Unveiling the Therapeutic Potential of Annelids as Thrombolytic Agents: A Research Investigation

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Abstract

Thrombosis, the development of blood clots in the circulatory system, presents a notable global health challenge. Encouragingly, natural sources derived from annelids, such as lumbrokinase found in earthworms and hirudin from leeches, and demonstrate potential as antithrombotic compounds. In this study, the modified fibrin plate method was employed to reveal the substantial specific fibrinolytic activity of 816 U/mg in *Eisenia fetida*. This was estimated from a standard fibrinolytic activity curve, demonstrating dose and time-dependent blood clot lysis activity in an *in-vitro* model. Incubating crude earthworm tissue extract at concentrations ranging from 0.055mg to 0.22mg resulted in clot lyses percentages of approximate 50.00% and 70.00% after 180 minutes. Historically esteemed for its medicinal value, the leech, *Hirudo medicinalis,* demonstrated notable efficacy in preventing blood clot formation compared to heparin in our investigation. We observed a significant APTT prolongation to 50 seconds, exceeding the normal range of 25 to 35 seconds, prompting an exploration of hemostatic disruptions. Furthermore, a dose-dependent relationship with medicinal leech tissue extract showed a systematic delay in blood clot formation. Hirudin activity, assessed through the thrombin titration method, revealed a specific activity of 13,000 ATU/mg. This comprehensive study underscores the medicinal significance of annelids, like earthworms and leeches as thrombolytic and anticoagulant agents in traditional medicine. Their accessibility, affordability, and minimal side effects further highlight their potential in addressing thrombosis-related disease.

1. INTRODUCTION

Annelida, a diverse phylum of invertebrates known as segmented worms, encompasses a wide array of species, ranging from earthworms to leeches. These creatures play crucial roles as decomposers, predators, and prey within various ecosystems, thriving in freshwater, marine, and terrestrial environments [1].

The phylum Annelida consists of three main classes: Oligochaeta, Clitellata, and Polychaeta. Earthworms and leeches belong to the Clitellata class. Leeches are renowned for their unique ability to draw blood, and their saliva contains anticoagulants, making them valuable in

various medical procedures. On the other hand, earthworms are widely acknowledged for their crucial role as decomposers in terrestrial ecosystems. Additionally, they are being explored for their potential application as antithrombotic agents, indicating a broader spectrum of significance beyond their ecological role [2].

The medicinal importance of leech can be clearly understood from the Anglo-Saxon word of "laece" means "Physician" that means from ancient civilization doctor and annelids were etymologically affiliated to each other [3]. In Islamic literature, the utilization of leech as therapy was precisely documented. In Book "Cannon of Medicine" Avicenna (980-1037 AD) wrote that leech have the ability to suck the blood from deep body vein that otherwise not possible with traditional wet cupping technique [4].

Notably, leeches like *Hirudo medicinalis* produce hirudin, a potent blood coagulation inhibitor discovered by Hay Craft [5].Anticoagulants in saliva are primarily proteins and peptides released by leech salivary glands [6]. Leech saliva includes over 100 bioactive chemicals with medicinal potential., Apart from the most prevalent bioactive molecule hirudin found in leech saliva, additional chemicals found in leech saliva include hyaluronidase, destabilase, Apyrase, Calin, Eglins, Bdellins, Carboxypeptidase A, Acetylcholine, and Histamine-like substance [7].

These active compounds have a variety of biological activities, including anticoagulants, vasodilators, anesthetics, antimetastatic, thrombolytics, anti-inflammatory, analgesic effect, slowing down adhesion and aggregation, repairing degraded tissue vascular permeability, decreasing hypoxia and healing stroke animals, exfoliating the organism via antioxidant pathways, degradation of the extracellular matrix, and antibacterial action[8-10].

Earthworms have a rich history in traditional Chinese medicine, where they've been utilized for pain relief, fever reduction, poison counteraction, hypertension treatment, and addressing various ailments such as migraines, joint pain, and insomnia [11].

These soil-dwelling creatures possess oxygen exchange and antioxidant properties and transfer health-beneficial elements from the soil to the body. They secrete antiseptic mucus, enabling the development of medicinal products. Extracts from earthworms are used to create pills, ointments, and lotions. These creatures are abundant in essential nutrients, including cholesterol, unsaturated fatty acids, proteins, lipids, and more, crucial for human health [12].

