Special Edition on "Frontiers of Biosciences in Sustainable Development (FBSD)"
CIBTech Journal of Zoology ISSN: 2319–3883; http://www.cibtech.org.htm
2023 Vol.12/S1, pp.239-247/Mubeen et al.
***Research Article** (Open Access)*

EXTRACTION OF CRUDE PROTEIN FROM EARTHWORM (*LAMPITO MAURITII*) AND ASSESSING ITS ANTI THROMBOLYTIC ACTIVITY

Mubeen sultana. D*[1], Subhashree V[2] and Rajeshwari T[1]
[1]*Research Department of Zoology, Justice Basheer Ahmed Sayeed College for Women*
[2]*P.G and Research Department of Zoology, University of Madras, Guindy Campus.*
[*]*Author for Correspondence: mubeensadiq@gmail.com*

ABSTRACT
Earthworms are beneficial residents of the soil. Earthworms break down dead and decaying organic matter into rich humus soil, thereby supporting plant growth. In the present study, the crude proteins are extracted from the earthworm and the antithrombotic activity of the same is assessed. The Earthworms were collected from the garden of J.B.A.S College in a perforated container along with some amount of soil. The protein was extracted from the earthworm by dissolving the lyophilized earthworm powder in HEPES Buffer and precipitating the protein using ammonium sulfate. The presence of protein was confirmed by total protein test and the presence of thrombolytic enzyme was confirmed using SDS-PAGE. Thrombolysis is the process of breakdown of blood clots formed in the blood vessels. The antithrombotic activity was assessed using clotted blood present in a microcentrifuge tube. Clot lysis was done by addition of different concentrations of the protein to the clotted blood. It is found that as the concentration of the active compounds in the extract increases, the percentage of clot lysis also increases. This assay confirms the antithrombotic activity of the protein extract. In future research, these crude proteins can be purified for further extensive studies.

Keywords: *Crude protein, Lampito maurutii, Protein precipitation, Antithrombotic activity, SDS-page and Clot analysis*

INTRODUCTION
Ischemic stroke occurs when a blood clot blocks or narrows an artery leading to the brain. A blood clot often forms in damaged arteries by the build-up of plaques (atherosclerosis). It can also occur in the carotid artery of the neck as well as other arteries. These kind of blood clots which pose a life-threatening risk are treated with Thrombolytics. It is also used to treat numerous pulmonary emboli and clots that form in shunts during renal dialysis. Interesting finding of thrombolytic activity by fibrinolytic protease termed Atroxase derived from snake venom reported up to 60% drop of rat's plasma fibrinogen level, indicating that purified and lyophilized form of the enzyme may be given to people in case of treating cardiovascular illness. Heparins, Vitamin K antagonists, and derivatives have been the main anticoagulant medications used in clinical settings for more than 50 years. Studies to develop new and enhanced recombinants variations regimen are in progress due to inadequacies of readily available thrombolytic medications. For triggering lysis and preventing reocculation, Aspirin and Heparin are notably effective. The most effective secondary prevention of ischemic cardiovascular disorders is still provided by selective antiplatelet agents and thrombin inhibitors, and medications like Aspirin is most well-established antithrombotic agents. However, safety is a major concern. Due to the medication's significant downsides of upper gastrointestinal haemorrhage and haemorrhagic events (Johnson, 2008). Several studies have been conducted over the past few decades in an effort to find new substances or sources to prevent platelet aggregations. The results of research in this field are positive for the development of the best thrombolytic therapy organically. The setting, idea and techniques for using natural materials to cure people have changed substantially over time (Sumi, *et al.,* 1990; Hesler, 1992). Natural medicines or traditional medicines made a revolutionary comeback with increased strength and play a vital role in human health (Demrow., *et al.,* 1995; Briggs, *et al.,* 2001; Rajapakse, *et al.,* 2005; Yamamoto, *et al.,* 2005). Natural medications are thought to be safer because they work naturally. Earthworms are good

candidates to produce antithrombotic substances because they have been used as a medicine for ages. It's protein and coelomic fluid were shown to have cytolytic agglutinating, proteolytic, haemolytic, mitogenic, antipyretic, tumorstatic, and antibacterial activity (M. Balamurugan *et al.*, 2007). The healing properties of earthworms, a type of macroinvertebrate found in soil, are well documented in ancient literature. In Fact, earthworm powder taken orally has shown to be capable of breaking down intravascular fibrin clots. According to Bhatnagar and Palta (2016), earthworms enhance body heat when consumed and are beneficial for treating rheumatoid arthritis, bronchitis, tuberculosis, and neurological illnesses. With that in mind we further investigated the antithrombotic properties of earthworm *lampito mauritii*.

