

Antitumor and Cytotoxic Protein Component from *Aporrectodea longa*: Purification, and Characterization Studies

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ABSTRACT

Background: Cancer, a metabolic disorder with multifactorial input, is the most complex human disease to study and find a cure. Over several decades, massive research effort leads to a better understanding molecular biology of cancer and its progression. Plants and animals both were explored for novel anti-tumor agents, and enzyme-based drugs have shown significant results. These animals evolved with an enzyme with promiscuous nature capable of catalyzing multiple biochemical reactions. Enzymes are an integral part of animal physiology and are reported excellent sources of the drug. **Objectives:** The study aimed to isolate, purify and characterize the protein component from *Aporrectodea longa* for anti-cancer potential. **Materials and Methods:** *A. longa* species earthworms were collected and processed for the extraction of total protein content. The anti-tumor protein component was purified via salt precipitation, charge-based purification, and size-based purification. SDS-PAGE was used to investigate molecular weight information. The anti-tumor activity was evaluated using different cell lines, including MCF 7, Hep G2, and HT 29 with Cisplatin, 5-fluorouracil, and tamoxifen as a standard anti-cancer drug. **Results:** The isolated and purified protein fraction had shown proteolytic activity against casein and fibrinogen. Protein fraction represents a serine protease and average molecular weight of nearly 42kDa. The result shows protein component possesses excellent anti-tumor activity analyzed by MTT assay. **Conclusion:** Anti-tumor activity of isolated and purified protein fraction from *A. longa* may serve as a potential candidate for anti-cancer therapy. However, molecular studies are required to explore the precise mechanism of action of candidate protein.

Key words: Antineoplastic agents, Cytotoxic activity, MTT assay, Chromatography, Cell lines, Protein/enzyme.

INTRODUCTION

According to recent reports from the WHO and CDC, there is an immense rise in the cancer burden. The increase in new cases is associated with infection, inflammation, and chronic illness.¹ Cancer continues to be a serious worldwide health problem that affects both advanced and emerging nations. Males and females are both at risk for cancer, and new incidences of cancer in children are on the rise. The global burden of cancer poses a challenge to the healthcare system.² Cancer progression is complex and involves several external and internal inputs. Inflammation is considered a major internal risk factor for the onset

of cancer and genetic abnormalities. It is difficult to enumerate the precise cause of cancer as it is highly complex and multifactorial. Male and females are at risk for separate cancer due to anatomical and physiological differences. Male are more prone to prostate and lung cancer; however, females are at higher risk towards breast and uterus cancer.³ Recent findings have shown that the rise in drug-resistant cancer cases posed a new level of challenge to clinical therapeutics. Anti-neoplastic therapeutics are incapable of controlling the growth of the drug-resistant tumor. Somatic and peripheral cancer is comparatively easy to

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diagnose and treat compared to the tumor in neuronal tissue.⁴ Several approaches have been proposed to deliver therapeutics, including anti-neoplastic agents across BBB but failed due to low permeability, low affinity and toxic effects, etc.⁵ The advantage of proteins as an anti-neoplastic agent is specific to target as higher affinity and least side effect.⁶

Researchers are investigating both animal and the microbial world for new generation anti-neoplastic agents. Proteins are highly promiscuous have shown tremendous scope for commercial applications.⁷ Invertebrates, especially worms, are rich in enzymes with broad substrate affinity and catalytic diversity. The earthworm has been known for its medicinal values for many centuries and is described in “Materia Medica.” Coelome of earthworm is rich in proteases with immense isoforms; nature provides a broad range of substrate affinity and catalytic diversity.⁸ The anti-tumor potential of enzymes from several earthworm species is being investigated by scientists.⁹ *Aporrectodea longa* is indigenous to tropical climates, but species are also found in other parts of the world. These enzymes do have medicinal potential; nevertheless, little is known about *Aporrectodea longa* species.^{10,11} The present work lies in the purification and characterization of proteolytic components from *Aporrectodea longa* species. The further study emphasizes enzyme activity and the determination of molecular weight. Verma *et al.* 2013, 2016, 2017 studied various proteolytic components, including thrombolytic, antibacterial, and anti-tumor of earthworm species *Pheretima posthuma*.¹²⁻¹⁴ The anti-tumor activity of proteolytic component aimed to evaluate stand anti-neoplastic drugs on various cancer cell lines.^{15,16}