In the late 18th century, Fredericq discovered proteolytic enzymes in earthworms' digestive systems, with later identification of enzymes capable of breaking down various substances [13].

Earthworm enzymes have been extensively studied since 1980. The proteases found in earthworms are multicomponent. Diverse earthworm species have diverse isozymes as a result of their different living conditions. Most of earthworm proteases reside in the anterior alimentary areas of the crop and gizzard. Earthworm oil was historically applied to address hemiplegia, paralysis, and muscle discomfort. Research also indicated the potential of an earthworm-derived fibrinolytic enzyme in wound healing, with no reported enzymes-related side effects. Powdered earthworms are highly valued in the Asia because of strong antipyretic, diuretic drugs, and fibrinolytic properties. A novel catalog of Earthworm Fibrinolytic Enzymes (EFEs) was unveiled by [14] showcasing multiple direct-acting fibrin(ogen)olytic enzymes with notable anti-oxidative and anti-bacterial properties.

2. MATERIALS AND METHODS

2.1 Sampling and Identification of Specie

Earthworms were collected from various locations in Bhimber, Azad Kashmir (Pakistan), between April and October 2021, including agricultural fields, cow manure, discarded areas, and soil. Leeches were gathered from the muddy areas of district Gujrat, Pakistan, in July and August 2021, using the hand sorting method. Species identification relied on keys from [16] for earthworms and morphological characteristics outlined by [17] for leeches.

2.2 Preservation after Collection

Worms were washed to remove surface debris, and earthworm guts were cleaned by soaking in cold water for 24 hours. Medicinal leeches, after rinsing and blood extraction, were sedated with 8% ethanol and morphologically identified by preserving them in 4% formalin, later transferring to 70% ethanol. Samples were stored at 4 °C for future use.

2.3 Zymogram of Earthworm and Leech

2.3.1 Sample preparation

Earthworm (1g) and leech tissues (0.9g) was homogenized in sterilized PBS extraction buffer (1.0 ml/100 mg of tissue). After centrifugation at 12000 rpm for 5 minutes at 4°C, the soluble supernatant containing lumbrokinase and hirudin served as the tissue extract for quantifying fibrinolytic enzymatic activity.

2.3.2 Preparation of native PAGE (Polyacrylamide Gel Electrophoresis)

For Zymogram analysis non-denaturing polyacrylamide gel (12 %) was prepared and loaded with 20 µl tissue extract supernatant. The gel was electrophoresed at 100 V for 2 hours until the bromophenol blue dye sinks to the gel bottom.

For overlaying gel, substrate (0.01%) was dissolved in PBS buffer supplemented with agar (1.5%), heated and allowed it to solidify in a Petri plate. After electrophoresis, the gel was overlaid on petri plate containing solidify substrate and incubated at 37°C for 2 hours. After incubation, the acrylamide gel was removed and petri plate was stain with staining solution (250 mg Coomassie Brilliant Blue RT 250, 270 ml Methanol, 45 ml Acetic acid, and distilled water to 500 ml) for 30 minutes. Then zymogram gel was de-stain with solution (50 ml methanol, 70 ml acetic acid, and distilled water to 1 liter) until clear. Distinct bands on a dark blue background indicate proteolysis sites.

2.4 Total Protein Estimation

Total protein concentration in the tissue extract was determined by Bradford assay, employing bovine serum albumin (BSA) as a standard [18]. Bovine Serum Albumin (BSA) standards were prepared in microcentrifuge tubes spanning concentrations from 0.125 to 1.5 mg/ml simultaneously; create three dilutions of the unknown crude tissue extract.

Bradford reagent was added at a 1:5 ratio (e.g., 1 ml reagent per 200 µl sample). Incubate for 60 minutes for color development, then measure absorbance at 595 nm against a Bradford reagent blank.