MATERIALS AND METHODS

MATERIALS

BLOOD SAMPLE:

Venous Blood sample was drawn in preparation for the procedure. Five healthy volunteers who had never used oral contraceptives or any other anticoagulant therapy were selected and blood drawn by a phlebotomist. Each of the five previously weighed micro centrifuge tubes received 500 µl of blood to create clots. This was used to analyse clot formation and lysis in the future.

EARTHWORM (LAMPITO MAURITII):

The earthworms were collected from the garden of JBAS College, along with some amount of soil in a sterile container and transferred to lab.

Figure 1: Showing the earthworm (*lampito mauritii*) with measurement.

METHODS:

IDENTIFICATION OF EARTHWORM:

The collected earthworms were identified by HetroGeneBiotech Laboratory, Pvt., Ltd., based on its morphological characters.

EXTRACTION OF PROTEIN:

The identified earthworms were washed with double distilled water twice. The washed earthworms were transferred to a pre-cooled mortar and pestle. Liquid Nitrogen was poured into the mortar and pestle containing the earthworms. The earthworms were ground into fine powder using mortar and pestle. The powdered material was then suspended in hepes buffer (pH 7.4). The suspension was then filtered using celite to remove other cell debris. The filtrate was then stored in -20°C until analysis.

ESTIMATION OF PROTEIN BY BIURET METHOD:

BIURET REAGENT:

3g of copper sulphate (CuSO4.5H2O) and 9g of sodium potassium tartarate was dissolved in 500ml of 0.2mol/litre sodium hydroxide. 5g of potassium iodide was added and made up to 1 litre with 0.2mol/litre sodium hydroxide. The protein standard taken was as 5mg BSA/ml.

PROCEDURE:

0.0, 0.2, 0.4, 0.6, 0.8 and 1ml of working standard was pipetted out in to the series of labelled test tubes. 1ml of the sample (filtrate) was pipetted out in another test tube. Volume of all test tubes was made to 1ml in all the test tubes. A tube with 1ml of distilled water served as the blank. 3ml of biuret reagent was added to all the test tubes including the blank test tube and sample test tube. The contents of the tubes were mixed well by vortexing / shaking the tubes and warmed them at 37°C for 10 min. Then the contents were allowed to cool at room temperature and the absorbance was read at 540 nm against blank. The standard curve was plotted by taking concentration of protein along x-axis and absorbance at 540nm along Y-axis. From this standard curve calculate the concentration of protein was calculated in the given sample.

DETERMINATION OF CLOT LYSIS:

Clot lysis approaches were carried out. 2.5 ml of venous blood was drawn from a healthy volunteer and disturbed in 5 pre-weighed sterile microcentrifuge tubes (0.5ml/tube) and incubated at 37°C For 45 minutes. After clot formation, serum was completely removed without disturbing the clot. The clot was weighed and then transferred to a well plate. 100µl of earthworm extract in the concentrations 10%, 25%, 50% and 100% was added to each well separately. To the control well, heparin was added to the clot. The well plate was then incubated at 37°C For 90 minutes and observed for clot lysis. Released fluid was removed and wells were again weighed to observe the difference in weight taken before and after clot lysis was expressed as percentage of clot lysis.