MATERIALS AND METHODS

All the chemicals and consumables used in the study were procured from Sigma Aldrich and Merck Ltd. The chemical and consumables used here of molecular biology grade. The cell lines for anti-tumor activity analysis were purchased from a local supplier, and anti-cancer drugs were procured from Sigma Aldrich. The standard molecular protocols were followed, and all the chemicals and consumables were stored as per supplier instructions; cell lines MCF 7, Hep G2, and HT 29 were maintained at liquid nitrogen and followed supplier protocols during the study. The concentration and dilution of anti-cancer drugs cisplatin, 5-fluorouracil, and tamoxifen were used in this study as per previous findings with slight modifications.¹²

Collection and processing of animals

Aporrectodea longa is a member of the Lumbricidae family habitat in the tropical region. These species of earthworms are widely distributed and evolved to habitats in arid and humid conditions. The idea behind choosing *Aporrectodea longa* as the source of a therapeutic protein (anti-cancer) is a wide range of habitat adaptation of animals and survival. There are growing reports demonstrating that earthworms are unique in immunity due to specific proteins/enzymes. I collected mature and grown animals 250gm from a local vermicomposting unit. The animals were characterized based on zoological findings. The animals were raised for two weeks in laboratory conditions proving ideal temperature (35-40°C), feed (humus) and humidity (40-60%). The fully-grown animals were used for crude protein preparation. The animals were washed repeatedly with fresh water and subjected to autolysis for 2 weeks. The processing and autolysis of earthworms were carried out as per a study carried out in 2017.¹⁴ As a bactericidal and bacteriostatic agent, I've added 1% sodium azide was added.¹³

Purification of Protein Component

The autolyzed animals were subjected to spin for 15 min at 5000 rpm at 4°C. The supernatant was collected in a fresh and sterile tube for further purification. The supernatant was filtered using Whatman filter paper (No 1). The clear supernatant was chromatographically purified in three steps: salt precipitation, ion-exchange chromatography, and size exclusion chromatography.¹⁷ Here, ammonium sulfate was used to precipitation total protein from crude and reconstituted in phosphate buffer saline (PBS, 50mM, pH 8.0). Dialysis was used to remove salt from the reconstituted protein and concentrate the protein. Here I used ready to use dialysis tube from Merck of molecular weight cut-off (MWCO) 20kDa. The dialyzed samples were subjected to ion-exchange chromatography. Here I used diethylaminoethyl-cellulose (DEAE-cellulose) resin as positive charged was pretreated as per GE Healthcare protocols (1N HCl) and alkali (1N NaOH). Charged DEAE Cellulose resin was saturated with an excess of PBS (50mM, pH 8.0) for an hour. The resin was packed on a glass column of dimension 30.0 x 2.0 cm and allowed to settle down overnight. The column was packed under gravity with an excess of PBS (50mM, pH 8.0). 10ml dialyzed crude protein was loaded into the column and allowed to travel into the column (Eluting knob was off). The PBS was used to equilibrate the column, and elution (3ml/min) was carried out using a combination of PBS (50mM) and KCl (250mM). A total of 15 fractions were collected and analyzed for

protein content and activity.¹⁶ The fraction with higher protein content was pooled and subjected to size exclusion chromatography. Here, I used Sephadex G50 beads for column packing under gravity. The column was packed and equilibrated with PBS (50mM, pH 8.0). A 5ml of DEAE Cellulose purified and pooled protein fraction was loaded into a column and eluted with an excess of PBS (50mM, pH 8.0). Elutes (10; 2 ml each) were collected and profiled for protein concentration and activity analysis.¹⁸ Lowery methods determined the protein concentration according to standard protocol and enzyme activity using protease assay.

