Cytotoxic Potential of Eudrilus eugeniae coelomcyte culture supernatant against tumor cells

Vidya.N[1] ,Dinesh.M.S[2], Ananda .S[3] and Radha.D.Kale[4]

[1,2] Department of Biotechnology, PES Institute of Technology, Bangalore-85

[3]Department of Biotechnology Sapthagiri college of engineering Bangalore-57

[4]Center for Scientific Research and Advanced Learning, *Mount Carmel* College (Autonomous), Bangalore-52

Abstract: The molecules causing cytotoxicity to tumor cells having major research interest and their application to develop drug against tumor. The Coelomocytes constitute immune component of earthworm coelomic fluid have the potential to produce biomolecules which causes cytotoxicity to tumors. The present study comprises of culturing coleomocytes isolated from coelomic fluid *of Eudrilus eugeniae* in HBSS as a culture media and with suitable conditions and testing the activity of coelomic fluid ,culture supernatant sample by MTT assay on tumor cell lines lung cancer cells (A549)and colorectal tumor cell lines(HTC 116) which showed significant cell death compared to respective control cell. The percent cytotoxicity in coelomic fluid ,coelomocyte culture supernatant and dialyzed sample were in the order 85% ,95% and 62% respectively. The highest cyotoxicity showed by culture supernatant .Supernatant enhanced its utilization as the major source of cytotoxic agent against tumor. The results of MTT assay were validated by performing more sensitive assay against the selected cell line used in the present study. The coleomocyte culture supernatant was administered in the concentration range 1-2mg/ml to induce cytotoxicity.The results showed 90% cytotoxicity in A549 Cell lines with concentration of 2mg/ml.The colorectal tumor cell showed moderate compare to referred standard values of Indian pharmacopeia/FDA .The findings of the study provide scientific evidence to utilize coleomocyte culture supernatant as a source to develop anticancer drug for treatment of cancer.

Keywords: Coelomocytes, *Eudrilus eugeniae*, lung ,colorectal cancer cell lines, cytotoxicity.

I INTRODUCTION

Earthworms Coelomocytes mainly comprised of phagocytic activity due to which foreign cells are prone for cell cytotoxicity.Coelomocytes comprises of enzymes like serine proteases, metalloproteases proteins like lysenin, lectin which are mainly responsible for cell death. Lysenin induces plasma membrane breakage and releases cytosolic contents inducing apoptosis. There are three main coelomocyte types those are eleocytes, free chloragogen cells or granular amoebocytes, both representing immune cells involved in wide range of defense mechanism. Several studies on cytotoxic , antitumor activity of various earthworm species have been studied. Anticancer activity of Indian earthworm lampito mauritii was tested against colorectal cancer cells.According to study conducted by Lourdmary AJB et,al in 2014 dry earthworm powder of lampito mauritii showed significant toxicity against HT29 colorectal cancer cells with 82.25% inducing cell death. Cell free coelomic fluid of earthworm possess apoptotic activity The studies revealed performed by M.K. Verma et,al (2013) showing 35%cell inhibition form serine protease isolated from earthworm of Pheritima phostuma on MCF-7 cell lines ..The present study involves the activity of coelomocyte culture supernatant of Eudrilus eugeniae to induce cytotoxicity to lung cancer and colorectal cancer cell lines against the tumor cells Activity coelomic fluid and coelomocyte culture supernatant was analysed for its cytotoxic activity.Clonogenic assay was performed to screen the potential of coelomocyte culture supernatant to suppress the tumor cells when incubated for long duration.

II MATERIALS AND METHOD

A. Culturing of earthworm *Eudrilus eugeniae.*

Seed culture of *Eudrilus eugeniae* has been collected from vermiculture unit of University of Agricultural Sciences, Bangalore. The collected worms were cultured in breeding polypropylene box 0.5*0.25m*0.25m box using partially decomposed cowdung for 30days. Earthworm *Eudrilus eugeniae* around 50 worms were fed with tissue paper for 24 hours and worm were washed thoroughly with water and rinsed with ca-LBSS media and then subjected to collection of coelomic fluid by chemical shock method (Nanditha madhusudan et,al 2010)

B. Culturing of coelomocytes by cell culture method. The tissue paper fed earthworms were collected from tank and subjected to washing in order with water ,distilled water and sterile water followed by wash with ca-lbss with antibiotics and bevastin and around 8-10worms were kept in clean sterilized beaker and 3ml of extrusion medium(25Mm EDTA ,5%Ethanol in Nacl) was added and exposed for 3min and continued upto 3sets.after exposure to extraction medium earthworms were released to natural feed for rejuvenation which takes 8days To this 3ml, 4ml of ca free lbss was added and centrifuged at 1500rpm for 15min. supernatant was discarded to the pellet 1ml of Ca- lbss was added and the procedure was repeated for 3times get a pool of coleomic fluid. To 5ml of HBSS 3ml of

coleomocytes were added and incubated at 37^0C for 3 days.After 3 days of incubation coelomocytes culture was centrifuged at 5000rpm for 15 min and supernatant was collected and protein content was estimated by Lowry's method to fix the dose for cytotoxic assay for tumor cells.,