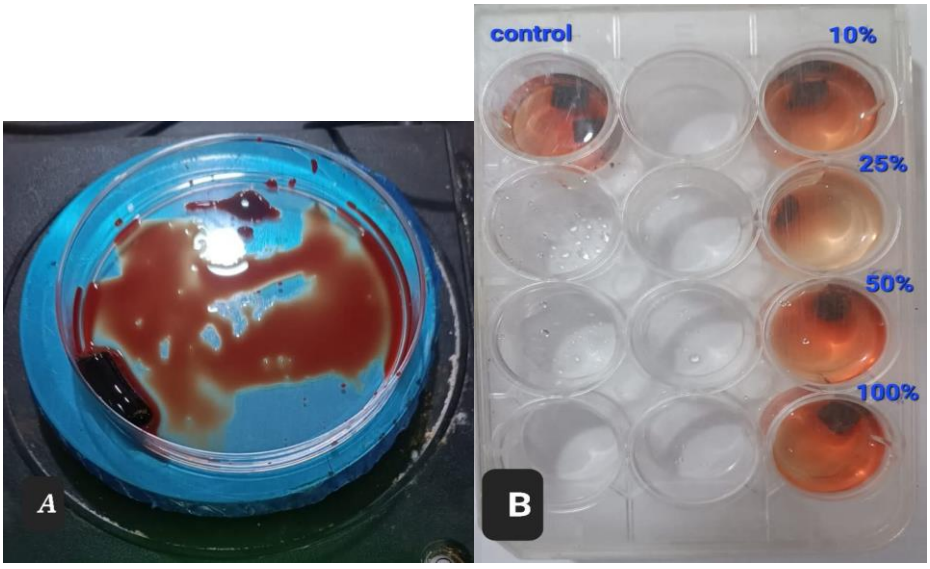

Figure 2: (A) showing the blood clot obtained from collected blood of volunteers (B)showing determination of clot lysis with different concentrations of extract along with control.

RESULTS AND DISCUSSION

Protein content:

In this study, the protein concentration was estimated using Biuret reagent. Standard Bovine serum albumin was taken of different volumes 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.0 ml of sample. The concentration of protein was observed as 0,1, 2, 3, 4, 5 and the concentration of the protein was unknown. The absorbance of the protein

was 0.00, 0.256, 0.384, 0.459, 0.512, 0.683 at 540 nm respectively (Table 1). The standard graph was plotted using the OD values (Figure 3). The absorbance of the the protein of the unknown sample was recorded as 0.602. In accordance with this value the concentration of the protein in unknown sample was estimated as 4.407mg protein/ml (Figure 4) from the standard graph.

Table 1: Standard graph for protein estimation by Biuret method.

Volume of standard BSA (ml)	Volume of distilled water (ml)	Concentration of protein (mg)	Volume of Biuret reagent (ml)	Absorbace at 540nm
0.0	1.0	0	3	0.00
0.2	0.8	1	3	0.256
0.4	0.6	2	3	0.384
0.6	0.4	3	3	0.459
0.8	0.2	4	3	0.512
1.0	0.0	5	3	0.683
1.0 sample	0.0	To be estimated	3	0.602

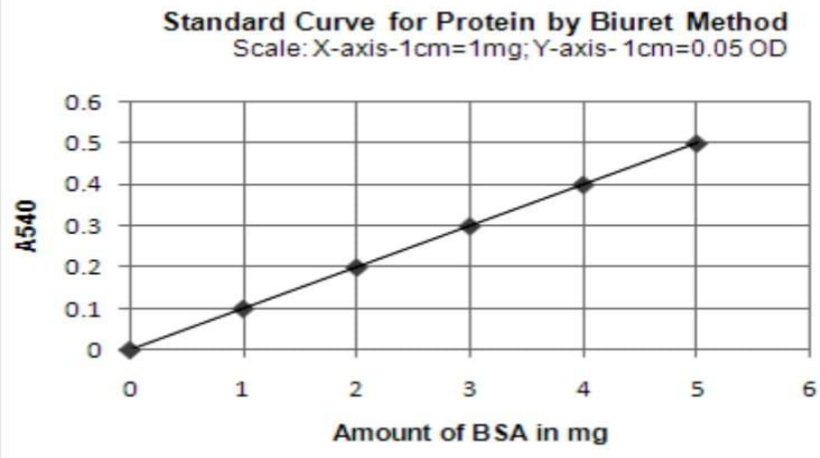

Figure 3: Standard curve for protein estimation by Biuret method

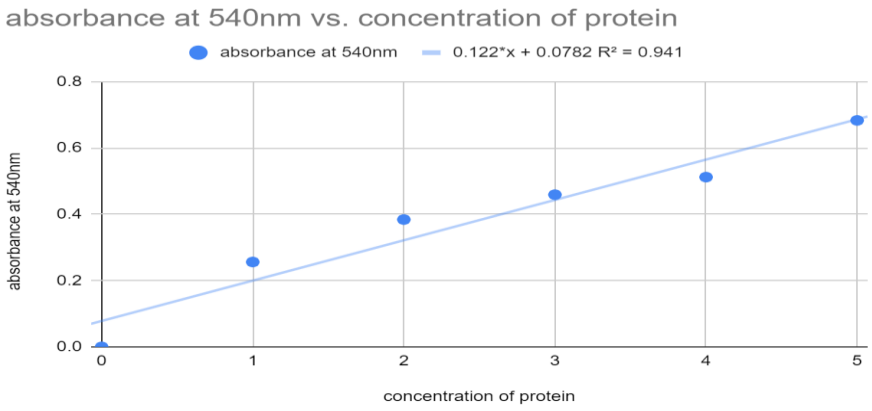

Figure 4: Absorbance at 540nm vs concentration of protein

Assessing Anti Fibrinolytic Activity of Earthworm (*Lampito mauritii*):