Characterization of purified protein

The available research literature on earthworm proteins and enzymes suggests that the earthworm's coelomic cavity possesses significant proteases. The study has also shown these proteases are generally serine proteases. The purified enzyme was subjected to proteolytic analysis using the casein hydrolytic test. The casein was used as a substrate on the agar plate, and the purified enzyme was added (5ml) into wells on the plate. Plates were incubated overnight at 37°C, and the zone of clearance was assessed as an indicator of enzyme activity.¹⁹ The protease experiment was performed for enzyme activity with specificity using casein as the substrate. SDS-PAGE analysis was used to measure the average molecular weight of the isolated protein component (enzyme). A 12 percent polyacrylamide gel was used for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Using standard protein ladder 8 µl purified protein component was loaded on polyacrylamide gel from different stages of purification.²⁰ Protein bands on the gel were seen using Coomassie Brilliant Blue G 250 staining. By comparing the average molecular weight of the protein component to the standard protein ladder, the average molecular weight of the protein component was determined.

Anti-cancer/anti-tumor activity analysis

Cell culture

Human cancer cell lines used in this study were procured from ATCC, India. I used three cancerous cell lines, including MCF 7, Hep G2, and HT 29, for anti-cancer activity analysis and Vero cell lines for cytotoxic studies. All cells were cultivated at 37°C in a 5% CO₂ incubator in minimal essential medium (MEM, GIBCO) supplemented with 4.5 g/L glucose, 2 mM L-glutamine, and 5% foetal bovine serum (FBS) (growth media). The cytotoxic activity of crude and purified protein components was carried out using Vero Cell lines.²¹

Standard Antineoplastic drugs and purified protein component

Three standard antineoplastic drugs, cisplatin, 5-fluorouracil, and tamoxifen, were used in different increasing concentrations i.e., 12.5, 25, 50, 100, 200 µg/ml. These antineoplastic agents were purchased from Sigma Aldrich at molecular biology grade, and dilutions were made as per instructions made by the supplier. The purified protein component was diluted in a similar concentration to determine anti-cancer/anti-tumor activity.²²

MTT assay

The MTT assay developed by Mosmann was modified and used to determine the inhibitory effects of test compounds on cell growth *in vitro*.²³ In brief, trypsinized cells from the T-25 flask were planted at a density of 5x10³ cells/well in growth media in each well of a 96-well flat-bottomed tissue culture plate and grown at 37°C in 5% CO₂ to adhere. After 48 hr incubation, the supernatant was discarded. The cells were pretreated with a growth medium before being mixed in triplicates with various doses of test substances (12.5, 25, 50, 100, 200 µg/ml) to achieve a final volume of 100 µl and cultivated for 48 hr. The compound was prepared as 1.0 mg/ml concentration stock solutions in DMSO. Culture medium and solvent were used as controls. Each well received 5 µl of fresh MTT (0.5 mg/ml in PBS) and was incubated at 37°C for 2 hr. To solubilize the colorful formazan product, the supernatant growth media was withdrawn from the wells and replaced with 100 µl of DMSO. The absorbance (OD) of the culture plate was measured using an ELISA reader, Anthos 2020 spectrophotometer, after 30 min of incubation.

RESULTS

Collection and Processing

We collected fully grown healthy *Aporrectodea longa* species animals 250gm from a local vermicomposting center. The complete autolysis yielded 500ml crude extract containing protein of interest. The crude protein component concentration and activity were determined via the Lowery method and protease assay using casein as substrate. At a crude level, protein component concentration was reported 1.54 mg/ml with enzyme activity and specific activity 3.1 U/ml and 2.01 U/mg.

