



**RESEARCH ARTICLE**

**INVITRO ANTICANCER POTENTIAL OF THE EARTHWORM PASTES OF *EUDRILUS EUGENIAE*, *PERIONYX EXCAVATES* AND *EISENIA FETIDA* AGAINST MCF 7, HCT 116 AND PC 3 CANCER CELL LINES**

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**ABSTRACT**

A novel area in modern day medicine termed as vermiceuticals include the production of pharmaceutically important compounds from earthworm extracts. Earthworm possess rich natural sources of antioxidants and are used with other herbs to treat a wide variety of conditions ranging from spasms and convulsions to pain relief, treatment of fever and certain types of arthritis. Focusing this, in our present study, the earthworm pastes prepared from three species of common earthworms were analyzed for invitro anticancer potential against three different cancer cell lines. MCF-7, PC-3 and HCT-116 cells were treated with earthworm pastes of *Perionyx excavates*, *Eudilus eugeniae* and *Eisenia fetida* and the effects on the cytotoxic nature of earthworm pastes of *Perionyx excavates*, *Eudilus eugeniae* and *Eisenia fetida* were determined using MTT cytotoxic assay. IC50 concentration was determined as the drug concentration that is required to reduce the half of the cells from the total population. From this analysis it was found that earthworm paste of *Perionyx excavatus* showed anticancer activity against all the three cancer cell lines used. It showed IC50 values of 87.45µg/ml, 104.8µg/ml and 239.1µg/ml in MCF-7, HCT-116 and PC 3 cells respectively. *Eudilus eugeniae* and *Eisenia fetida* also showed IC50 values of 320.9µg/ml, 321µg/ml and 14.18µg/ml, 25.95µg/ml in MCF-7 and HCT-116 cancer cells respectively but no activity against PC 3 cells. DNA ladder assays, clonogenic assays and cell cycle studies were also performed on PC3, MCF-7, HCT-116 cells by treating them with earthworm pastes of concentration- 320µg/ml, which showed results in a dose dependent manner and a maximum cell cycle arrest as 25.85 % , 27.88 %,30.03 % for *P.excavatus*, *E. eugeniae* and *E. fetida* at G2/M phase respectively and induced apoptosis. Further research on the principal active components of earthworm pastes of the three mentioned species leads to development of drug to treat human cancer.

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**INTRODUCTION**

Earthworms are ancient invertebrate animals on earth. They play an important role not only in the food chain of ecosystem, but they also influence physical chemical properties of soil composition and enforce microbiological processes in soil itself (Edwards C A et al, 1996; Liu YQ et al, 2004). There are about 3920 named species of earthworm so far reported worldwide (Cooper E L., 2005; Vohora S B et al, 1978). In India, so far, 509 species, referable to 67 genera and 10 families, have been reported.(Kale R.,1991) . Earthworms play an important role in agro-ecosystem like enhancing decomposition, humus formation, nutrient cycling and soil structural development (Kladivko E.J et al, 1986). Recently earthworm protein and its coelomic fluid were reported to have cytolytic, agglutinating, proteolytic, haemolytic, anti-pyritic, tumorstatic and antibacterial activities (Franken N. A.et al, 2006; Daniel C et al, 2013). Earthworms were found to have healing effect on wounds, chronic folds, piles and sore throat (Ramesh P. et al, 2011). Earthworm's anti-pyretic properties were reportedly

tried in China and Japan in reducing fever. Studies on the medicinal value of indigenous earthworms are limited (Masahiro Takada et al, 2011; Crouch, S.P.M. et al 1993). The present study was aimed to test the anti-cancer activity of the earthworm pastes of the common indigenous earthworms *Perionyx excavatus*, *Eisenia fetida* and *Eudrilus eugeniae* in MCF-7 breast carcinoma cells, PC3 Prostate cancer cells and human colorectal cancer cell line HCT 116. In the present study, earthworm pastes showed a dose dependent effect on different cancer cell lines detected with MTT assays. DNA ladder assay was performed which showed that DNA laddering was much more apparent in HCT 116 cells as compared to other two cells. Clonogenic studies showed dose dependent inhibition of growth of MCF-7, HCT116 and PC-3 cells by earthworm samples. Cell cycle studies showed that treatment of earthworm samples on cancer cells showed maximum cell cycle arrest at G2/M phase and induced apoptosis. From the present, it is proposed that earthworm pastes may be a future candidate for treating tumors. The outcome of the finding

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would suggest further directions in the search of anticancer agents in the treatment of cancer.