Heparin was used to prevent or treat certain blood vessels, heart and lung conditions. Heparin was also used to prevent blood clotting during open heart surgery, by-pass surgery, kidney dialysis, and blood transfusion. The effect of clot lysis using 100µl of earthworm protein extract was quite significant. The concentrations of the protein enhanced clot lysis in a dose dependent manner along with incubation time factor. The weight of the clot was noted as 0.28, 0.26, 0.25, 0.26, 0.27g before clot lysis. The extract was taken in 10%, 25%, 50%, 100% and heparin was taken. The weight of the clots was weighed as 0.27, 0.24, 0.22, 0.20, 0.16 after clot lysis (Table 2).

Table 2: Analysis of clot lysis using various extract concentrations

Concentration of the extract	Weight of the clot before (g)	Weight of the clot after (g)	Percentage of clot lysis (%)
10%	0.28	0.27	3.57
25%	0.26	0.24	7.69
50%	0.25	0.22	12.00
100%	0.26	0.20	23.08
Control (Heparin)	0.27	0.16	40.74

The percentage of clot lysis was calculated by using the formula: Percentage of the clot lysis = Weight of clot x 100. The percentage of clot lysis in standard versus the percentage of clot lysis in 5 different concentrations was given as a bar graph (Figure 6 and table 3)

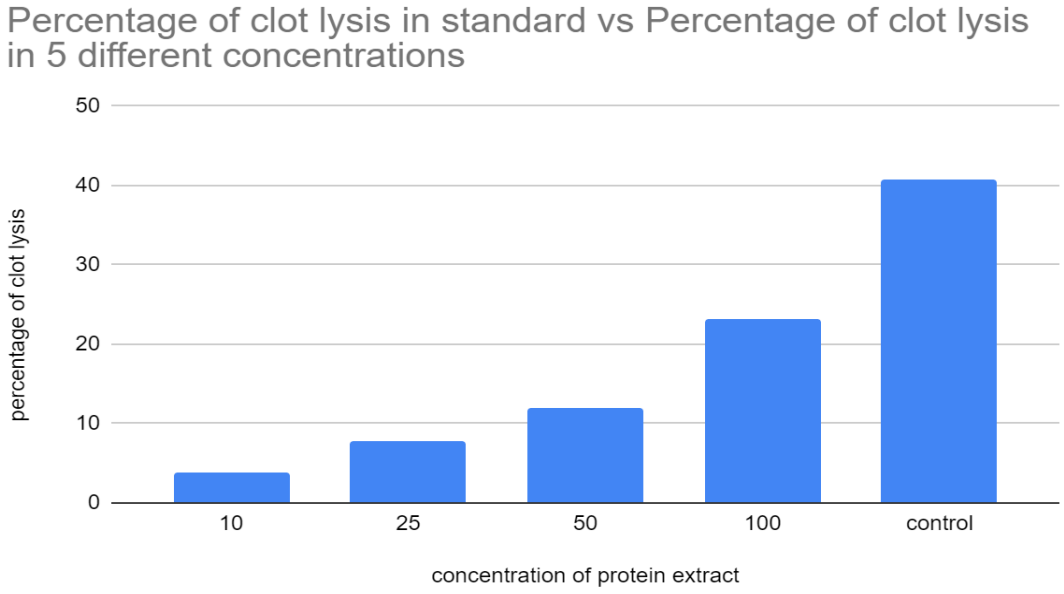

Figure 6: Graph showing percentage of clot lysis vs concentration of protein extract in both control and extract.