Purification of the protein component

Crude protein was subjected to three steps chromatographic purification, including salt precipitation, ion-exchange chromatography and size

exclusion chromatography. Dialysis was performed to remove excess salt and to concentrate purifying protein. Here we used ammonium sulfate for protein precipitation, and at 65% w/v total protein was pellet down. The precipitated protein was reconstituted into 20 ml PBS (50mM, pH 8.0). The concentration and activity were determined. As a result, shown in Table 1 and Figure 1, the precipitated protein component was active in high concentrations. We report 1.14 mg/ml total protein with enzyme and specific activity 2.8 U/ml and 2.45 U/mg with a purification fold of 1.21. The caseinolytic assay shown in Figure 1, demonstrates an active enzyme. Repeated dialysis further improved purification fold (1.46) with 0.85 mg/ml and enzyme and specific activity 2.5 U/ml and 2.94 U/mg, respectively. SDS-PAGE was used to assess the average molecular weight of isolated protein components (Figure 2). The DEAE cellulose ion-exchange chromatography yielded four active elutes as fractions 18-21 (Figure 3). These elute fractions were pooled and evaluated for concentration and activity analysis. These pooled fractions were then submitted to size exclusion chromatography. The purification yield in ion-exchange chromatography was 1.59, with a total protein concentration of 0.59 mg/ml and enzyme activity and specific activity of 2.1 U/ml and 3.2U/mg. A size-based chromatography offered

three active elute with higher protein content and activity, i.e., elute fraction no 12-14. These elute fractions were pooled and evaluated for protein concentration and enzyme activity. The total protein present in pooled elute from the Sephadex column was reported 0.25 mg/ml, with a purification yield of 2.76 (Figure 4 and Table 1). The enzyme and specific activity reported 1.45 U/ml and 5.8 U/mg, respectively. The protein component was active during the purification process with higher activity (Figure 1).

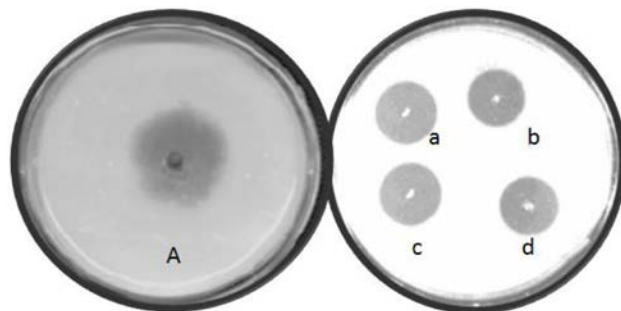


Figure 1: Proteolytic analysis of crude and purified protein component using casein as substrate. Plate A represents the lysis zone with crude extract on the milk agar plate. The second plate represents a clear lysis zone of casein on the casein agar plate; a- salt precipitate, b- dialyzed sample, c- pooled fractions of DEAE cellulose, and d- fraction 13 from Sephadex column.

$$\text{conc. of protein} \left(\frac{\text{mg}}{\text{ml}} \right) = \frac{\text{OD of Test}}{\text{OD of Std.}} \times \frac{\text{Conc. of Std.}}{\text{Vol. of sample used ml}}$$

$$\text{Enzyme activity} \left(\frac{\text{U}}{\text{ml}} \right) = \mu\text{M of tryosine released} \times \frac{V}{pqr}$$

$$\text{Specific activity of enzyme} \left(\frac{\text{U}}{\text{mg}} = \frac{\text{Enzyme activity}}{\text{Protein content (mg)}} \right)$$

$$\text{Purification fold} = \frac{\text{Specific activity}}{\text{Crude specific activity}}$$

Table 1: Purification table; enzyme activity, specific activity and purification yield.

Purification Steps	Conc. of Protein (mg/ml)	Enzyme Activity (U/ml)	Specific Activity (U/mg)	Purification Fold
Crude extract	1.54	3.1	2.01	--
Salt Precipitation	1.14	2.8	2.45	1.21
Dialysis	0.85	2.5	2.94	1.46
DEAE-Cellulose	0.65	2.1	3.2	1.59
Sephadex G50	0.25	1.45	5.8	2.76

Where V is the total volume of assay in ml, p - volume of enzyme used, q denotes the time of assay and r is volume used for colorimetric determination.