2.5. Activity Assays for Enzymes

2.5.1 Fibrinolytic activity assay for lumbrokinase

Fibrinolytic enzyme activity in earthworm tissue extract was detected by modified fibrin plate method. For plate preparation, a 1.0% agarose solution in 1xPBS buffer was heated, combined with 1 IU/ml thrombin and 1 mg/ml fibrinogen in TBS pH7.4, and allowed it to solidify in a petri dish. Well of 5 mm was made by punching in plates, loaded with 50 µl crude tissue extract, and incubated overnight at 37°C. Hydrolysis around wells was assessed by measuring clear zone diameter with ruler (calculate the mean values from three replicates n = 3) sample activity relative to controls.

2.5.2 Blood-clot lysis activity assay

An *in-vitro* experiment was performed in accordance with the methodology outlined elsewhere [19], 500 µl of fresh blood was incubated in pre-weighed Eppendorf tubes for 2 hours at 37°C. After clot formation, serum was removed, and clot weight determined. Various concentrations of crude tissue extract from earthworm enzyme in the range of 50 µl to 200 µl were added into the blood clot and incubated at 37°C for different time interval. After incubation, clot lysis was measured by weight change. Results (mean ± SD, n=3) were analyzed using a t-test ($p < 0.01$).

2.5.3 Hirudin activity assay

2.5.3.1 Thrombin standard curve

Antithrombin activity of hirudin was quantified through titration with a thrombin solution, the activity was express in Anti thrombin Units (ATU) by comparing them to standard anticoagulants such as heparin. [19]. In the experiment, 100 µl anticoagulant was mixed with 200 µl 0.05% bovine fibrinogen in a 50 mM Tris-Cl buffer (pH 7.5) and 5 µl thrombin solution (100 NIH/mL) was added gradually, mixed, and incubated at 37°C for 1 minute. The experiment was stopped if a fibrin clot forms within 1 minute or continued to add more thrombin solution if no clot appears [20].

2.5.3.2 Blood coagulation assays

The aPTT test (activated partial thromboplastin time) assay confirmed the functionality of hirudin utilizing a Rayto RT-2201C coagulation analyzer [21].

2.5.3.2.1 Citrated Plasma Preparation

Venous blood (5.0 ml) was collected and combined with buffered sodium citrate in a citrate tube (ratio of 9 parts bloods to 1 part sodium citrate). The mixture was centrifuged at 3000 rpm at room temperature for 10 minutes. The resulting citrated plasma supernatant was stored at 15-25°C for use within four hours.

2.5.3.2.2 Control Plasma Preparation

Reconstitute a Control plasma vial with 1.0 ml distilled water, gently agitate, and incubate for 15 minutes at 15-25°C. The reconstituted plasma remains stable for four weeks at -20°C.

2.5.3.2.3 Thrombin time assay

A 100 µl aliquot of citrated plasma was incubated with 100 µl of crude tissue extract and 50 µl of reconstituted aPTT reagent at 37°C for 4 minutes in a coagulation tube provided with the coagulometer, aPTT time was measured by Rayto RT-2201C coagulation analyzer after adding 50 µl of 0.1 M $CaCl_2$ pre-warmed to 37°C. Different dilutions of the tissue extract was combined with citrated plasma (final volume: 100 µl) and were evaluated. A control plasma test was conducted before each experiment to evaluate reagent and coagulation analyzer precision and accuracy.

3. RESULTS

3.1 Sample Collection and Specie Identification of Earthworm

Earthworms were collected from various areas in Bhimber District, Azad Kashmir, Pakistan. The most prevalent species, *Eisenia fetida*, was selected for the study, supported by [22].

It exhibited distinctive features, including a reddish-brown tubular body with approximately 115 segments, a saddle-shaped clitellum, a dorsal pore between segments 4/5 and 5/6, and seminal vesicles in pairs in segments 11 and 12.