Table 3: Percentage of clot lysis vs concentration of protein extract in both control and extract.

concentration of protein extract	percentage of clot lysis
10	3.75
25	7.69
50	12
100	23.08
control	40.74

Discussion

In the current study, a clot lysis test was used to assess the thrombolytic activity of the earthworm (*Lampito mauritii*) as an antithrombotic agent against the majority of cardiovascular diseases. Humans have always relied on nature to treat a variety of illnesses. Modern phytopharmacological research has revived interest in traditional medicine and opened up a new area for the discovery of animal derivative drugs that are effective in treating specific diseases. Many studies have been done to identify the plants, natural foods, and their supplements that have antithrombotic activity. About 30% of pharmaceuticals are thought to be made from animal derivatives. Platelets play a significant role in the onset of atherothrombosis and cause endothelial surface damage (produced by reactive oxygen species). A class of drugs called thrombolytics or fibrinolytics is used to manage and treat intravascular clots that are dissolving. These medications belong to the plasminogen activator class. Acute peripheral arterial occlusion, deep vein thrombosis, pulmonary embolism, acute ischemic stroke, intracardiac thrombus formation, and acute myocardial infarction are all treated with this class of medication. The use, mechanism, and limitations of thrombolytics are discussed in this activity. Acute myocardial infarction, acute ischemic stroke, and other conditions that involve intravascular clots will be treated using this activity, which will also highlight the mechanism of action, adverse event profile, monitoring, and toxicity relevant for interprofessional team members. Drugs used to treat venous thrombosis block the coagulation cascade. This has historically included unfractionated heparin (UFH), which promotes antithrombin's inhibition of coagulation serine proteases (Damus *et al.,* 1973). Only an IV injection makes UFH bioavailable, which restricts its application to hospital care. One of the many coumarine derivatives, which are vitamin K antagonists, has typically been used to maintain long-term prophylaxis (e.g. warfarin, [trade name Coumadin], acenocoumarin [trade name Sintrom]. Vitamin K antagonists prevent the vitamin K-dependent coagulation serine proteases from undergoing post-translational processing, which in turn inhibits the coagulation cascade as a whole (Furie *et al.,* 1990). Small molecules that directly inhibit factor Xa or thrombin belong to a new class of anticoagulant medications. Compared to LMWH or fondaparinux, many of these are being developed as oral medications, which may be a significant advantage. The only direct factor Xa inhibitor that is currently approved is rivaroxaban, but it has not yet received approval in the US. Similar medications that are still undergoing clinical trials include apixaban, betrixaban, and edoxiban. These are initially being tested for relatively brief prophylaxis before/following orthopaedic surgery, but the hope is that they will eventually prove to be secure for long-term prophylaxis. In this study, the protein concentration was estimated using Biuret reagent. The unknown sample of earthworm contains 4.407 mg protein / ml. Bakhtawer (et al.,) 2021 conducted a study on Isolation and purification of thrombolytic enzyme extracted from earthworm which shows that 50µl of enzyme showed lysis, partial hydrolysis on 150µl enzyme and complete hydrolysis on 250µl of enzymes. Comparing to study conducted on the determination of the thrombolytic activity of the Natto extract done by Masada (2004). The main active substance was bacillopeptidase secreted by natto bacillus. The protein absorbance was seen to be 1.20. And the maximum level of clot lysis is shown as 20%. This shows that the earthworm exhibits more thrombolytic activity than natto bacillus. Comparing to study

conducted on the potential of fibrinolytic protease enzyme from tissue of sand sea cucumber (*Holothuria scabra*) as thrombolysis agent done by N Hidayati *et al.,* (2021) had made a review study on thrombolytic activity of different species of sea cucumber. The highest protein content is seen in *Holothuria scabra* that is 76.64% . Development of antithrombotic agents using fibrinolytic protease from the tissues of *Holothuria scabra* offers novelty for the treatment of cardiovascular diseases. Comparing with the study conducted on Fibrinolytic Serine Protease isolated from Lampito mauritii done by A.J. bhorgin Lourdumary *et al.,* (2014). The determination of protein was done by spectrophotometric method. The concentration of protein is seen be high in crude extract (1.8g). The fibrinolytic activity is seen to be 53.50%.

CONCLUSION

In conclusion from the recorded data, it is evident from the data that has been collected that the findings may have significant effects on cardiovascular health. This discovery may also point to the potential for producing novel thrombolytic compounds from the extracted crude protein of earthworm (Lampito mauritii). More research is being done to identify and characterize the substances that are responsible for thrombolytic activity. Additionally, research must be done to identify the secondary metabolites or bioactive elements and their functions. The use of these natural extracts and compounds will usher in a new era of drug docking with novel target molecules and the development of advanced therapy. In this study, it was shown that using synthetic versions of the same basic principles, folk medicine could be just as effective as modern medicine in lowering the risk of cardiogenic problems as well as other risk factors like deep vein thrombosis and side effects. Even though the study is only a preliminary analysis, it opens the door for future molecule development.