Characterization of purified protein

The purified protein component was characterized for its nature and average molecular weight. The casein hydrolytic assay shows protein fraction is serine protease in nature. The protein component was active in both the form, i.e., crude and purified (Figure 1). The average molecular weight of purified protein components was determined by SDS-PAGE using a standard protein ladder and reported nearly 42kDa (Figure 2). Mass spectroscopic studies are required for precise molecular weight and 2D gel electrophoresis for the isoforms nature of the protein. The cytotoxic activity of crude and purified protein components was carried out using

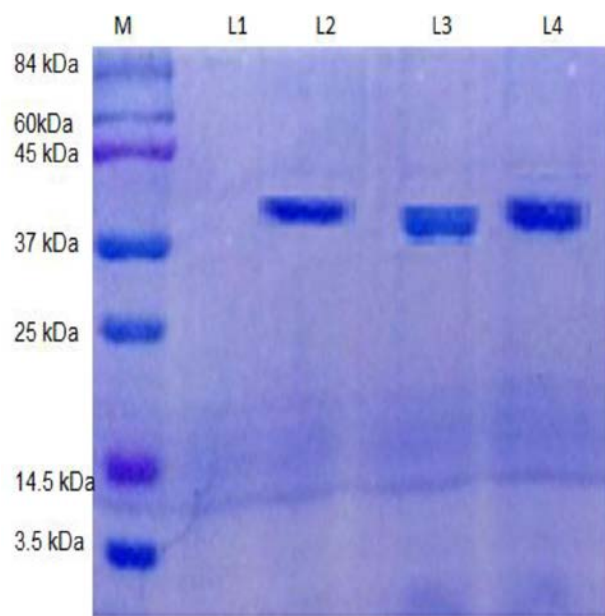


Figure 2: Average molecular weight of purified fraction of protein component from *Aporrectodea longa*. The average molecular weight of purified protein was observed nearly 42kDa.

Vero cell lines. The enzyme activity was calculated as per the formula (under Table 1). As purification process progress the yield and specific activity of protein component increases (see Table 1). On the contrary, total protein content and enzyme activity decrease with the progression of the purification process.

Anticancer/anti-tumor activity

The purified protein fraction had shown tremendous anti-cancer activity against tested cell lines MCF 7, Hep G2 and HT 29 with cisplatin, 5-fluorouracil, and tamoxifen. MTT assay was used to determine anti-cancer/anti-tumor potential of purified protein fraction. The effect of purified protein fraction on different cell lines was reported significantly differently (Tables 2, 3, and 4, Figure 5, 6 and 7). With colon cancer cell lines HT29 and Cisplatin as a typical antineoplastic treatment, the effect of pure protein fraction showed maximum percent inhibition (81.65 percent) in a dose-response curve. Other cancer cell lines have also demonstrated a positive result in cell grown inhibition with purified protein fraction i.e. Hep G2 78.8% and MCF 7 with 74.5%. Earlier Verma *et al.*, 2013 reported a similar

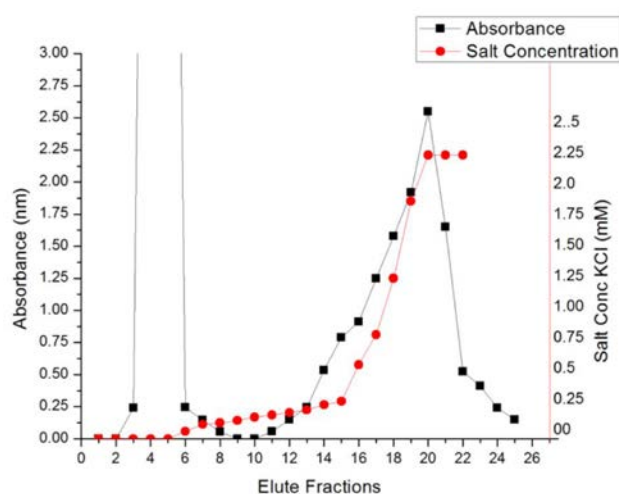


Figure 3: A DEAE Cellulose chromatogram is showing the elution pattern of the active protein component.

Table 2: Dose Response of protein component on HT-29 (Colon Cancer).