Table1 Morphological Characteristics of *Eisenia fetida*

Habitat	Body shape/ color	Number of segments	Length	Anatomy
Manure worm thrive in rotting vegetation, manure	Tubular Reddish brown	Approximately 115	120mm length, 5mm diameter	Clitellum start from segments 25

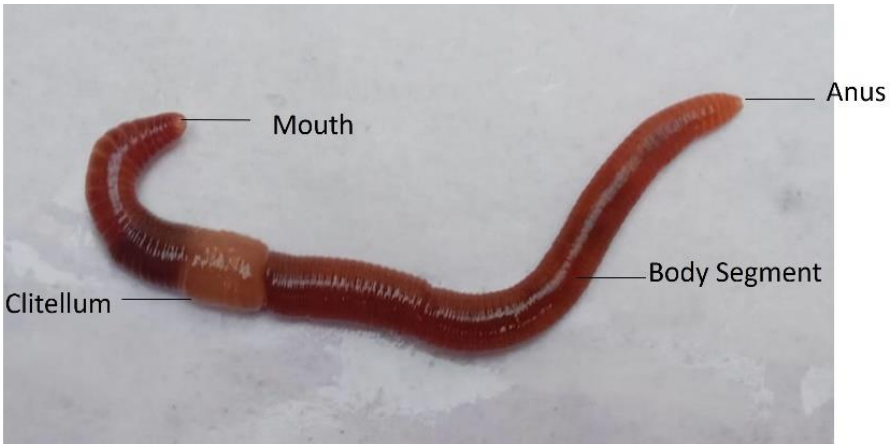

Fig.1: Morphological representation of sampled earthworm (*Eisenia fetida)*

3.2. Sample collection and Specie Identification of Leech

Hirudo medicinalis was selected for the study. These leeches possess muscular, bilaterally symmetrical bodies, segmented and dorsoventrally flattened into thirty-three parts. They display distinctive features, including speckles on the underside, six reddish-brown stripes on a dark background on top, and disk-shaped posterior and anterior suckers.

Table 2 Morphological **c**haracteristics of *Hirudo medicinalis*

Habitat	Body shape/color	Number of segments	Length	Anatomy
Pond with a muddy bottom	Cylindrical dorso-vertically flattened dark brown	34	Up to 10 cm	Anterior and posterior disc shape sucker

Fig.2: Morphological representation of leech (*Hirudo medicinalis)*

3.3. Dissection and Viscera Collection

Earthworm's digestive tract was recover by dissecting earth worm for extraction of fibrinolytic enzymes, focusing on the anterior portions like the crop, gizzard, and initial part of the intestine. Hirudo species leeches, yielded bioactive proteins in their saliva, notably hirudin from salivary gland secretions. Leech salivary gland samples were collected for hirudin extraction.

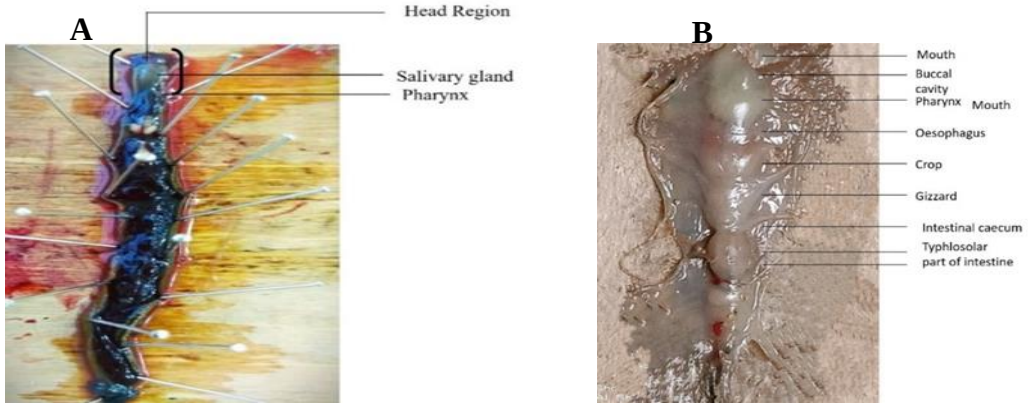

Fig.3: Tissue collection after dissection (A) visceral organ of leech (B) visceral organs of earth worm

3.4 Casein Zymography

The presence and approximate size of fibrinolytic enzymes in tissue extracts of *E. fetida* digestive tract and *H. medicinalis* salivary gland were studied by Zymography. Casein hydrolysis were observed and zymography was applied to *E. fetida* digestive tract and *H. medicinalis* salivary gland tissue extracts. Distinct proteolytic activity was observed with clear bands against a dark blue background. The approximate molecular weight of proteolytic enzymes was found to be 30-40 KDa (Fig. 4B) and 10-20 KDa (Fig. 4A) in case of *E. fetida, H. medicinalis* respectively.

