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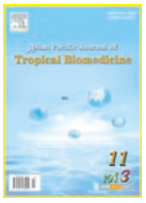

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Evaluation of cytotoxic and anti-tumor activity of partially purified serine protease isolate from the Indian earthworm *Pheretima posthuma*

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PEER REVIEW

Peer reviewer

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Comments

The present study on isolation, partial purification and evaluation of cytotoxic and antitumor activity of a serine protease from the chosen Indian Earthworm *P. posthuma* is important in medical research.
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ABSTRACT

Objective: To isolate, partially purify and evaluate the cytotoxic and antitumor activity of a serine protease from the chosen Indian earthworm *Pheretima posthuma*.
Methods: Whole animal extract was prepared and purified its protein constituents by size and charge based chromatographic separation techniques using Sephadex G-50 and DEAE-Cellulose resin respectively. Average molecular weight of the protein isolate was determined and analyzed for its cytotoxic property against Vero cells in different dilutions (1: 20 and 1: 40) and anti-tumor activity by MTT assay (a colorimetric assay) using breast cancer cell line MCF-7, with tamoxifen as standard.
Results: One of the protein constituents after purification was characterized as serine protease by Caseinolytic plate diffusion assay. Average molecular weight of this purified isolate was determined, by SDS-PAGE analysis with standard protein ladder, as of 15 kDa. The performed tests suggested that the 15kDa fraction has potent cytotoxic activity and satisfactory antitumor activity as well *in vitro*.
Conclusions: Exact molecular mechanism of the cytotoxic and antitumor activities is yet to be explored and currently we are working on ultra-purification and biophysical characterization of this fraction. Further investigation into the mechanism(s) of cytotoxic and antitumor activities at molecular level would be useful in treatment of various classes of cancer and viral infections in future.

KEYWORDS

Earthworm fibrinolytic enzyme, Cytotoxicity, Antitumor activity, Serine protease, Minimum inhibitory concentration

1. Introduction

Earthworm, a natural scavenger, happily crawls/burrows into soil and furnishes enormous benefits to agricultural land[1]. Despite this, earthworm is also known for its therapeutic potential since ancient times and prescribed as complete medicine in China, Korea and Japan for treatment of many diseases[2]. Crude extract of earthworm is recommended for the management of liver, blood and gynecological problems especially in eastern Asian countries[3]. The salt water in which the earthworms are soaked is often given to manage post-partum complications in women[4]. Additionally, earthworm paste, since ancient times, is used clinically for wound heal and as antimicrobial in surgeries[5]. In Chinese Materia Medica earthworm is considered as complete medicine and used for various therapeutic purposes such as blood purification, blood disorders, against jaundice, wound healing, and as antimicrobial, anti-inflammatory and antioxidant agent[6]. With the advancement of technology, especially in molecular biology and proteomics in last few decades, numerous

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compounds have been isolated and characterized for their therapeutic applications from various species of earthworm[7]. Among these therapeutic potentials, fibrinolytic property of earthworm proteases is explored significantly[8]. The credit goes to Mihara and coworkers in 1983, for isolation of fibrinolytic component from *Lumbricus rubellus* and characterization as serine protease[9]. Further, fibrinolytic component isolated was found as isoform of 6 proteases and called as lumbrokinase together. This was a breakthrough in earthworm based medicine and concurrently various species of earthworms are explored for isolation of earthworm fibrinolytic enzyme (EFE) by different researchers. Further, with molecular mechanisms and physiochemical properties evaluated, it has been found that EFE has tremendous potential to dissolve thrombus in circulatory pipelines with minimal complications[10]. Recent studies have confirmed clinical significance of EFE in cerebral ischemia and more emphasis is given to refine earthworm serine protease as external fibrinolytic agent, efficiently working in neuronal tissues[9].