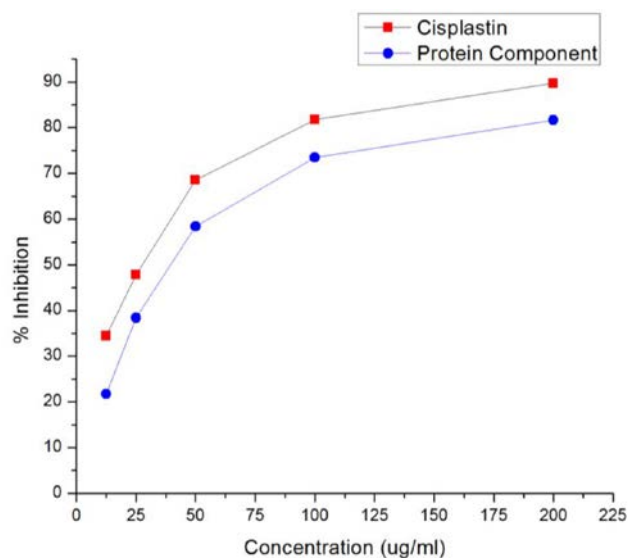
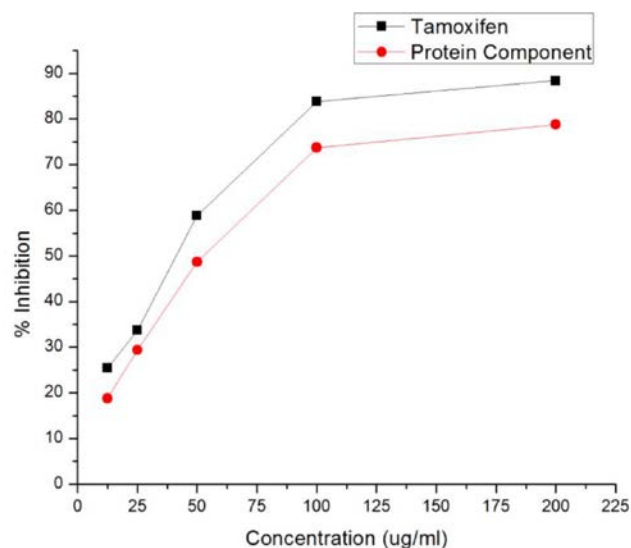
Conc (µg/ml)	OD of cisplatin at 572 nm	% Cell Survival	% Cell Inhibition	Protein OD 572 nm	% Cell Survival	% Cell Inhibition
12.5	0.280	63.6	36.4	0.242	78.22	21.78
25	0.231	52.2	47.8	0.216	61.58	38.42
50	0.145	31.5	68.5	0.202	41.6	58.40
100	0.106	18.2	81.8	0.181	26.52	73.48
200	0.091	10.3	89.7	0.164	18.35	81.65

Table 3: Dose response of protein component on Hep-G2 (Liver Cancer) cell line.

Conc (µg/ml)	OD of tamoxifen 572 nm	% Cell Survival	% Cell Inhibition	Protein OD 572 nm	% Cell Survival	% Cell Inhibition
12.5	0.922	74.6	25.4	0.342	81.2	18.8
25	0.814	66.3	33.7	0.324	70.6	29.4
50	0.535	41.2	58.8	0.302	51.3	48.7
100	0.220	16.2	83.8	0.281	26.25	73.75
200	0.170	11.6	88.4	0.260	21.2	78.8

Table 4: Dose response of protein component on MCF 7 (Breast Cancer) cell line.