## METHODS AND METHODOLOGY

**Collection of earthworms** Fully matured earthworms *Eudrilus eugeniae*, *Perionyx excavatus* and *Eisenia fetida* were collected from Camson Biotech, Hebbal, and Karnataka Compost Development Corporation, Bangalore.

**Preparation of earthworm paste** (M. Balamurugan *et al.*, 2007): The earthworms were washed in running tap water in order to remove the sand particles from the surface of earthworms and then fed with wet blotting paper for 18 to 20 hours to clear their gut. The gut cleared worms were again washed with distilled water. The worms were kept in plastic troughs, covered tightly with polythene cover, and exposed to sunlight for 3 days to kill them. Mucus and coelomic fluid that oozed out digested the dead worms forming a brown colored paste earthworm paste (EP) (Balamurugan *et al.*, 2007). The earthworm pastes were filtered and the filtrates obtained were condensed in water-bath at 35°C. All the above mentioned common earthworms were made into pastes for studying anticancer potentials in various cancer cell lines *invitro*.

**Cancer cell lines:** MCF-7 breast carcinoma cells, PC3 Prostate cancer cells and human colorectal cancer cell line HCT 116 were obtained from the ATCC, USA and maintained in RPMI 1640 medium at Skanda life science, Bangalore. The cell lines were maintained in RPMI 1640 (Himedia, India) medium supplemented with 10% FBS (Foetal Bovine Serum) and Penstrep (Penicillin-Streptomycin) (1000 units/ml). The cells were grown in the Humidified CO<sub>2</sub> incubator (Binder's, USA) at 5% CO<sub>2</sub> level at 37 °C. 25 Sq. cm flasks (Corning) were used to culture the cells. Passage of cells was carried out by 0.05% trypsin- EDTA at 70-80% confluency. The cells were cryopreserved with DMSO and stored at -80 °C for further stocks.

**In-vitro Cytotoxicity assay of earthworm pastes** (Mosmann, 1983): The cells (HCT 116, PC-3 and MCF-7 cancer cells) were cultured in 96-well plates (Corning, USA) with a cell density of  $5 \times 10^4$  cells/well<sup>1</sup>. Cells were allowed to grow in a monolayer and examined under the inverted microscope for confluency and regular morphology. The RPMI 1640 medium was discarded from the wells and treated with reconstituted RPMI 1640 medium that contained earthworm pastes (EP) in a series of concentrations from 0-320 µg/ml [2 fold variations] . Control wells were also maintained simultaneously which did not receive EP. All the treatments were performed in triplicates. After incubation with compounds, the media is removed from the wells and 100 µl/well (50 µg /well) of the MTT (5 mg/10ml of MTT in 1X PBS) working solution was added and incubated for 3 to 4 hours at 37°C. After incubation with MTT reagent, the media was removed from the wells and 100 µl of DMSO was added to rapidly solubilize the formazan. The Absorbance was measured at 570 nm. IC<sub>50</sub> value was calculated from the observed optical density in comparison with the untreated control. The inhibition rate of the EP on

cancer cells was calculated by the formula % of inhibition =  $100 - (\text{Sample/Control}) * 100$ .

**DNA Ladder Assay:** DNA ladder assay was done according to the protocol mentioned elsewhere (Takada *et al.*, 2011). Adherent and detached cells were harvested and washed with phosphate buffered saline (1X PBS). Approximately  $2 \times 10^5$  cells were lysed with a lysis buffer composed of 50 mM Tris-HCl, 10 mM ethylene diamine tetra acetic acid (EDTA)-4Na and 0.5% sodium-*N*-lauroyl sarcosinate (pH 7.8). The lysates were incubated in the lysis buffer containing 0.33 mg/ml RNase A at 50 °C for 30 min and then further incubated in the lysis buffer containing 0.33 mg/ml proteinase K at 50 °C for 30 min. Equal amount of DNA was electrophoresed on 2.0% agarose gel. Gels were stained with 0.5m g/ml ethidium bromide for 15 min and visualized under UV light.

**Clonogenic survival assay:** Clonogenic survival assay was done as mentioned in the protocol (Franken *et al.*, 2006). This assay serves as a useful tool to test whether a given cancer therapy can reduce the clonogenic survival of tumor cells. 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 with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents. MCF-7, HCT116 and PC-3 cells were plated at  $2 \times 10^3$  cells/well (6-well plate) containing DMEM or RPMI-1640 complete media and incubated at 37°C, 5% CO<sub>2</sub> for 24 h. After 24 h, cells are treated with various concentrations (0, 160 and 320 µg/ml) of earthworm samples. Cells are grown for 14 days. Fresh media is 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. Finally take the picture at 4X by using Olympus inverted microscope.