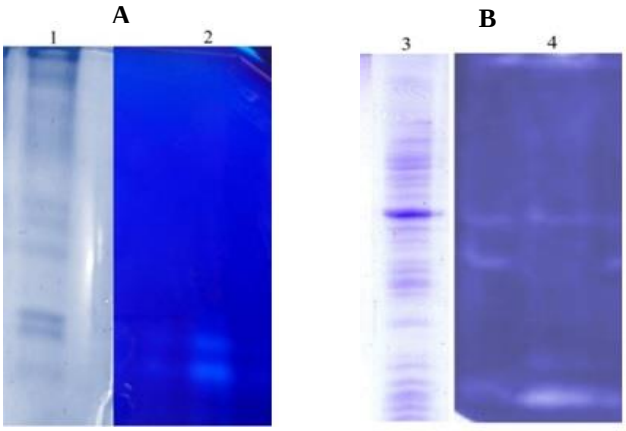

Fig. 4: Zymogram analysis of protein extraction from earthworm and leach viscera. **A:** Protein extracted from mouth organ of leach **B:** protein extracted from digestive tract of earth worm. Lane 1 and 3, native-PAGE of protein samples; lane 2 and 4 are the corresponding hydrolysis on casein agar plate.

3.5 Total Protein Assay

The total protein concentration in the crude tissue extract was determined by comparing the absorbance of unknown with of BSA standard solutions and concentration was determined. The total protein concentration of the crude tissue extract collected from the earthworm and leach was found to be 1.10 ±2.75 mg/ml and 1.5±3.908 mg/ml respectively. All measurements were conducted in triplicate, and the results were expressed as the mean ± standard deviation.

3.6 Activity assay

3.6.1 Fibrinolytic activity of lumbrokinase

The fibrinolytic assay was performed in order to measure the break down fibrin, a key blood clotting protein. In our study, a modified fibrin plate method was used to measure enzyme activity in tissue extracts. Protein samples showed fibrinolytic activity by forming a clear zone of degradation after incubated overnight at 37°C. However, lumbrokinas standard curve

was generated in order for precise quantitative measurement, offering insights into effectiveness of fibrinolytic enzymes. Fibrinolytic activity was calculated with the help of lumbrokinase standard curve regression equation (y = 5.2897x + 7.758 R^2 = 0.986) Fig. 5. Crude earthworm tissue extracts in our study was found to exhibit activity of 816 U/mg on fibrin plate, as determined from control standard curve.