REFERENCES

Adams, David & Griffin, L & Nachajko, W & Reddy, V & Wei, C. **(1991)**. A synthetic DNA encoding a modified human urokinase resistant to inhibition by serum plasminogen activator inhibitor. *The Journal of biological chemistry*. **266**(13) 8476-82. 10.1016/S0021-9258(18)92999-6.

Alquwaizani M, Buckley L, Adams C, Fanikos J (June 2013). Anticoagulants: A Review of the Pharmacology, Dosing, and Complications. Current Emergency and Hospital Medicine Reports. 1 (2): 83–97. doi:10.1007/s40138-013-0014- 6. PMC 3654192. PMID 23687625

Alkjaersig, N., Fletcher, A. P., & Sherry, S. (1959). The mechanism of clot dissolution by plasmin. *The Journal of clinical investigation*, **38**(7), 1086– 1095. https://doi.org/10.1172/JCI103885

Arslan, Elif & Cotuk, Ayşın. **(2008).** Antibacterial and haemolytic activity of the coelomic fluid of *Dendrobaena veneta* (Oligochaeta, Lumbricidae) living in different localities. *IUFS J Biol*. 67. 23-32.

Astrup T. (1956). Fibrinolysis in the organism. Blood, 11(9), 781–806. λ Balamurugan, Mariappan & Parthasarathi, K & Cooper, Edwin & Ranganathan, L. (2006). Earthworm paste (*Lampito mauritii*, Kinberg) alters inflammatory, oxidative, haematological and serum biochemical indices of inflamed rat. *European review for medical and pharmacological sciences.* **11**(2)77-90. PMID :17552137

Baruah, D. B., Dash, R. N., Chaudhari, M. R., & Kadam, S. S. (2006). Plasminogen activators: a comparison. Vascular pharmacology, 44(1), 1– 9. https://doi.org/10.1016/j.vph.2005.09.003 31

Basta, Giuseppina & Lupi, Cristiana & Lazzerini, Guido & Chiarelli, Piero & L' Abbate, Antonio & Rovai, Daniele. (2004). Therapeutic effect of diagnostics ultrasound on enzymatic thrombolysis. A in vitro study on blood of normal subjects and patients with coronary artery disease. Thrombosis and haemostasis.91.(16):1078-83.https://doi.org/10.1160/Th03-11-0684 PMID: 15175792

Bhorgin & Murugaian, Perumal. (2014). Fibrinolytic Serine Protease Isolated from Earthworm *Lampito mauritii*. *International journal of Science and Research*. **3**(10). 961-966.

Cho, I. H., Choi, E. S., Lim, H. G., & Lee, H. H. (2004). Purification and characterization of six fibrinolytic serine-proteases from earthworm *Lumbricus rubellus*. *Journal of biochemistry and molecular biology*, 37(2),199–205. https://doi.org/10.5483/bmbrep.2004.37.2.199

Damus P., Hicks M. & Rosenburg, R(1973). Anticoagulant Action of Heparin. Nature 246, 355–357. https://doi.org/10.1038/246355a0