In addition to fibrinolytic property, studies of late suggest potent cytotoxic and anticancer activity of earthworm serine protease(s)[11]. Cancer/tumor is considered as exacerbating condition for current healthcare system and medicine subsequently. Diversity, drug resistance and metastasis of tumors make the problem even more complicated. Earthworm serine protease was evaluated for anticancer activity in 2007 by Chen Hong that isolates from *Eisenia foetida* (*E. foetida*) were analyzed for the said activity, both *in vivo* and *in vitro*[11]. In this study, human hepatoma cell lines: HLE, Huh7, PLC/PRF/5 and HepG2 were treated with purified serine protease from *E. foetida* and promising anti-tumor activity was confirmed by Western blot analysis of matrix metalloproteinase (MMPS) and flow cytometric assay[12]. Many more studies were performed to reconfirm potential of earthworm serine protease in crude and purified form for their innate antitumor activity. Many researchers' successful trials on antitumor activity of earthworm protease gave tremendous scope to design novel efficient antitumor agents with biological origin[12,13].

2. Materials and methods

The chemicals used in current study were procured from HiMedia, GE Healthcare and Sigma Aldrich and are of molecular biology Grade. Indian earthworm species *Pheretima posthuma* (*P. posthuma*) as source of serine protease were procured from Vermicomposting Center situated near Mangalagiri, Guntur District, Andhra Pradesh, India (16.41°N and 80.45°E). We have followed standard protocols while preparing chemical reagents and carrying out experiments.

2.1. Collection and processing of earthworm

Fully grown and mature Indian earthworm species *P. posthuma* approximately 1 kg collected from local vermicomposting center were further grown for one week in soil with excess humus and repeated water spray under laboratory conditions. After one week, healthy and mature earthworms were subjected to autolysis after repeated washes with sterile distilled water. Autolysis of cleaned earthworms was performed for 3 h at 60 °C in 20 mmol/L phosphate buffer pH 7.5 with 0.02% sodium azide as bacteriostatic. Further to complete autolysis, earthworms were subjected to 15 °C for one week in refrigerator. After one week of autolysis, earthworms were subjected to successive high speed centrifugation at 16000 r/min for 30 min at 4 °C. Clear supernatant was transferred into sterile tubes under aseptic conditions. Supernatant of 500 mL volume was filtered several times with Whattman filter paper to remove tissue debris, if any and then subjected for purification[14].

2.2. Isolation and purification of serine protease

Purification of earthworm protease was carried out as per protocol designed by Mihara *et al*. with slight modifications[15,16]. Earthworm coelomic fluid was purified with successive chromatographic purification steps: salt precipitation, dialysis, size based and charge based separations. Total protein content of crude extract of 500 mL was precipitated with ammonium sulphate and 65% salt employed to recover 100% of protein[17]. Precipitated protein part was separated from supernatant by high speed centrifugation at 20000 r/min for 30 min at 4 °C. The precipitate was suspended in 25mL of 20 mmol/L phosphate buffer pH 7.5 and subjected to dialysis. Dialysis was performed to remove salt from crude protein by using dialyzing membrane (HiMedia) of molecular weight cut off (MWCO) of 12 kDa. Dialysis was done against 20 mmol/L phosphate buffer pH 7.5 for 8 h with gentle stirring at 4 °C. Buffer was replaced with fresh one at an interval of 2 h during dialysis. After dialysis crude protein sample was transferred into sterile eppendorff tubes and subjected to next step of purification.

Sephadex-G50 (G E Healthcare) was used to prepare column for size based separation. Sephadex-G50 beads were allowed to swell for 48 h in phosphate buffer at room temperature with mild agitation and sonicated to remove entrapped air. Sephadex-G50 beads were loaded in glass column (60×2 cm) and allowed to pack under gravity for 3-4 h. We have packed Sephadex-G50 matrix of dimension 20×2 cm and dialyzed sample was loaded in column along the sides, allowed to saturation and eluted with excess of 20 mmol/L phosphate buffer pH 7.5 with 3mL per min speed. Approximately 25 fractions were collected and assayed for caseinolytic activity with total protein content of each fraction. SDS-PAGE was run with 12% acrylamide concentration and each fraction was loaded with standard low range protein ladder. Fractions having average molecular weight of the range 15-20 kDa and caseinolytic activity were pooled up and subjected to charge based separation[18].

DEAE-Cellulose (G E Healthcare product), an anion resin, was used for separation of protein on charge basis. Prior to column preparation, resin was treated with HCl 30 min, NaOH 30 min and distilled water 30 min to charge. Further, resin was treated with excess of 20 mmol/L phosphate buffer pH 7.5 to remove any traces of acid or alkali. Now charged resin was allowed for packing in glass column and we have marked 20×2 cm under gravity packing. Pooled fractions from Sephadex-G50 were

loaded in column and eluted with 20 mmol/L phosphate buffer pH 7.5 and increased concentration of NaCl with phosphate buffer (0.1%, 0.2%, 0.3%, 0.4% and 0.5%). We have collected 20 fractions with a speed of 3mL per minute. Each fraction was assayed for caseinolytic activity and total protein content[19].