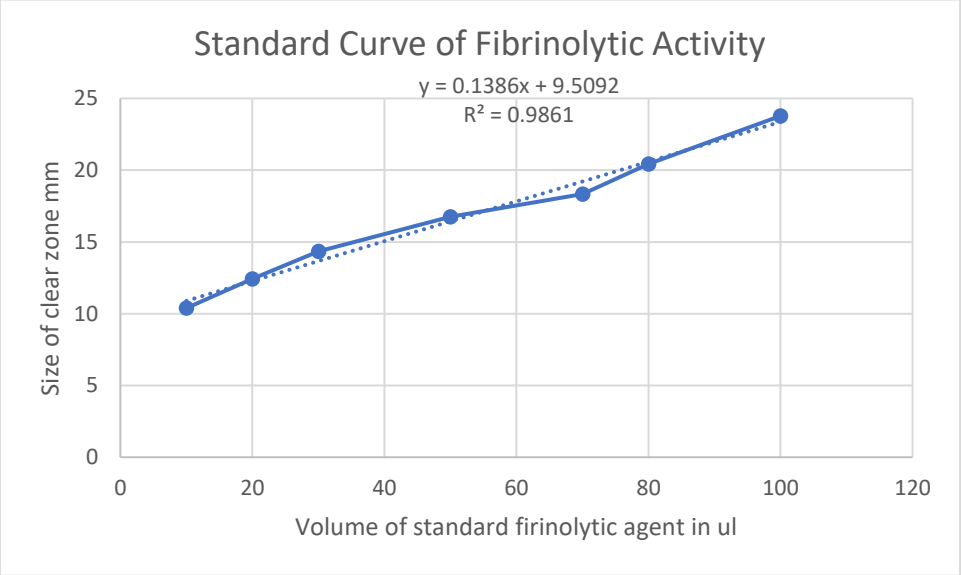

Fig. 5 Standard curve for fibrinolytic activity

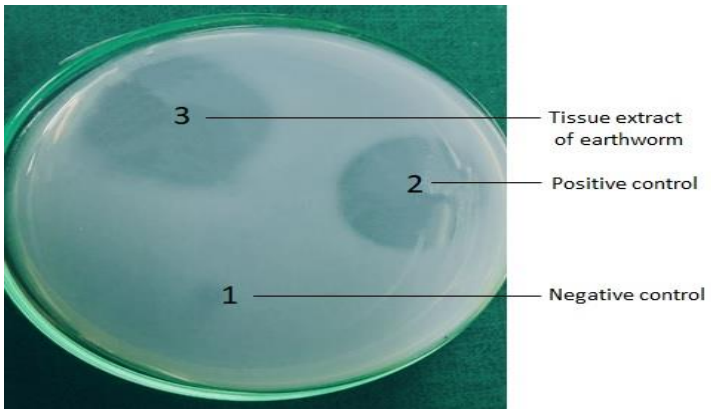

Fig. 6 Fibrin plate assay: 1. negative control 2. Positive control 3. Crude tissue extract (earthworm).

3.6.2 Percentage clot lysis activity

(Dose-Time Dependent Blood Clot Lysis of lumbrokinase)

The investigation demonstrated a direct correlation between the percentage of blood clot lysis activity, dosage, and incubation time (Fig 8). The minimum 50 (µl) and maximum 200 (µl) concentration of crude earthworm tissue extract resulted in the clot lysis of 50%, 70% respectively, after 180 minutes of incubation.

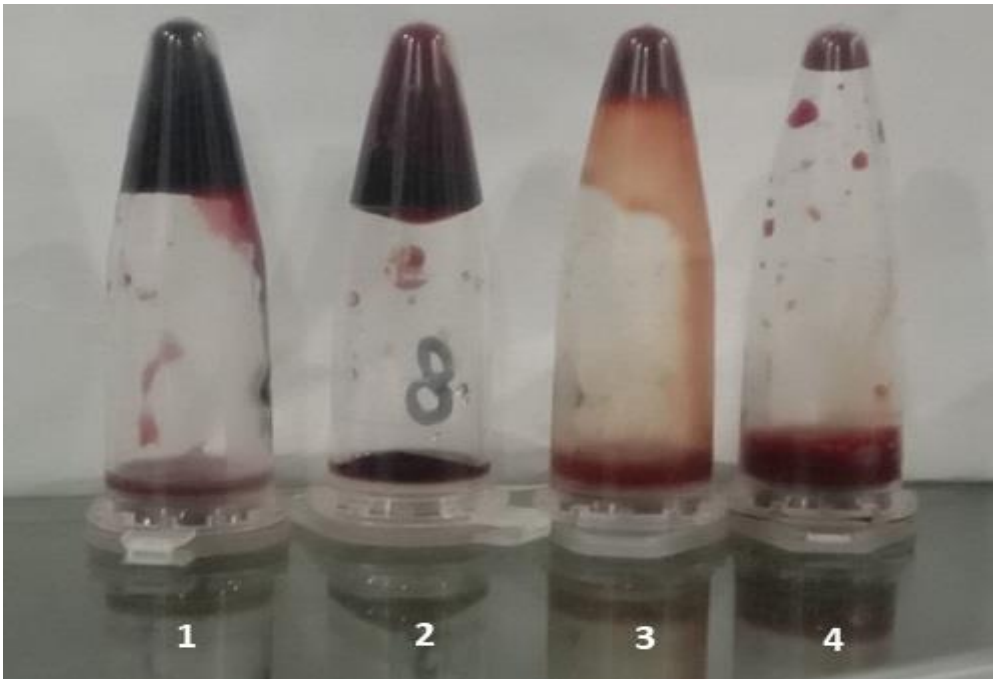

Fig. 7 Blood clot lysis activity after addition of crude enzyme extract 1 (50 µl), 2 (100 µl) 3 (150 µl), 4 (200 µl) incubated at 37°C for 180 minutes.