Debnath, M., Saha, S., & Kumar Sil, S. **(2018).** First report on fibrinolytic and thrombolytic activity of *Eutyphoeus gammei* an earthworm species collected from Tripura, Northeast India. *Asian Journal of Pharmaceutical and Clinical Research*, 11(11), 236 240.https://doi.org/10.22159/ajpcr.2018.v11i11.27739.
Edwards, C. A. & Lofty, J. R. (**1977)**, Biology of earthworms / C. A. Edwards, J.R. Lofty Chapman and Hall; Wiley London : New York.
Edwards, C.A., Arancon, N.Q. (2022). Earthworm Morphology. In: Biology and Ecology of Earthworms. Springer, New York, NY. https://doi.org/10.1007/978-0- 387-74943-3_1
Gerald, Bhorgin & Murugaian, Perumal. **(2014).** Fibrinolytic Serine Protease Isolated from Earthworm *Lampito mauritii*. *International Journal of Science and Research*. 3. 961-966. 32
Hidayati, N., Fuad, H., Munandar, H., Zilda, D. S., Sulistyaningtyas, A. R., Nurrahman, N. & Ethica, S. N. (2021). Potential of fibrinolytic protease enzyme from tissue of sand sea cucumber (*Holothuria scabra*) as thrombolysis agent. In IOP Conference Series: *Earth and Environmental Science* Vol. 743, No. 1, p. 012007). IOP Publishing.
Hrzenjak, T., Popović, M., Bozić, T., Grdisa, M., Kobrehel, D., & Tiska- Rudman, L. (1998). Fibrinolytic and anticoagulative activities from the earthworm Eisenia foetida. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology*, 119(4), 825–832. https://doi.org/10.1016/s0305-0491(98)00060-1
Joyia, F., Zia, M., & Taskeen Raana, Muhammad Sarwar Khan, G. **(2018).** Isolation, purification and functional characterization of fibrinolytic Protease from an Earthworm *Eisenia foetida*.Pure *And Applied Biology (PAB)*, **7**(2), 906- 909.
Malik, M. F., & Afsheen, S. (2021). Isolation and Purification of Thrombolytic Enzyme Extracted from Earthworm Punjab, Pakistan. *Abasyn Journal of Life Sciences*, 4(1), 127-135.
MasadaM.(2004). Determination of the thrombolytic activity of Natto extract. food style, 8(1), 92-95.
Mathur, Abhishek & Verma, Satish & Singh, Santosh & Prakash, Archana & Prasad, Gbks & Dua, Virendra (2011). Anti- inflammatory activity of earthworm extracts. IJPSR. **2**(2).278.
Mihara, H., Sumi, H., Yoneta, T., Mizumoto, H., Ikeda, R., Seiki, M., & Maruyama, M. (1991). A novel fibrinolytic enzyme extracted from the earthworm, Lumbricus rubellus. *The Japanese journal of physiology*, 41(3), 461–472. https://doi.org/10.2170/jjphysiol.41.461 33
Monroy, F., Aira, M., Gago, J. A., & Domínguez, J. (2007). Life cycle of the earthworm *Octodrilus complanatus* (Oligochaeta, Lumbricidae). Comptes rendus biologies, **330**(5), 389–391. https://doi.org/10.1016/j.crvi.2007.03.016
Nakajima, N., Ishihara, K., Sugimoto, M., Sumi, H., Mikuni, K., & Hamada (1996). Chemical modification of earthworm fibrinolytic enzyme with human serum albumin fragment and characterization of the protease as a therapeutic enzyme. *Bioscience, biotechnology, and biochemistry*, 60(2), 293–300. https://doi.org/10.1271/bbb.60.293
Nakajima, N., Mihara, H., & Sumi, H. (1993). Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus*. *Biosciencebiotechnology, and biochemistry*, **57**(10), 1726–1730. https://doi.org/10.1271/bbb.57.1726
Nasrullah, Bakhtawer & malik, Muhammad & Afsheen, Sumera. (2021). Isolation and Purification of Thrombolytic Enzyme Extracted from Earthworm, Punjab, Pakistan. *Abasyn Journal Life Sciences*. 127-135. 10.34091/AJLS.4.1.15
Reynolds, J.W. 2017. *Lampito mauritii* (Oligochaeta: Megascolecidae) first earthworm record for Sharjah, U.A.E. **22**(5): 131-134. [paper in English; abstracts in English, French, Spanish, German]. *Megadrilogica*. 22. 131-134.
Sivasankari, Balayogan (2016). Study on life cycle of earthworm *Eisenia foetida*. *International Research Journal of Natural and Applied Sciences*, 3(5), 83-93.
Smith B. J. (1984). SDS Polyacrylamide Gel Electrophoresis of Proteins. *Methods in molecular biology* (Clifton, N.J.), 1, 41–55. https://doi.org/10.1385/0- 89603-062-8:41

Wang, Feng & Wang, Chao & Li, Mei & Gui, Lulu & Zhang, Jiping & Chang, Wenrui (2003). Purification, characterization and crystallization of a group of earthworm fibrinolytic enzymes from *Eisenia fetida*. *Biotechnology letters*. 25.1105-9. 10.1023/A:1024196232252.34
Zhou, Yuan & He, Rong-Qiao (2013). Earthworm Fibrinolytic Enzymes. Handbook of Proteolytic Enzymes. 30. 2953-2958. 10.1016/B978-0-12- 382219- 2.00647