2.3. Determination of total protein content

Protein content of each fraction was determined by Lowry's method[20]. Bovine serum albumin (BSA) was used as standard for the plot of standard graph between optical density and concentration. The crude and purified fractions, each of 100 μL mixed with 0.5 mL Lowry's reagent and 5 mL alkaline copper sulphate were incubated at room temperature for 30 min in dark, and optical density was measured at 660 nm. The concentration of earthworm protease was measured at every stage of purification cascade.

2.4. Determination of average molecular weight

Each fraction collected from DEAE-Cellulose chromatography matrix was evaluated for its average molecular weight. A 12% acrylamide matrix was prepared and chromatographic fractions were loaded (20 μL) with standard protein ladder low range and run for three hours. Gel after completion of electrophoresis was stained with Coomassie brilliant blue G250 and destained with destaining solution with excess methanol. Average molecular weight was determined as per standard protein ladder. The samples exhibiting average molecular weight of 15 kDa were pooled and subjected to further analysis: caseinolytic activity, antitumor activity and cytotoxic activity.

2.5. Determination of caseinolytic activity

Proteolytic activity of purified fractions was determined by well diffusion plate assay using casein (Sigma) as substrate for casein hydrolysis[20]. Casein agar plates were prepared with 5% casein dissolved in 50 mmol/L phosphate buffer pH 7.5. A total of 25 μL of each purified sample was loaded in wells punched previously in casein agar plates. Plates were incubated for 16 h at 37 °C[21].

2.6. Determination of cytotoxic activity

We performed a preliminary analysis for cytotoxic activity of earthworm protease employing Vero cells. These were epithelial cells from kidney, isolated from African green monkey. Both crude and purified fractions of earthworm protease were tested for cytotoxic activity *in vitro*. We have analyzed five samples in total from different steps of the purification cascade *viz*., crude, after dialysis, Sephadex-G50 fraction and DEAE-Cellulose (two) fractions in different dilutions[22,23].

2.7. Determination of antitumor activity

Human cancer cell lines used in this study were procured from National Centre for Cell Science, Pune. All cells were grown in minimal essential medium (MEM, GIBCO), supplemented with 4.5 g/L glucose, 2 mmol/L L-glutamine and 5% fetal bovine serum (growth medium) at 37 °C in 5% CO_2 incubator. The MTT assay developed by Mosmann was modified and used to determine the inhibitory effects of test compounds on cell growth *in vitro*[22]. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5×10^3 cells/well in growth medium and cultured at 37 °C in 5% CO_2 to adhere. After 48 h incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (12.5, 25, 50, 100, 200 μg/mL) in triplicates to achieve a final volume of 100 μL and then cultured for 48 h. The compound was prepared as 1 mg/mL concentration stock solution in DMSO. Culture medium and solvent were used as controls. Each well then received 5 μL of fresh MTT (0.5 mg/mL in phosphate buffer solution) followed by incubation for 2 h at 37 °C. The supernatant growth medium was removed from the wells and replaced with 100 μL of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate was read at a wavelength of 572 nm on an ELISA reader, Anthos 2020 spectrophotometer. Assay was carried out in triplicate using all the chemicals and consumables with molecular biology grade in absolute sterile conditions[10].

3. Results

3.1. Isolation and purification of serine protease

Approximately, 500 mL crude extract after autolysis of mature earthworm was taken and subjected to a cascade of purification techniques. Salt precipitation recovered 100% crude protein with use of 65% ammonium sulphate and excess salt was removed by brief dialysis. About 10 mL of dialyzed protease was recovered after brief dialysis against 20 mmol/L phosphate buffer pH 7.5. All Sephadex-G50 fractions: 25 were analyzed for caseinolytic activity and SDS-PAGE analysis. Fraction number 18, 19 and 20 were pooled and further purified with DEAE-Cellulose resin. Fraction number 11 and 12 were pooled up and subjected to further activity analysis. Basic criteria for selection of fractions in between molecular weight 15-20 kDa is based on the experimental study carried out using *E. foetida*[24].