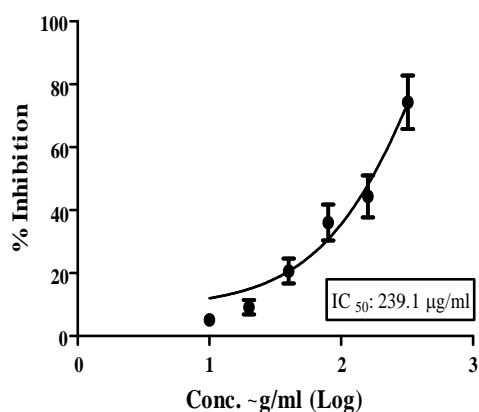
**Cell Cycle Analysis:** Cell Cycle Analysis was done as mentioned in the protocol (Ramesh P. *et al.*, 2011).  $5 \times 10^5$  cells (HCT116, MCF-7 & PC-3) were cultured in a 12-well plate containing 1 ml of complete media. After 24 hrs of incubation, the spent media was removed and washed one time with 1X PBS. Different concentration (0, 160 and 320 µg/ml) of control, earthworm samples were added in 1 ml / well and incubated for 24 hrs. After 24 hrs of treatment, the media was removed, washed one time with 1X PBS and finally the cells were collected by Trypsin-EDTA. ( both floating and adherent cells were collected).  $5 \times 10^5$  cells / ml were centrifuged at 1500 rpm for 5 minutes at room temperature and the supernatant was discarded. The cells pellet was resuspended gently with 2 washes in 1XPBS. Cells pellet was fixed overnight at 4 °C in a 500 µl of Fixing solution (containing 15% FBS and 15% PBS in 70% ethanol). Centrifuged at 1500 rpm for 5 min at room temperature and the supernatant was discarded. Cells pellet was washed two times with cold 1XPBS. Cells were incubated for 1 h at room temperature in 500 µl of propidium iodide (PI) solution containing 0.05mg/ml PI, 0.1 mM EDTA, and

0.05mg/ml RNase A in PBS. The percentage of cells in various stages of cell cycle in compounds treated and un-treated populations were determined using FACS Canto II (BD Biosciences, San Jose, CA) and analyzed by Flow Jo 7.5.5 (Tree Star Ashland OR).

## RESULTS

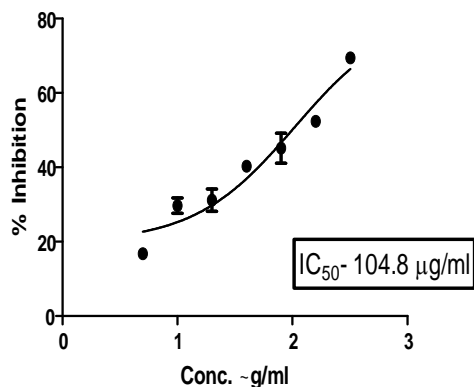
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### In-vitro evaluation of sample A in PC3 Prostate cancer cells

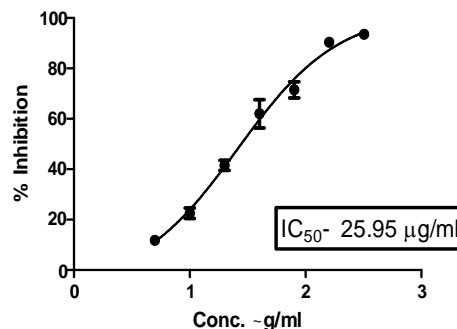


**Figure 1** Graph showing the results of percent inhibition of the extracts on the cell lines. Left: % inhibition on MCF7 Cell lines. Right: % inhibition on PC3 cell lines. The concentration of the samples were expressed in µg/ml. all the values were average of triplicates. Sample A: earthworm paste of *Perionyx excavatus*; Sample B: earthworm paste of *Eudrilus eugeniae*; Sample C: earthworm paste of *Eisenia fetida*.

