂. The eppendorf tube incubates at 37°C for 30 min. in water bath, allowed to stand until firm clot was obtained.

Determination of Thrombolytic activity

Determination of thrombolytic activity was carried out according to the method of Prasad et al., (2007). The clot containing eppendorf tube labeled as control load with sterile distilled water, standard load with Streptokinase (is one of the recognize plasminogen activator) at 1000 IU/ml concentration and two test sample tubes Eudrilus eugeniae extract (EEE) loaded with earthworm extract at the concentrations 0.05 and 0.10 mg/ml. All these eppendorf tubes were incubated for 90 min at 37°C in water bath. After incubation
measure the released clot and weigh the remaining clot. Following formula was used to calculate the clot percentage.

\[
\text{% of clot lysis} = \left(\frac{\text{weight of released clot}}{\text{weight of clot}}\right) \times 100.
\]

Where, Weight of released clot = deduction of weight of eppendorf tube after clot lysis from weight of eppendorf tube before clot lysis

Weight of clot = subtracts the weight of empty eppendorf tube from the weight of clot containing eppendorf tube.

III. STATISTICAL ANALYSIS

The significance % of clot lysis was compared with control. The data was expressed as mean ± standard error. Statistical analysis was carried out by one way analysis of variance (ANOVA) followed by “Bonferroni multiple comparison test” at p<0.05 level of significance using SPSS software.

IV. RESULTS

The test earthworm extract at concentration 0.05 and 0.10 mg/ml showed 29.95 ± 1.96 %, 31.70 ± 0.57 % clot lysis respectively. On the basis of results we conclude that the activity is not dose dependent. The standard streptokinase at 1000 IU/ml of dose showed 31.80 ± 0.20 % clot lysis. All doses are significantly increases the percentage of clot lysis as compared to the control. What is significant to note is that, EEE at the concentration 0.10 mg/ml (31.70 ± 0.57%) exhibits parallel activity to that of standard streptokinase at 1000 IU/ml (31.80 ± 0.20 %).

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Group</th>
<th>Concentration</th>
<th>% of clot lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>--</td>
<td>04.31 ± 0.84***</td>
</tr>
<tr>
<td>2.</td>
<td>Streptokinase</td>
<td>1000 IU/ml</td>
<td>31.80 ± 0.20***</td>
</tr>
<tr>
<td>3.</td>
<td>EEE – I</td>
<td>0.05 mg/ml</td>
<td>29.95 ± 1.96***</td>
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<tr>
<td>4.</td>
<td>EEE – II</td>
<td>0.10 mg/ml</td>
<td>31.70 ± 0.57***</td>
</tr>
</tbody>
</table>

***P < 0.001, Vs control.

V. DISCUSSION

Now-a-days, epidemiological studies have revealed that increase in cases of atherosclerosis is due to several important environmental and genetic risk factors. The most critical clinical aspect of atherosclerosis is plaque rupture and thrombosis, the term is called atherothrombosis (Aldons, 2000). According to World Health Organization, now a day, the cardiovascular diseases are the largest threatened issue. To control this issue there are some fibrinolytic agents are available in market namely, Urokinase, tP-A, however, these fibrinolytic agents are expensive and short life in in vivo etc., The Streptokinase is cost effective; however, the immunogenicity restricts the treatments, if the antistreptokinase presents in circulation (Banerjee et al., 2004). Earthworm is the medicinally important was reported by number of research workers those know the earthworm's long association with medicine. The Jen et al., in 1989, studied on natural substance of hematology and evaluate the animal crude extract from 37 type of animals, and 120 drugs derived from plant for their fibrinolytic activity using fibrin plate method, their findings showed that water extract of animal crude drugs of Scolopendra, Agkistrodon, Hirudo, Sepia Os, Lumbricus exhibit 115, 114, 107, 107, 101 % of fibrinolysis respectively, the MeOH extract of Hirudo also showed 105% fibrinolysis activity. In present study, crude extract of earthworm (EE) were studied for their in vitro thrombolytic potential and streptokinase used as standard for comparison, in plasma clot model. Mihara et al., in 1991 firstly reported and isolated the earthworm Fibrinolytic enzyme “Lumbrokinase” from Lumbricus rubellus, and evaluated fibrinolytic activity using fibrin plate method, firstly they demonstrated that the anterior fragment of earthworm has good activity as compared to posterior fragment of earthworm and then compared the Earthworm Proteolytic Enzyme, (EPE) 50- days solution to thrombolytic agent- urokinase, which indicate equivalent fibrinolytic activity, moreover in subsequent characterization of EPE concluded that the enzyme activity retained in pH range between 3 to 10 at 37°C and pH 6 to pH 9 at 60°C. Our results, earthworm extract of Eudrilus eugeniae evaporated at 60°C at the concentration 0.10 mg/ml showed 31.70 ± 0.57 % clot lysis, are in agreement with that of Mihara et al., (1991). Feng et al., in 2003, purified, characterized and crystallized fibrinolytic enzymes from Eisenia fetida and compared the lumbrokinase from Lumbricus rubellus and fibrinolytic enzyme from Eisenia fetida, and found that N-terminal sequence of both enzymes homologous to each other. They state that it may be due to difference in Genera, which supports evolutionary trend. In current study, the Eudrilus eugeniae, other genera of earthworm, extract is used. On the basis of results, Eudrilus eugeniae extract has capability to dissolve clot as like lumbrokinase and...
fibrinolytic enzyme from *Eisenia fetida* reported by Mihara et al., (1991) and Feng et al., (2003) respectively, and, hence our findings are counterpart to that of cited above; which may also represent the descendent hereditary fibrinolytic potency. Therefore, to prove this, further study on isolation and characterization is warranted.

**VI. CONCLUSION**

The potential results reveal that further, study is needed for isolating the particular active principle and characterizes the specific molecule, which is responsible for clot lysis from earthworm, *Eudrilus eugeniae*. This may also supports pharmaceutical industries and farmers for their sustainable livelihood.

**VII. REFERENCES**