Conc (µg/ml)	OD of 5-Fluorouracil at 572 nm	% Cell Survival	% Cell Inhibition	Protein OD 572 nm	% Cell Survival	% Cell Inhibition
12.5	0.812	72.2	27.8	0.327	84.3	15.70
25	0.748	65.4	34.6	0.313	72.54	27.46
50	0.582	42.5	57.5	0.298	58.0	42.0
100	0.311	15.8	84.2	0.276	32.54	67.46
200	0.168	11.4	88.6	0.253	24.36	75.74

**Figure 5: Dose-response curve of purified protein component on colon cancer cell line HT 29 with standard antineoplastic drug cisplatin.****Figure 6: Dose-response curve of purified protein component on liver cancer cell line Hep G2 with the standard antineoplastic drug tamoxifen.**

finding in another species of earthworm. Considering the size of the purified protein component, it resembles antibacterial and cytotoxic components from *Lumbricus rebus*. Similarly, *E. fetida* a well-studied earthworm species had reported similar molecules of protein origin with immense cytotoxic and antimicrobial activity. The anti-tumor activity is very close to standard antineoplastic drugs used in the present study (Table 2-4).

DISCUSSION

The present study reports a 42 kDa protein component with immense anti-cancer activity tested in different cell lines, including MCF 7, Hep G2, and HT 29. The protein component was isolated and purified from *Aporrectodea longa*, an earthworm species habitat in tropical countries. The protein component was

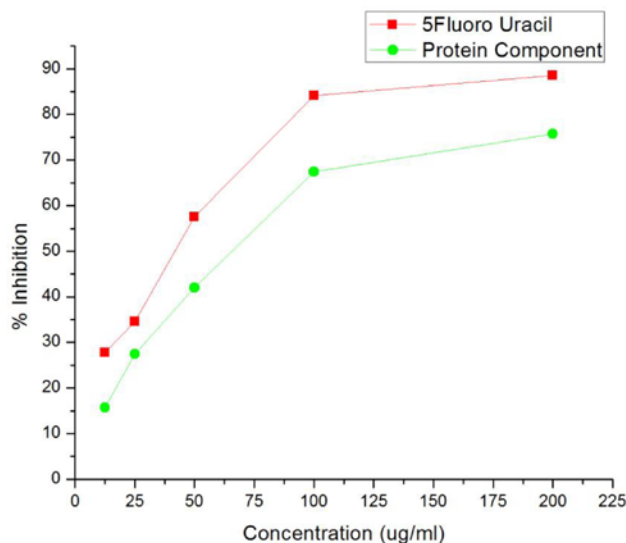


Figure 7: Dose-response curve of purified protein component on breast cancer cell line MCF 7 with standard antineoplastic drug 5-fluorouracil.

purified using chromatographic techniques. The protein was reported as serine protease in nature, as shown in clear casein hydrolysis on the milk agar plate. The anti-cancer activity of purified protein components was reported close to standard antineoplastic drugs, including cisplatin, 5-fluorouracil, and tamoxifen. Exploring the clinical importance of such enzymes requires animal and human validation. The result shown in the present study demonstrates the concentration and activity of protein components at the crude level.¹³ The protein had shown a significant cell lysis activity (cytotoxic) in an *in vitro* cell culture setup.¹² These findings suggest that protein/enzyme from *Aporrectodea longa* possesses an excellent anti-tumor activity requiring clinical validation.²⁴ Verma *et al.*, 2013 used a partially purified protein fragment fraction for anti-cancer activity analysis against MCF7. Here, I report a higher percentage of cell inhibition.¹² However; animals offer a new class of anti-cancer molecules, which may turn into effective antineoplastic therapeutics in the future. The earthworms are known for a wide range of active proteases with broad substrate affinity and catalytic diversity. These molecules differ in earthworm species, and the nature of such molecules depends on animal habitat. Invertebrates, including lower worms, possess a unique immune system regulated by proteins/enzymes and peptides. There is overwhelming research evidence clearly demonstrating that the Annelids genus is rich in several proteases as a key molecule in earthworm physiology that possesses anti-tumor activities.^{25,26} These molecules offer better protection against a wide

range of infections and foreign invaders. The protein from earthworm coelomic cavity profiled for several therapeutic applications, including clot-dissolving, antiviral, antibacterial, and wound healing capacity. The study confirms an active anti-tumor/anti-cancer protein molecule from *Aporrectodea longa*.