### Antiproliferative activity for human Colorectal cancer cell line HCT 116

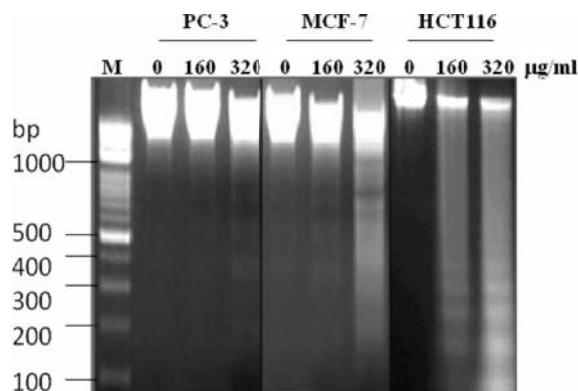


### Antiproliferative activity for human Colorectal cancer cell line HCT 116



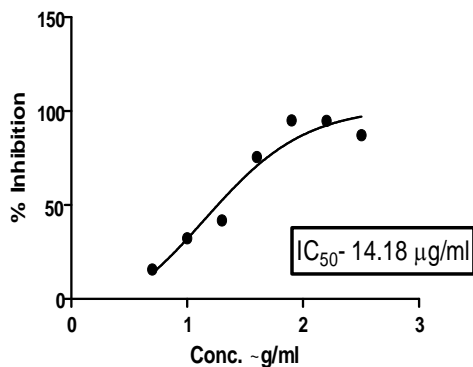
**Fig 2** Graphs showing the results of Antiproliferative activity for human colorectal cancer cell line HCT 116. Left: % inhibition of sample A Middle: % inhibition of sample B; Right: % inhibition of sample C. The concentration of the samples are expressed in µg/ml. all the values were average of triplicates. Sample A: earthworm paste of *Perionyx excavatus*; Sample B: earthworm paste of *Eudrilus eugeniae*; Sample C: earthworm paste of *Eisenia fetida*.

**DNA Ladder Assay:** DNA fragmentation is a biochemical hallmark of apoptotic cell death. Data shows that the formation of DNA laddering, occurred in PC-3, MCF-7 and HCT116 cells incubated in the presence of earthworm samples at indicated concentrations for 24h. DNA laddering was much more apparent in HCT 116 cells as compare to other two cells, where it was much less apparent in cells incubated in the absence of samples (control).



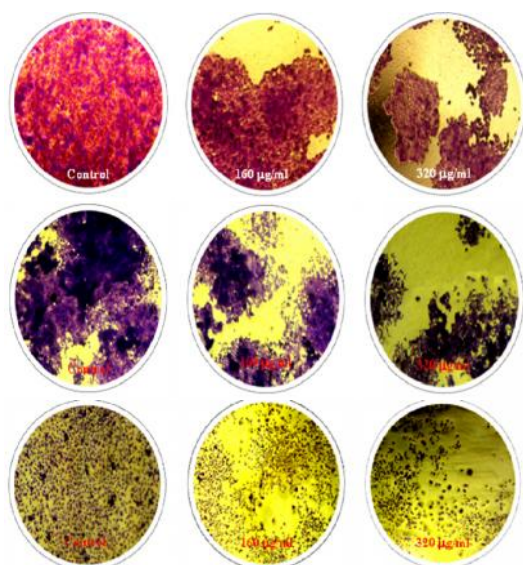
**Figure 3** Figure showing the effect on cell viability. Both treatments reduced cell viability in a dose dependent manner. Left: Treatment of PC-3 cell lines with sample A. The treatments are made at 160 and 320µg/ml. Middle: Treatment of MCF-7 cell lines with sample B; Right: Treatment of HCT-116 with Sample C. Control is represented by 0µg/ml. Lane M: Molecular marker 100bp. Sample A: earthworm paste of *Perionyx excavatus*; Sample B: earthworm paste of *Eudrilus eugeniae*; Sample C: earthworm paste of *Eisenia fetida*.

