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Anticancer Potentials of Peptides of Coelomic Fluid of Earthworm *Eudrilus eugeniae*

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Production of compounds of Pharmacological importance from earthworms is a novel area that has gained importance in many of the South East Asian countries in modern day medicine termed as vermiceuticals. Earthworms were maintained in plastic tubs containing decomposed organic matter that served as substrate and their feed. Cancer cell were cultured according to standard cells culture method. The cancer cells were treated with different concentrations of proteins from coelomic fluid of *Eudrilus eugeniae* to study the concentration effect on cytotoxicity using MTT assay. The result revealed that the coelomic fluid of *Eudrilus eugeniae* induced cell death in 48hrs and the activity was concentration dependent. In the present study Cytotoxic effect of coelomic fluid from *Eudrilus eugeniae* was studied on HeLa cell, Colon cancer cells, WBC malignant tumor cells and Brain tumor cells. This confirms the presence of cytotoxic molecules in the coelomic fluid of earthworms. This work suggests that some of the coelomic fluid components might be useful for pharmaceutical applications in the treatment of cancer.

Key words: Coelomic fluid, cytotoxicity, *Eudrilus eugeniae,* MTT.

Earthworms have been widely used in traditional Chinese medicine for thousands of years. However, it is only during the past few decades, with the development of biochemical technologies that research on the pharmaceutical effects of earthworms has been initiated. Fibrinolytic enzymes were first isolated from earthworms in 1980's. Coelomic fluid of the earthworm *Eisenia fetida* was shown to induce apoptosis of HeLa cells *in vitro* (Yanqin, Yan, Zhenjun, Shijie, Chong, Yan, Yuhong, 2007). Cytotoxic effect of coelomic fluid of earthworm *Eudrilus eugeniae* was studied on Baby hamster Kidney 21 (BHK_{21}) cells (Rudrammaji, Suma Sridhar, Dinesh and Sonole., 2008). Coelomic fluid (CF) of earthworms contain cytolytic, antibacterial and agglutinating components (Roch *et al*, 1989; Valembois *et al*, 1982; Lassegues *et al*, 1989 Mohrig *et al*, 1996). The primary function of this cytolytic system may destroy membranes of foreign cells which may cause cell death by cytosol release. This is a preliminary report on cytotoxic activity of coelomic fluid proteins from the earthworms *Eudrilus eugeniae* by MTT assay method. The cytotoxicity was checked using Hela cell, Colon cancer cells, WBC malignant tumor cells and Brain tumor cells.

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MATERIALS AND METHODS

Media and other reagents

RPMI-1640 media, Dolbeccos minimum essential medium (DMEM), Fetal bovine serum (FBS), Trypan blue dye, MTT, dimethyl sulphoxide (DMSO), Laminar air flow, CO_2 incubator, hemocytometer, HPTLC, microtitreplates Micropipettes, earthworm (*Eudrilus eugeniae*), ice cubes.

Earthworm Culture

The Earthworms were collected from a commercial vermiculture unit near the city and maintained in plastic tubs containing decomposed organic matter until used for study.

Coelomic fluid collection by heat and cold shock method

Earthworms were washed in distilled water. They were placed on ordinary wet filter paper in plastic boxes with lids having fine pin holes. After 48 hrs, the gut was cleared of organic matter as they feed on filter paper. They were thoroughly washed with distilled water and then placed in glass funnel. Warm water (45-50^{o}C) in a glass beaker (10ml) was used to give heat shock and in a similar way ice cubes were used to give cold shock. The treatment was alternated at a gap of three minutes to overcome the shock effect. This caused agitation for the coelomic fluid to ooze out from dorsal pores and mouth of earthworms. The fluid released was collected into the eppendorf tubes. Earthworms were released back to the culture tubs after collection of coelomic fluid.

Coelomic fluid was also collected by placing the earthworms in Petri plates held in a slanting position in the palm. Their body surface was rubbed with wet finger and later with ice cubes taken in a beaker. The released fluid from their body was collected by Pasteur pipette.

Purification of coelomic fluid by ammonium sulphate fractionation

Coelomic fluid was subjected to various percentage of ammonium sulphate fractionation (20%, 30%, 45% and 60%). The precipitated protein was dissolved in PBS buffer. The fractionation was subjected to dialysis to remove the salt. The dialysed fraction was tested for the cytotoxic activity against different tumour cell lines taken in microwell titre plates. The absorbance for the incubated cell suspension was read in Elisa reader at 570nm as indicated in Fig. 1, Fig. 2, Fig. 3, and Fig. 4.

Biochemical characterization of coelomic fluid was done by HPTLC to analyse the the different components of the coelomic fluid.

Maintenance and Culture of HeLa , Colon , WBC malignant tumor and Brain tumor cell lines

HeLa, colon cancer cells, WBC malignant tumor cells and Brain tumor cells were purchased from NCCS, Pune. The cells were stored in liquid nitrogen after sub culturing. The stored cells were taken out and kept at -80^{0}C for few hours and thawed at 37^{0}C in water. Revived cells were taken to LAF for sub culture maintaining sterile conditions.