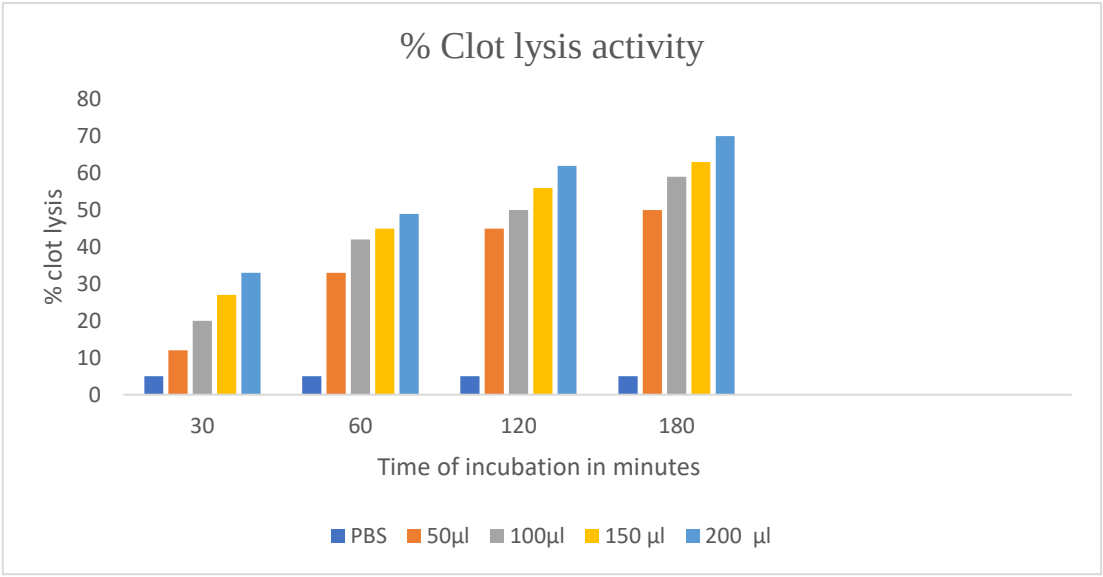

Fig 8. Graphical representation of clot lysis activity of lumbrokinase in time and dose dependent manner

3.6.3 Hirudin activity assay

3.6.3.1 Thrombin titration method

Hirudin's biological activity was assessed through thrombin titration, an analytical method for quantifying a substance in a sample. In vitro testing demonstrated that hirudin is more effective anticoagulant than commonly used reagents, notably delaying blood clot formation. The increased volume of thrombin employed for clot formation in the hirudin-based thrombin titration method confirms its enhanced potency as an antithrombin reagent.

Table 3 Thrombin volume for clot formation in thrombin titration method

Negative control	Heparin	Hirudin
5 µl	160.65 µl ± SD 1.24 (n=3)	173.45 µl ± SD 1.69 (n=3)

3.6.3.2Antithrombin activity assay

The crude salivary tissue extract was the subject of the anti-thrombin activity assay. Results revealed that the antithrombin activity (aPTT prolongation) of the crude saliva extract was a linear function with the volume of the crude mixed with the citrated plasma.