### Antiproliferative activity for human Colorectal cancer cell line HCT 116



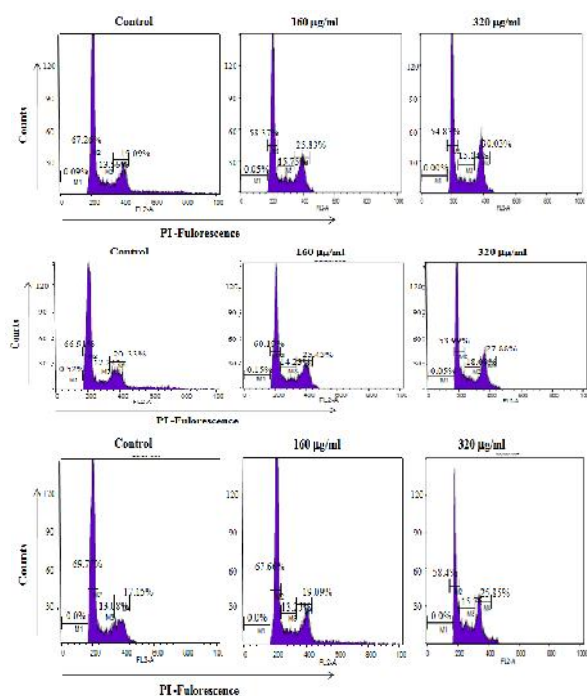
**Clonogenic survival assay:** Samples B, C and A showed dose dependent inhibition of growth of MCF-7, HCT116 and PC-3 colonies or cells respectively.

The Sample B inhibits 25 % and 45 % growth of MCF-7 cells at the concentration of 160 and 320µg/ml respectively, similarly sample C inhibits 30% and 50% growth of HCT116 cells at the concentration of 160 and 320µg/ml respectively where as sample A inhibits 20% and 35% growth of PC-3 cells at the concentration of 160 and 320µg/ml respectively.



**Figure 4** pictures showing the results of Clonogenic assay on the three different cell lines with three different extracts. Left: Growth inhibition on MCF-7 with sample B; Middle: Growth inhibition on HCT116 with sample A; Right: Growth inhibition on PC-3 with sample A; Sample A: earthworm paste of *Perionyx excavatus*; Sample B: earthworm paste of *Eudrilus eugeniae*; Sample C: earthworm paste of *Eisenia fetida*.

**Cell Cycle Analysis:** Treatment of Samples C, B and A on HCT116, MCF-7 and PC-3 cells respectively showed cell cycle arrest in a dose dependent manner. All these samples when treated with above cells at 320µg/ml concentration showed maximum cell cycle arrest as 30.03 %, 27.88 % and 25.85 % at G2/M phase respectively. This result suggested that treatment of the above three samples C, B and A on HCT116, MCF-7 and PC-3 cells showed maximum cell cycle arrest at G2/M phase and induces apoptosis.



**Figure 5** Cell Cycle studies using HCT116, MCF-7 and PC-3 cells for earthworm samples. Top: HCT116 with sample C; Middle: MCF-7 with sample B; Down: PC-3 with sample A. Sample A: earthworm paste of *Perionyx excavatus*; Sample B: earthworm paste of *Eudrilus eugeniae*; Sample C: earthworm paste of *Eisenia fetida*.

## CONCLUSION

The earthworm pastes of the common earthworms showed a potent anticancer activity. In the above MTT cytotoxicity assay earthworm pastes of all the earthworms used have given better results for the above mentioned cancer cell lines. And earthworm paste of *Perionyx excavatus* was found to be highly potent as an anticancer agent as it has shown anticancer activity in all the three cell lines (MCF-7, HCT116 and PC-3). It showed IC50 values to be 87.45µg/ml in MCF 7 breast carcinoma cell lines, 239.1µg/ml in PC3 prostate cancer cell lines and 104.8µg/ml in HCT116 human colorectal cancer cells. *Eudrilus eugeniae* and *Eisenia fetida* also showed good inhibitory effect for HCT 116 (Human colorectal cell lines). DNA fragmentation had occurred in PC-3, MCF-7 and HCT116 cells when incubated in the presence of earthworm pastes of *Perionyx excavatus*, *Eudrilus eugeniae* and *Eisenia fetida* respectively for 24 h. In clonogenic assays earthworm pastes of *Perionyx excavatus*, *Eudrilus eugeniae* and *Eisenia fetida* showed dose dependent inhibition of growth of PC-3, MCF-7, HCT116 colonies or cells respectively. The *Eudrilus eugeniae* inhibits 25 % and 45 % growth of MCF-7 cells at the concentration of 160 and 320µg/ml respectively, *Eisenia fetida* inhibits 30 % and 50 % growth of HCT116 cells at the concentration of 160 and 320µg/ml respectively and *Perionyx excavatus* inhibits 20 % and 35 % growth of PC-3 cells at the concentration of 160 and 320µg/ml respectively. Also cell cycle analysis of earthworm pastes of all the three earthworm species showed maximum cell cycle arrest at G2/M phase on all the three cancer cell lines and induces apoptosis.

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