RPMI-media and fetal bovine serum were used for culturing and maintenance of the HeLa, colon and cancer cells. Dulbecco's minimum essential medium was used for culturing and maintenance of the WBC malignant, and Brain tumor cells.

Measurement of cytotoxicity of coelomic fluid proteins on different cell lines.(MTT assay)

The HeLa cells, Colon Cancer cells, WBC malignant tumor cells, Brain tumor cells were seeded onto separate 96 well flat bottom micro titer plates at a concentration of 1.0X10^{4}cells/well in duplicates. Cells were allowed to grow for 24h in RPMI medium, centrifuged for 10 min at 3000 RPM. Supernatant was removed and pellet was treated with 100µl of 0.1mg/ml, 0.2mg/ml, 0.3mg/ml, 0.4mg/ml, and 0.5mg/ml of coelomic fluid of *Eudrilus eugeniae* respectively. Each concentration was set up in duplicates. Incubated for 48h after which 10µl MTT with a concentration of 4mg/ml was added to each well. Incubated for 4h at 37^{0}C, centrifuged for ten min at 2000 RPM, supernatants were carefully removed and 100µl of DMSO was added. After insoluble formazon crystals were dissolved completely, absorbance was read at 490nm to determine the inhibition rate of coelomic fluid against HeLa cells, Colon Cancer cells, WBC malignant tumor cells and Brain tumor cells.

Inhibition rate = [(1-mean OD of treated group/ mean OD of untreated control) x100%] [1]

of coelomic fluid protein and percentage of inhibition is shown in table 1.

RESULTS AND DISCUSSION

Total Proteins were estimated by the method of Lowry *et al*., (1951) using bovine serum albumin (BSA) as standard (0-75µg).

The HP-TLC band shows the presence of different compounds in the coelomic fluid of the earthworm *Eudrilus eugeniae* which may be responsible for the cytotoxicity of the cancer cells.

HP-TLC (High performance Thin layer Chromatography)

Cytotoxicity assay

The cytotoxic effect of coelomic fluid proteins was observed on tested cell lines and it was found to be concentration specific. Cytotoxicity of different cell lines at different concentrations

Table 1. Cytotoxicity of earthworm (Eudrilus eugeniae)coelomocytes on different cancer cell lines

Coelomic fluid Protein concentration (mg/ml) cells	Cytotoxicity on different cell lines			
	HeLa cells	Colon cancer tumor cells	WBC Malignant cells	Brain tumor
0.1	15.1%	10.8%	9.8%	5.4%
0.2	19.0%	14.25%	13.87%	9.7%
0.3	24.5%	18.24%	18.68%	14.19%
0.4	33.0%	23.7%	23.38%	19.3%

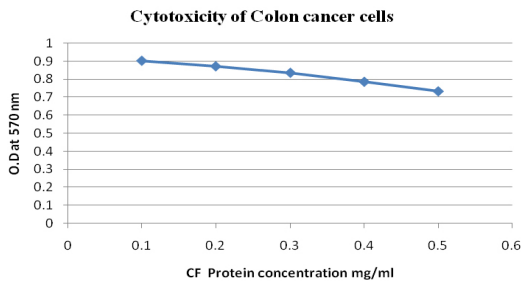

Fig. 1. Cytotoxicity of CF of *Eudrilus eugeniae* on HeLa cells using Elisa reader

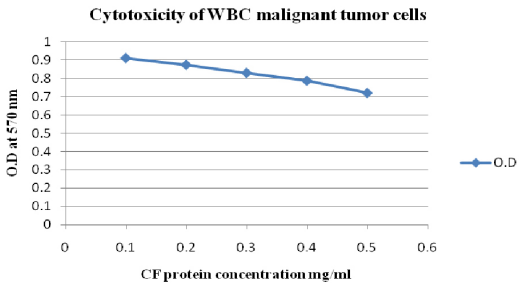

Fig. 2. Cytotoxic activity of CF of *Eudrilus eugeniae on c*olon cancer cells using Elisa reader

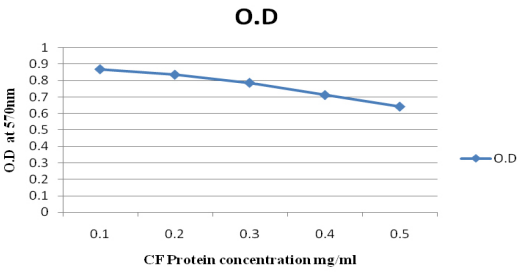

Fig. 3. Cytotoxicity of CF of *Eudrilus eugeniae* on WBC malignant tumor cells using Elisa reader