3.2. Quantification of protease

Quantification of protease was done by Lowry's method at every step of purification cascade. Crude extract and salt precipitates have shown maximum protein content 800 μg and 1000 μg. Among 25 fractions collected from Sephadex-G50 matrix, fractions 18, 19 and 20 were pooled up and protein amount was 360 μg. The DEAE-Cellulose fractions 11 and 12 shown protein content 290 μg were pooled up and subjected to cytotoxic and antitumor activity analysis *in vitro*.

About 65% ammonium sulfate was employed to recover maximum protein content and 1 mg/mL concentration protein was recovered in crude form. Protease concentration

after Sephadex-G50 and DEAE-Cellulose based purifications was found to be 0.27 mg/mL and 0.22 mg/mL respectively (Figure 1, Table 1).

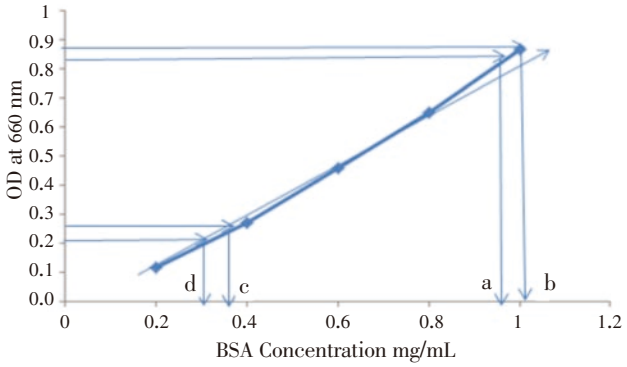

Figure 1. BSA standard curve plotted with concentration of BSA (mg/mL) against absorbance at 660 nm.

Table 1
Quantification of protease with BSA as standard.

Sample	Concentration of BSA (mg/mL)	OD at 660 nm
Blank	0.0	0.000
1	0.2	0.116
2	0.4	0.270
3	0.6	0.460
4	0.8	0.651
5	1.0	0.869
Crude extract (a)		0.823
Ammonium sulphate precipitation (b)		0.898
After dialysis		0.351
Sephadex-G50 fraction 18, 19, 20 (c)		0.272
DEAE-Cellulose fraction 11, 12 (d)		0.218

3.3. Caseinolytic activity

Fractions collected at different points of the purification process were analyzed for proteolytic activity using casein as substrate. Candidate fractions with average molecular weight of 15-20 kDa showed strong caseinolytic activity at 37 °C (Figure 2), confirming serine protease nature of earthworm protease, which exhibits cytotoxic and anticancer activity.

3.4. Determination of average molecular weight

Multiple SDS-PAGES were run to determine average molecular weight of candidate protease (Figure 3). Average molecular weight of partially purified protease with presumed cytotoxic and anti-cancer activity was 15 kDa based on standard low range protein ladder. Fractions with average molecular weight of 15 kDa were pooled up and subjected to further analysis: cytotoxic and antitumor activity.

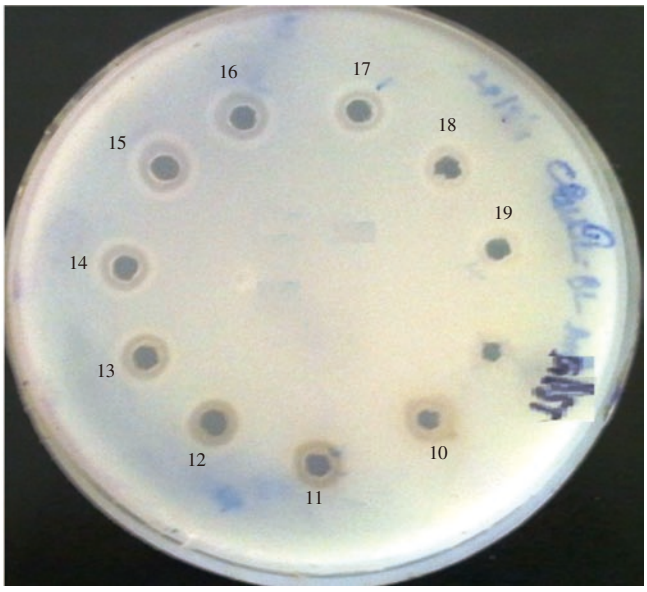

Figure 2. Caseinolytic activity of fractions collected during purification steps of earthworm protease.