CONCLUSION

The author reports a 42 kDa protein component with immense anti-cancer activity tested in different cell lines, including MCF 7, Hep G2, and HT 29. The protein component was isolated and purified from *Aporrectodea longa*, an earthworm species habitat in tropical countries. The protein component was purified using chromatographic techniques. The protein was reported as a serine protease, as shown in clear casein hydrolysis on the milk agar plate. The anti-cancer activity of purified protein components was reported close to standard antineoplastic drugs, including cisplatin, 5-fluorouracil, and tamoxifen. Exploring the clinical importance of such enzymes requires animal and human validation. Animals, on the other hand, provide a new class of anti-cancer compounds that could one day become useful antineoplastic treatments. Earthworms have a large number of active proteases with a wide range of substrate affinity and catalytic variety. These molecules differ in earthworm species, and the nature of such molecular depends on animal habitat. Invertebrates, including lower worms, possess a unique immune system regulated by proteins/enzymes and peptides. These chemicals provide improved defense against a variety of diseases and foreign invaders. The protein from earthworm coelomic cavity profiled for several therapeutic applications including clot dissolving, antiviral, antibacterial and wound healing capacity. The study confirms an active anti-tumor/anti-cancer protein molecule from *Aporrectodea longa*.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

MCF 7: Michigan Cancer Foundation-7; **Hep G2:** Human hepatoma cell line; **HT 29:** Human colorectal adenocarcinoma cell line; **SDS-PAGE:** sodium dodecyl sulphate–polyacrylamide gel electrophoresis; **kDa:** kilodalton; **PBS:** Phosphate Buffer Saline; **FBS:** Foetal Bovine Serum; **DEAE:** Diethylaminoethyl; **OD:** Optical Density; **WHO:** World Health Organization; **CDC:** Centers for Disease Control; **BBB:** Blood Brain Barrier.

SUMMARY

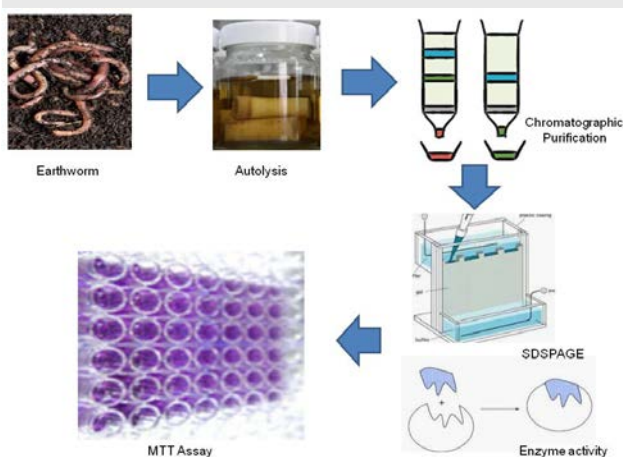
The protein component was isolated and purified from *Aporrectodea longa*. The protein component was evaluated for anti-cancer activity. The isolated and purified protein fraction had shown proteolytic activity against casein and fibrinogen. The newly isolated protein fraction represents a serine protease, and the average molecular weight was observed at nearly 42kDa. The anti-cancer activity of the protein component was evaluated using three distinct cancer cell lines, including MCF7, Hep G2, and HT 29. MTT Assay was carried out for % inhibition using three different standard anti-cancer drugs including, 5-Fluorouracil, tamoxifen, and cisplatin. The protein component showed excellent anti-cancer activity.

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PICTORIAL ABSTRACT



SUMMARY

Earthworms *Aporrectodea longa* species were collected and processed for the extraction of total protein. The anti-tumor protein component was purified via three steps chromatographic purification processes. The purified protein characterized for enzyme activity and average molecular weight was found 42kDa. Cytotoxic activity of purified protein component was carried out using Vero Cell lines. The antitumor activity of the purified protein component was carried out by MTT assay using different cell lines, including MCF 7, Hep G2 and HT 29 with cisplatin, 5-fluorouracil, and tamoxifen. The results showed a 42 kDa protein component with significant anti-tumor activity.

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