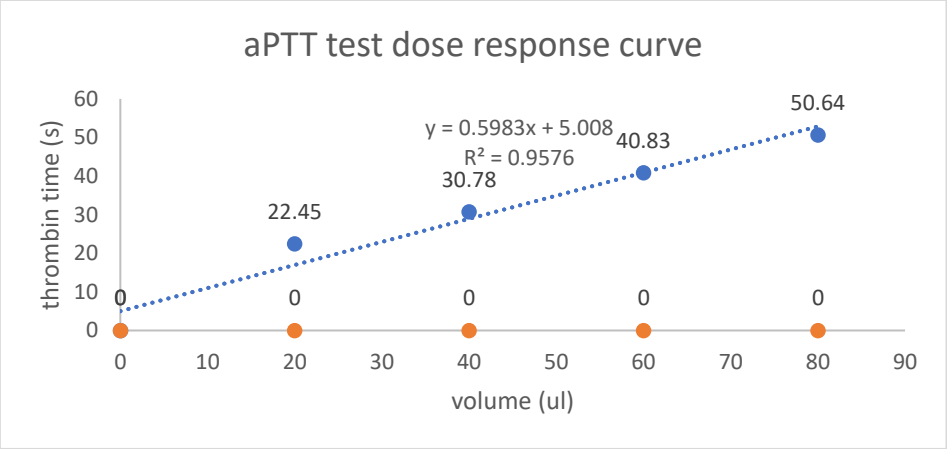

Fig.9 Thrombin time and dose responsive curve of hirudin

As a result, it was observed that the addition of 80 µl of the crude saliva extract increased a PTT appreciably.

4. DISCUSSION

Many studies reported the earthworm's fibrinolytic activity using the standardized fibrin plate method. Various species of earthworm including *Etyphoeus gammiei*, *Eisenia fetida*, *Lampitomauritii*, and *Perionyx excavates* were found to exhibit robust fibrinolytic

capabilities. *Eisenia fetida*'s glycolipoprotein complex G-90, containing P I (34 kDa) and P II (23 kDa) serine peptidases, demonstrated significant anticoagulant and fibrinolytic attributes. These isozymes, applied in capsule-based medication, provide stable and effective treatment for clotting disorders, minimizing hemorrhagic side effects [23-26].

In the current investigation, by employing the modified fibrin plate method, we unveiled the noteworthy specific fibrinolytic activity of 816 U/mg in *Eisenia fetida* present in local habitat. The fibrinolytic enzymes demonstrated dose and time-dependent blood clot lysis activity in an *in vitro* model. Incubating the crude earthworm tissue extract at ranging from 50 µl to 200 µl resulted in clot lysis percentages of approximate 50 % and 70 % after 180 minutes.

The medicinal leech, *Hirudo medicinalis*, is highly esteemed for its potent anticoagulant, hirudin. Extracted from local leeches, hirudin effectively inhibits thrombus formation by directly interacting with thrombin [27]. Its natural thrombin inhibitory properties and monospecific, co-factor-independent mechanism contribute to its popularity as an anticoagulant. Hirudin's high specificity for thrombin, coupled with the accessibility of recombinant and synthesized variants, makes it a favorable choice. Notably, hirudin's therapeutic doses are associated with minimal side effects on platelets and a lack of bleeding complications [28]. Hirudin is advised as an anticoagulant for in vitro investigations assessing the hemocompatibility of biomaterials or surface modifications. Regarding their anticoagulant efficacy, both heparin and hirudin have demonstrated greater potency than low-molecular-weight heparin (LMWH) [29]. At equivalent doses, both heparin and hirudin have proven effective in inhibiting platelet and fibrin deposition. It's worth noting that, in comparison to heparin and LMWH, hirudin displays minimal anti-Xa activity [30]. Hirudin, a selective thrombin inhibitor, is the preferred anticoagulant for in vitro studies on platelet aggregation in whole blood, highlighting the importance of extracellular calcium for platelet function [31].

In the current investigation, it was noted that the locally found species of medicinal leech exhibits a pronounced efficacy in preventing blood clot formation compared to conventional anticoagulants like heparin. Additionally, a linear relationship was observed between the dose of medicinal leech extract and the prolongation of activated partial thromboplastin time (aPTT), indicating a systematic and efficient delay in blood clot formation with increasing doses. The activated partial thromboplastin time (aPTT), increased by more than two-fold with the elevation of the tissue extract dosage. Hirudin activity, determined through the thrombin titration method, showed a specific activity of 13,000 ATU/mg.

This study confirms the medicinal importance of earthworms and leeches as thrombolytic and anticoagulant agents in traditional medicine, highlighting their accessibility, affordability, and minimal side effects.

5. CONCLUSION AND RECOMMENDATION

This recent investigation confirms the significant role of annelid earthworms and leeches as thrombolytic and anticoagulant agents, highlighting their medicinal importance as traditional sources of remedies. These organisms serve as accessible and cost-effective medicinal resources, offering the added advantage of minimal or negligible side effects. The widespread availability and affordability of these natural sources underscore their potential as valuable contributors to traditional medicine, emphasizing their relevance in healthcare practices with a favorable risk profile.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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