



## Isolation and determination of biochemical nature of water soluble anticoagulant from earthworm

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### Abstract

Earthworms are commonly known as farmer's friend. Various previous studies have confirmed the anti-inflammatory, analgesic, antipyretic and anticancer effects of earthworm extract. In the present study selected species of earthworm was used to study the anticoagulative activity of earthworm extract by APTT test. The study involves the extraction and isolation of anticoagulant from earthworm which was found to be in the form of DNA. In order to uncover the biochemical nature of this molecule, the anticoagulant was processed with various hydrolases such as Proteinase-K, Dnase, Rnase and lysozyme. Simultaneously APTT test and agarose gel electrophoresis were performed to confirm the results. Standard herring sperm DNA and yeast RNA were also used to compare the anticoagulative activities with that of anticoagulant purified from earthworm. Individual components of nucleotide were also checked which might be responsible for the anticoagulative capability.

**Keywords:** Anticoagulant activity, APTT, DNA, *Eudrilus eugeniae*, Hydrolases

### Introduction

Earthworm has been recognized as an anti-inflammatory, analgesic and antipyretic agent (Noda *et al.*, 1996). It shows anticancerous effect by preventing excess glucose uptake (Nagasawa *et al.*, 1991). It is also implicated in hemostasis by acting either as a fibrinolytic or anticoagulatory agent which results in the facilitation of blood circulation (Wang *et al.*, 1989). Anticoagulation activity was reported by Woo (1996) on an earthworm species *Lumbricus rubellus*. The earthworm has been suspected to contain proteases which specifically dissolve the fibrin clots or anticoagulant(s) which selectively interfere with the intrinsic pathway of the blood coagulation cascade (Mann *et al.*, 1990; Davie *et al.*, 1991; Leipner *et al.*, 1993; Kim *et al.*, 1995; Woo, 1996). Antimicrobial and Anti-inflammatory activities of earthworm, *Eudrilus eugeniae* were investigated by Mathur *et al.* (2010 a and b) Pharmaceutical significance of earthworm *Eudrilus eugeniae* was

also reported by him. *Eudrilus eugeniae* is an African earthworm specie and is having good reproduction capability. As no work like the present investigation is reported till yet thus we have emphasized our study on this specie. The aim of our study was to investigate the anticoagulant activity from earthworm specie, *Eudrilus eugeniae* and to determine the biochemical nature of the anticoagulant.

### Materials and Method

#### Collection of material

Adult earthworms were provided by Jai Bharat Nursery, Rani Pokhari, Rishikesh (U.K), India. The worms were washed in order to remove the sand debris and were kept in N-saline for washing the gut. The step was repeated several times until the gut gets thoroughly cleared.

#### Purification of Anticoagulant

A detailed purification procedure (Woo, 1996) was adopted. The earthworms were homogenized in distilled water in the ratio 1:1 (w/v) followed by heat extraction at 100 °C for 30 minutes after centrifugation, the supernatant was subjected to ammonium sulphate fractionation at final concentration of 80 %. The precipitate was suspended in a minimum volume of 50 mM Tris

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HCl (pH 8.0) and the anticoagulatory activities were measured by Activated partial thromboplastin time (APTT) test.

#### Activated Partial Thromboplastin time (APTT) test

An *in vitro* coagulation test of APTT was performed according to the manufacturer's instruction. The data was analyzed in percent coagulation time.

#### Agarose gel electrophoresis

1% agarose gel was prepared in 1X TAE buffer according to the standard protocol and the sample was loaded along with marker in the wells along with gel loading buffer. The observation of the bands was done under UV-transilluminator.

#### Treatment of anticoagulant with various hydrolases

The concentrated sample of 50  $\mu$ l (0.92 mg/ml) was separately incubated with Proteinase-K (2  $\mu$ g), DNase (5  $\mu$ g) in the presence of 2mM MgCl<sub>2</sub>, Rnase (5  $\mu$ g) and lysozyme (20  $\mu$ g) at 37°C overnight in a total volume of 150  $\mu$ l adjusted with 50mM Tris HCl (pH 8.0). Later on APTT test and agarose gel electrophoresis of the hydrolases digested samples were carried out.

#### APTT of Standard DNA, RNA and individual components of nucleotide

APTT test was performed according to the manufacturer's instruction available in the kit. Calf thymus DNA and Yeast RNA were used as positive standards. Pentose sugars (Deoxyribose and Ribose sugars), phosphoric acid nitrogenous bases (purines and pyrimidines) were used for the determination of APTT in order to assess the individual component responsible in anticoagulant activity of DNA.