C. Antitumor activity of coelomic fluid and Coelomocyte culture supernatant.

*MTT assay :*Antitumor activity of earthworm coelomic fluid ,coelomocytes culture supernatant and dialyzed sample were tested on two tumor cell lines lung cancerA549 and colon cancer HCT Cell lines. Proceured from ATCC USA and maintained in RPMI 1640 medium with 10% FBS at Skanda life sciences pvt ltd Bangalore.These cells were grown under maintained CO_2 at 5% CO_2 level and cells were grown in T-25 flasks. Cells were passaged with 0.05% trypsin –EDTA at 70-80% confluency. viability of cell were checked and cell were centrifuged. The cells with density 50000 cells/well of A549 and HCT116 were seeded in 96well plate and incubated for 24hrs at 37^oC, 5 % level in CO_2 incubator. Coelomic fluid, culture supernatant and dialyzed samples was incubated ranging from 0.2mg/ml of protein concentration. After incubation with samples, the media was removed from the wells and 100µl/well (50 ug /well) of the MTT (5 mg/10ml of MTT in 1X PBS, the solution is filtered through a 0.2 µ m filter and stored at 2–8 °C for frequent use or frozen for *extended periods)* working solution was added and incubated for 3to 4 hours.After incubation with MTT reagent, the media is removed from the wells and add 100 µl of DMSO to rapidly solubilize the **formazan**. Absorbance was measured at 590 nm(Gonzalez, R.J et,al,2001)

D. Clonogenic assay

Clonogenic assay serves as a useful method to test whether a given cancer therapy can reduce the clonogenic survival of tumor cells.(Franken N.A et ,al, 2006) A colony is defined as a cluster of at least 50 cells which can often only be determined microscopically. Clonogenic assay is the method to determine cell reproductive death after treatment used to determine the cytotoxicity of coelomocyte culture supernatant. The cell strain A549 cells were plated at $2x10^3$ cells/well (6-well plate) containing DMEM complete media and incubated at 37^0C, 5% CO_2 for 24 h. After 24 h, cells are treated with various concentration of C. fluid and culture supernatant with protein concentration ranged from 0 to 2 mg/ml. Cells are grown for a period of 14 days. Fresh media was added on the seventh day. On the fourteenth day to produce colonies of >50 cells/ colony, media was removed from the wells and washed once with ice-cold PBS. The colonies were stained with 1 ml of 1% crystal violet in 80% methanol for 30 minutes on a rocking platform. The wells were rinsed three times with PBS and air-dried, and the colonies were counted. The stained cells were captured using inverted microscope with 4x magnification.

III RESULTS AND DISCUSSION

A. Antitumor activity of coelomic fluid and Coelomocyte culture supernatant on A549 Cells by MTT assay.

Anitutmor activity of coelomic fluid and coelomocyte culture supernatant was tested on A549lung cancer cell line and HCT116 Colon cancer cell line. Coelomic fluid and coelomocyte culture supernatant protein concentration ranging from lmg/ml ,1.25 mg/ml 1.50 mg/ml ,1.75 mg/ml and 2 mg/ml were added to both tumor cell lines. Maximum tumor cell inhibition for A549 cells were found to be 89.85% at protein concentration of 2 mg/ml and 93.91% for coelomocyte culture supernatant.

Both coelomic fluid and culture supernatant induce tumor cell suppression when administered at different concentration Table.1. The inhibition increases as the protein concentration in coelomic fluid and culture supernatant increases.Inhibition rate was same for coelomic fluid and culture supernatant at initial concentration of protein start from 1to1.50mg/ml later culture supernatant showed higher inhibition than coelomic fluid at protein concentration ranges from 1.75 to 2mg/ml.

Morphological changes in tumor cells after treating with coelomic fluid and coelomocyte culture supernatant which showed visible changes in cell morphology in which rounding of tumor cells detaching from the bottom of the flask can be seen clearly with both tumor cells A549 and HCT116.are shown in Fig 2 and 4. Coelomic fluid and supernatant showed dose dependent cytotoxic effect on both A549 and HCT116 cells. Interestingly *C. supernatant* had significant cytotoxic effect on both the cells about 93% at concentration of 2 mg/ml. Its IC_{50} values are determined as shown in the table 1 and 2.