3.5. Cytotoxic and anti-tumor activity

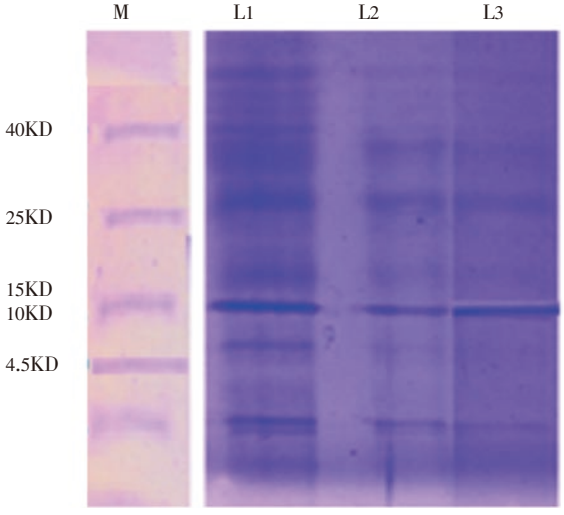

Figure 3. SDS-PAGE analysis of partially purified protease. M-protein ladder low range, L1-dialyzed sample, L2 and L3-DEAE Cellulose fractions 11, 12.

Earthworm protease showed considerable cytotoxic and antitumor activity *in vitro* in crude and purified form (Figure 4, Table 2). There is slight enhancement of cytotoxic and

Table 2
Dose response of earthworm protease (crude and purified) on MCF- 7 (breast cancer) cell line.

Conc. μg/mL	OD of tamoxifen at 572 nm	% Cell Survival	% Cell Inhibition	OD of crude protease at 572 nm	% Survival	% Inhibition	OD of purified protease at 572 nm	% Cell Survival	% Cell Inhibition
12.50	0.468	89.30	10.70	0.383	98.80	1.20	0.345	88.90	11.10
25.0	0.366	66.00	34.00	0.347	88.50	11.50	0.324	82.80	17.20
50.0	0.201	33.60	66.40	0.328	83.00	17.00	0.310	78.70	21.30
100.0	0.126	17.90	82.10	0.289	71.80	28.20	0.287	72.00	28.00
200.0	0.110	14.60	85.40	0.262	63.90	36.00	0.251	61.50	38.50

antitumor activity in purified form of earthworm protease. Crude protease has shown significant cytotoxic activity up to dilution of 1:20 while DEAE Cellulose fractions was much higher and we noticed that cytotoxic activity was up to dilution of 1:40. For analysis of antitumor activity MCF-7 cell lines were treated with various increasing concentration of earthworm protease in crude and purified form. Maximum percent cell inhibition was 38.5% in case of purified earthworm protease. IC_{50} values for protease in crude and purified form were found to be 276.04 and 263.14 μg/mL, which were much higher than IC_{50} standard 39.67 μg/mL for tamoxifen. Survival percentage of MCF-7 cells under exposure to different concentrations of earthworm protease (crude and purified) was 64% and 62% respectively.

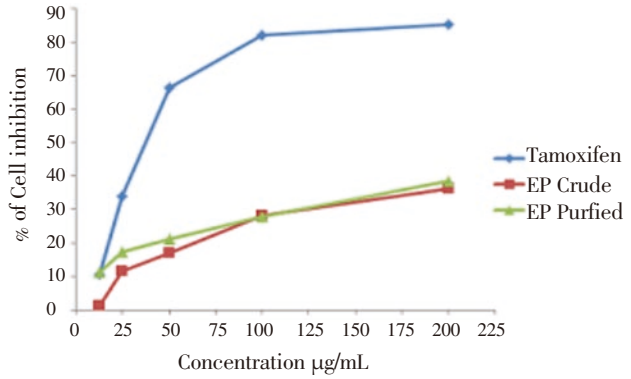

Figure 4. Anticancer activity of earthworm protease (samples EP crude and EP purified) with tamoxifen as standard on MCF-7 (breast cancer) cell lines.