#### Results and Discussion

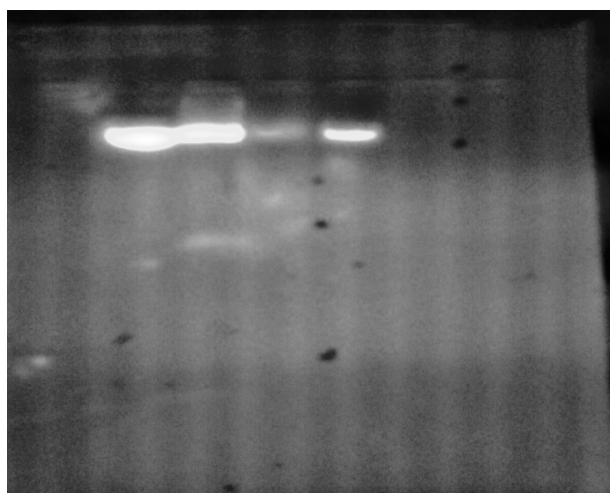
From the present investigation it was revealed that the anticoagulant purified from earthworm extract was in the form of DNA which produced reddish-orange colored bands with ethidium bromide under UV transilluminator. When APTT test was performed of this extracted anticoagulant it showed 76.66% coagulation time (Table-1) with respect to Standard Diagnos Thrombo reagent (available in the kit).

To confirm our studies, when the anticoagulant was treated with various hydrolytic enzymes at 37°C

overnight, a diffused band of DNA was observed on treatment with Dnases under UV light, which confirmed our studies that the anticoagulant is in the form of DNA (Fig.1). When such DNase treated sample was subjected for APTT the anticoagulant activity gets reduced. This decrease in value of APTT might be due to the digestion of anticoagulant (DNA) by Dnases (Table-2).

**Table-1: Activated prothrombin thromboplastin time (APTT) of purified anticoagulant from earthworm extract**

Reagent\Earthworm extract	Mean values of APTT (seconds)	% Coagulation time
Diagnos Thrombo Reagent	120	130.43
Earthworm extract	92	76.66



**Fig.1: Bands of digested DNA after the treatment of hydrolases. (1, proteinase-k treated; 2, lysozyme treated; 3, Dnase treated; 4, Rnase treated)**

**Table-2: Activated prothrombin thromboplastin time (APTT) of hydrolases treated purified anticoagulant**

Reagent\ Earthworm extract	Mean values of APTT(seconds)			
	Hydrolases treated Dnase	Proteinase Lysozyme -k	Rnase	
Diagnos Thrombo Reagent	62	56	66	62
Earthworm extract	136	51	70	154

Our next question was whether this effect of the DNA was unique for the earthworm, *E.eugeniae*. When herring sperm DNA was used to measure its effect on the coagulation, the APTT value gets reduced *viz.* 45 seconds (Table-3) which confirmed our findings that anticoagulant (DNA) purified from earthworm, *E. eugeniae* is a potent anticoagulant. When the results of APTT of standard yeast RNA were compared with that of anticoagulant (DNA) purified from earthworm and herring sperm DNA, the coagulum appeared in no time in the plasma treated with RNA sample. Simultaneously no values of APTT were observed in pentose treated plasma sample (Table-3). It may be due to the fact that single stranded RNA could be more compact than double stranded DNA. We assumed therefore that the effect of anticoagulation was due to negatively charged matrix provided by the extended DNA. If this assumption is valid, the DNA could be compared with heparin in terms of their anticoagulatory mechanisms. It has been already shown that thrombin inhibition by AT-III was accelerated in the presence of heparin since it provides a negatively charged matrix as a template (Ehrlich *et al.*, 1991; Nesheim, 1983).

**Table-3: Activated prothrombin thromboplastin time of Standard DNA, RNA and components of nucleotide**

Samples	Mean values of APTT (seconds)
Herring sperm DNA	45
Yeast RNA	NA
Nitrogenous bases	90
Phosphoric acid	36
Pentose sugar	NA

NA= No activity

We further confirmed that nitrogenous bases present in the DNA are the active components responsible for anticoagulant activity. The mean value of APTT was found to be 90 seconds much higher than that of phosphoric acid *viz.* 36 seconds (Table-3) while pentose sugar showed no values of APTT. Further studies are needed to refine the technique. Thus the present investigation revealed the fact that DNA could be considered as alternative thrombotic agent to heparin and various other anticoagulants used routinely in pathological labs.

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