Earlier studies showed the cytotoxic activity of the earthworm coelomic fluid and protein purified from earthworm extracts.the percentage cell inhibition was found to be 82.25%(Lourdmery et,al 2014),cell inhibition was found to be 78.52% with coelomic fluid (Mohamed jaabir et,al 2011) and many others. These studies used earthworm either as powder or coelomic fluid was isolated by sacrificing the worms.In the present study after extraction of coelomic fluid worms were released back to natural feed where worms were revived back to normal after a week and further can be reused.

Table 1.Absorbance values of effect of earthworm samples on A549 cells by MTT assay

Earthworm samples	Conc.mg/ml	Absorbance at 590 nm	% Inhibition	IC_{50}
	Control	1.1082	0.00	
Coelomic fluid	1.00	0.8933	19.39	
	1.25	0.7064	36.25	1.469
	1.50	0.3822	65.51	
	1.75	0.2833	74.43	
	2.00	0.1125	89.85	
Coelomocyte culture supernatant	1.00	0.9433	14.88	
	1.25	0.7517	32.17	
	1.50	0.4011	63.80	1.881
	1.75	0.1683	84.81	
	2.00	0.0675	93.91	
	conc. µg/ml			
Dialyzed				
	150.00	0.6246	43.64	
	300.00	0.3497	68.44	

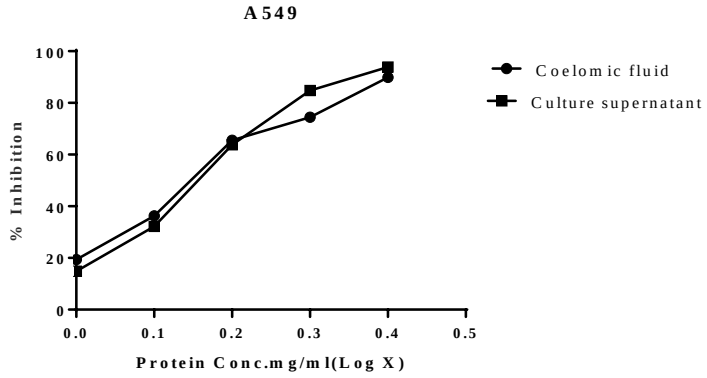

Fig1: Represents the percentage inhibition of A549 tumor cells when treated with coelomic fluid(89.85% inhibition) and culture supernatant(93.91% inhibition).

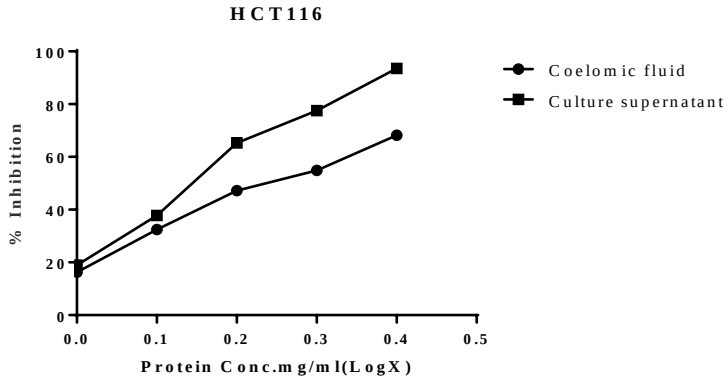

Fig 3: Represents the percentage inhibition of HTC116 tumor cells when treated with coelomic fluid(68.17% inhibition) and supernatant(93.58% inhibition)

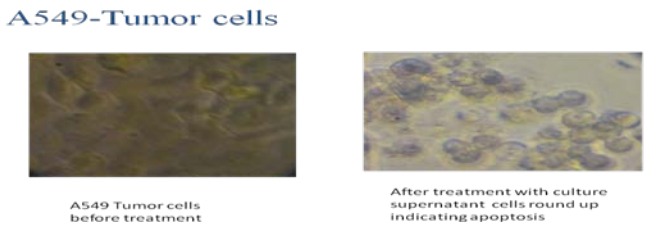

Fig 2:.Morphology of A549 cells left picture indicates A549 cells seen attached to surface before treatment and after treatment right side indicated round up of cells and they are detached.

Table 2.Absorbance values of effect of earthworm samples on HCT116 cells by MTT assay

Earthworm Samples	Conc.mg/ml	Absorbance at 590 nm	% Inhibition	IC_{50}
	Control	0.8381	0.00	
Coelomic fluid	1.00	0.7014	16.31	1.72
	1.25	0.5664	32.42	
	1.50	0.4422	47.24	
	1.75	0.3785	54.84	
	2.00	0.2668	68.17	
Coelomocyte culture supernatant	1.00	0.68	18.96	1.93
	1.25	0.5217	37.75	
	1.50	0.2911	65.27	
	1.75	0.1883	77.53	
	2.00	0.0538	93.58	
	conc. µg/ml			
Dialyzed				
	150.00	0.6246	25.47	
	300.00	0.3497	58.27	

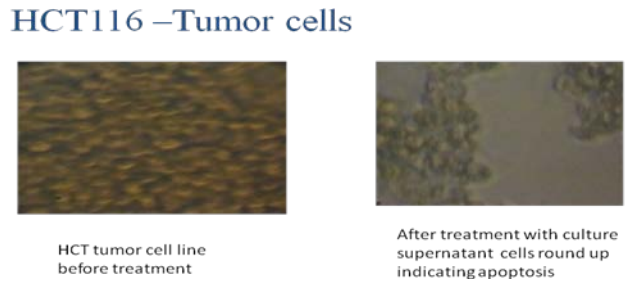

Fig.4:.Morphology of HTC116cells seen attached to surface before treatment and after treatment right side indicated round up of cells and they are detached.