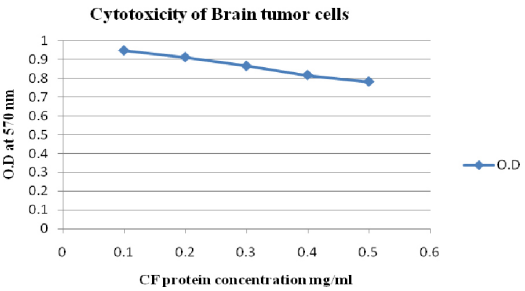

Fig. 4. Cytotoxicityof CF of *Eudrilus eugeniae* on Brain tumor cells using Elisa reader

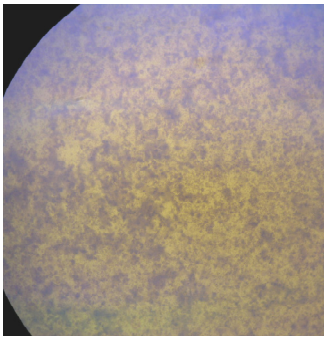

Plate 5(a): Normal HeLa Cells

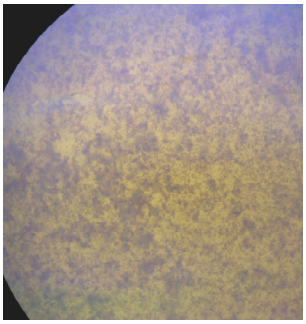

Plate 5(b). Coelomic fluid proteins inducing cytotoxicity in HeLa cells

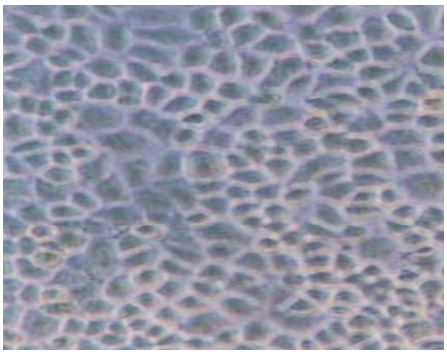

Plate 6(a). Normal Brain tumor cells

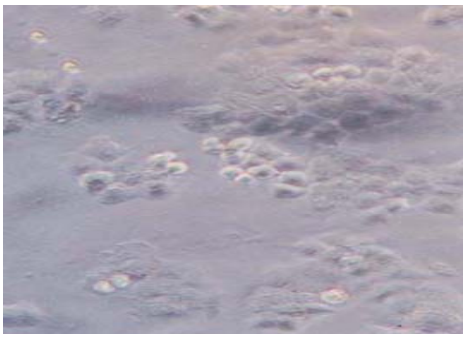

Plate 6(b). Coelomic fluid proteins inducing Cytotoxicity in brain tumor cells

Plate 7(a). Colon cancer cell lines

Plate 7(b). Coelomic fluid proteins inducing Cytotoxicity in Colon cancer cells

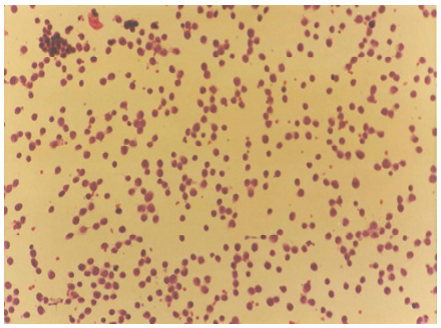

Plate 8(a). WBC Malignant tumor cell lines

Plate 8(b). Coelomic fluid proteins inducing Cytotoxicity in WBC Malignant tumor cells

CONCLUSION

One of the most striking medical practices of the 21st century is the chemoprevention of cancer. Currently, over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them. In recent years, increased attention has been focused on the evolution of the invertebrate immune system. Biologically active molecules are present since early phases of evolution. The ceolomic fluid of earthworms exhibits different biological functions, including, bacteriostatic, proteolytic, cytolytic and mitogenic activities.

In the present study, coelomic fluid treated different cancer cell showed cytotoxic effect up to a concentration of 0.5 mg/ml. Coelomic fluid showed a dose dependent effect on above said cancer cells on detection with MTT assay. These results which show that HeLa cell inhibition at high concentration of coelomic fluid were similar to those reported by Yan (2005) which stated that mass concentration of coelomic fluid of earthworm *Eisenia eugeniae,* can kill HeLa cells by cell necrosis and lysis in a dose dependent manner. Kauschke and Mohrig, summarized that *Eisenia eugeniae* has a toxic effect on a variety of cell types, such as chicken fibroblasts . According to (Englemann *et al* 2003) the presence of cytotoxic molecules are released from a characteristic coelomocytes type from *Eisenia eugeniae* The coelomic fluid of earthworms contains cytolytic and hemagglutinating molecules, which can be released from various Coelomocytes .Identication of different component proteins by HP-TLC technique was according to the method of Ueda *et al* (2004) The cytotoxic effect of coelomic fluid of earthworms may be related to many components like proteins and peptides within the coelomic fluid. Fibrinolytic activities, anti tumor activities and microbial activities were found in coelomic fluid by many researchers. .

The study was carried out using the entire protein moiety of the coelomic fluid. Further studies have to be carried out to test the different macromolecules independently to identify the specific compound/compounds responsible for cytotoxicity.

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