4. Discussion

Earthworms, the first metamerically segmented and eucoelomate worms belonging to the class oligochaeta of phylum Annelida have inspired researchers through their diverse functions in addition to playing the role of "friend of farmer". From the time of Charles Darwin till date, earthworms have been fascinating organisms by virtue of their behavior, as source of food with richness of proteins, minerals and fatty acids, and wide use in traditional medicines of different countries for their several lead compounds bringing relief to human ailments. Very few people are aware of the earthworm's long association with medicine despite the availability of relevant literature right from 14th century. Doctors who practice folk/traditional medicine in countries like India, Burma and China claim that earthworms are sources of bioactive compounds, which are found new uses in production of 'life saving medicines' for cardiovascular diseases, cancer, and inflammation[25,26]. Several other functions like bacteriostatic, proteolytic, cytolytic and mitogen activities have been attributed to the coelomic fluid of the earthworms[4,26]. There are also reports suggesting hemolytic and anti-tumor activity of earth worms [27]. But the actual chemical nature and composition of the constituents responsible for these observed effects are hitherto unexplored at molecular level and therefore is the need for in depth investigations, if scientifically validated pharmaceutical applications are to be established.

Current study demonstrated that the whole animal extract of *P. posthuma* has active principles involved in strong fibrinolytic activity and considerable cytotoxic and anti-tumor activity. We attempted to isolate and purify four distinct protein fractions from the crude extract, of which 15 kDa fraction reported in this study has been found to possess strong protease function and aid in suppression of tumor cell lines and viral infected cells. Efforts are underway to carry out ultra purification and perform biophysical characterization of this fraction before attempting *in vivo* studies. Further experiments with all the isolated protein fractions would possibly unravel the therapeutic potential and subsequent scientific drug formulations of *P. posthuma* in treatment of human ailments.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Cancer/tumor is considered as exacerbating condition for current healthcare system and medicine subsequently. However, some methods such as surgery, chemotherapy, radiation therapy, and immunotherapy are available. They are far from reaching the goal of complete removal of the cancer cells without damage to the rest of the body. Earthworm serine protease is evaluated for antitumor activity. Furthermore, it has been proved that the earthworm proteases enhance the curative effects by both radiation therapy and chemotherapy. In the present study isolation, partial purification and evaluation of cytotoxic and antitumor activity of a serine protease from the chosen Indian Earthworm *P. posthuma* has been reported.

Research frontiers

Recently, there has been increased interest in the anti-tumor activity of serine protease of earthworm *P. posthuma*. In many studies it showed significant anti-tumor activity in hepatoma cells both *in vitro* and *in vivo*, which could be of inducing apoptosis of hepatoma cells and inhibit the expression of MMP-2.

Related reports

Earthworm protease showed considerable cytotoxic and antitumor activity *in vitro* in crude and purified form. There is slight enhancement of cytotoxic and antitumor activity in purified form of earthworm protease. Crude protease has shown significant cytotoxic activity up to dilution of 1:20 while DEAE Cellulose fractions were much higher and we

noticed that activity was up to dilution 1:40. For analysis of antitumor activity, MCF-7 cell lines were treated with various increasing concentration of earthworm protease in crude and purified form. Current study demonstrated that the whole animal extract of *P. posthuma* has active principles involved in strong fibrinolytic activity and considerable cytotoxic and anti-tumor activity.

Innovations and breakthroughs

There are scarce reports on anti-tumor activity of earth worms extracts. But the actual chemical nature and composition of the constituents responsible for these observed effects are hitherto unexplored at molecular level and therefore is the need for in depth investigations, if scientifically validated pharmaceutical applications are to be established. In the present study they isolated and purify four distinct protein fractions from the crude extract, of which 15 kDa fraction reported in this study has been found to possess strong protease function and aid in suppression of tumor cell lines and viral infected cells.

Applications

The present study paves way to conduct ultra purification and perform biophysical characterization of this fraction before attempting *in vivo* studies. Further experiments with all the isolated protein fractions would possibly unravel the therapeutic potential and subsequent scientific drug formulations of *P. posthuma* in treatment of human ailments.

Peer review

The present study on isolation, partial purification and evaluation of cytotoxic and antitumor activity of a serine protease from the chosen Indian earthworm *P. posthuma* is important in medical research.

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