B. Clonogenic assay

Clonogenic assay was performed on lung cancer cell line A549. This assay proves the ability of coelomic fluid and coelomocyte culture supernatant to restrict the multiplication of tumor cells when incubated for 14 days. *C. fluid and supernatant* showed does dependent inhibition of growth of A549 colonies /cells. The *C. fluid* inhibits 25 %, 40% and 90 % growth of A549 cells at concentration of 1, 1.5 and 2 mg/ml respectively, where as *C. supernatant* inhibits 20 %, 62 % and 96 % growth of A549 cells. From the obtained result both the samples ceolomic fluid and culture supernatant showed a promising result to inhibit the growth of tumor cells(Fig 5). As this study gives an insight that the growth of tumor cells can be controlled when coelomic fluid and culture supernatant were incubated with tumor cells as these samples did not allow the tumor cells to replicate and grow indicating positive hope to develop antitumor drug from earthworm immune cells as source will be biological and extensive studies need to be carried out to understand the process how the tumor cell growth is restricted. As earlier studies indicate the use of earthworm paste for the study were in the present study earthworms were not sacrificed as coelomic fluid is isolated and coelomocytes were cultured for the study. As this work is first of its kind to report coelomocyte culture supernatant to show antitumor activity.

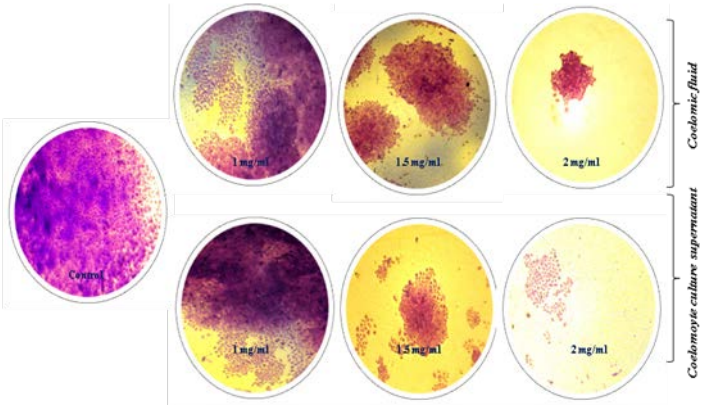

Fig 5 .Represents pictures treated with above row indicating the cell growth inhibition on A549 Lung cancer cell line when treated with coelomic fluid at different concentrations and below row represents the treatment with coelomocyte culture supernatant.

IV CONCLUSION

The present study throws a spotlight where earthworm *Eudrilus eugeniae* coelomic fluid as well as cultured supernatant of coelomocytes showed its cytotoxicity towards both A549 Lung cancer and HCT116 colorectal cancer cells. Coelomocyte culture supernatant showed higher cytotoxicity compare to coelomic fluid which indicates the higher concentration of active components in the culture supernatant compare to coelomic fluid.The presence of higher concentration of metabolites in culture supernatant plays a major role in cytotoxic process. Clonogenic assay results showed the inhibition in the culture supernatant treated tumor cell lines indicating the sustainable potential of metabolites to permanently suppress the tumor cell replication in the targets. This supports the application of these principal molecules to develop drug against tumor. Further collaboration with industry to elucidate the structural functional relationship with tumor targets with these active ingredients helps in development of anticancer drug against specific targets.

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Authors

First Author – Vidya.N, Ph.D Research scholar , PES Institute of Technology, Bangalore .Email : vidyavs13@gmail.com..
Second Author –M..S Dinesh Ph.D ,PDF(IISc), Assistant professor , PES Institute of Technology.
Email:dineshms@pes.edu
Third Author –Ananda .S,Ph.D ,HOD , Department of Biotechnology Sapthagiri college of engineering Bangalore.
Email:ipranand@gmail.com
Fourth Author- Radha.D.Kale ,Ph.D Center for Scientific Research and Advanced Learning, *Mount Carmel* College Bangalore,Email-dr.rdkale@gmail.com

Correspondence Author – M..S Dinesh, dineshms@pes.edu ,dineshms_74@yahoo.com
Contact No.:9686612818