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Evaluation of antitumor activities of different epigeic earthworms

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ABSTRACT

Earthworms have a long association with the medicinal property as the biomolecules/compounds produced by the earthworms are of pharmacological importance with high potential in the eradication of various diseases with very low cost. Researchers have proved that earthworms are immune to malignant diseases such as different kinds of cancers. Hence, the present study was undertaken to evaluate the antitumor activities of different epigeic earthworms, such as *Eudrilus eugeniae*, *Eisenia fetida*, and *Perionyx excavatus*. The cytotoxicity assay was tested through 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay on Michigan Cancer Foundation-7 (MCF-7) cells by exposing them at various concentrations (200, 400, 600, 800, and 1,000 µg/ml) of different epigeic earthworm powders and standard antitumor chemotherapy drug Cisplatin (15 µg/ml). The percent growth inhibition/percent viability of MCF-7 cells varies with different concentrations of earthworm powder. The IC_{50} value was more prominent with *E. fetida* (113.97 µg/ml), followed by *E. eugeniae* (825.67 µg/ml) and *P. excavatus* (1,617.31 µg/ml). Based on the above results, it can be concluded that the tissues of the earthworm, *E. fetida*, seems to be a very good anticancer agent against MCF-7 cells as compared to other two earthworm species. Therefore, such studies could be useful in the future for the development of novel therapeutic agents against different types of cancers, further molecular level experimental studies are required to ascertain the pathways and genes responsible for the anticancer effect, and thereby, we can exploit the beneficial aspects of various earthworm species in drug delivery research and also in pharmaceutical applications.

1. INTRODUCTION

The main problem encountered in combating cancer is the uncontrolled proliferation/division of cancer cells and metastasis. It is influenced by either the inherent properties of tumor cells, systemic nature, or local environmental factors [1]. About 90.50 million people had already different types of cancers, and about 14.10 million new cases occur every year, which leads to a death count of 8.8 million people [2]. The most common type of cancers in male is lung, prostate, colorectal, and stomach cancers, whereas in females are breast, colorectal, lung, and cervical cancers [3–5]. The financial costs to treat all these cancers were estimated at about $1.16 million USD/ year as of 2010 [6].

Over the past few decades, researchers have explored alternative therapies and remedies to prevent cancer progression but have reached very low success rates. Chemotherapy is the only option but plays like a double-edged sword. Apart from killing cancer cells, it also kills certain other adult cells that divide rapidly in the body, for example, the cells of gastro-intestinal linings, bone marrow, and hair follicle cells, thereby causing a significant influence on other parts of the body [7].

Natural products have been regarded as important resources that could produce potential chemotherapeutic agents. Since 1960, over 50% of anticancer drugs approved by the Food and Drug Administration, USA, were originated from natural resources [8]. Most of the animal resources have been used in medicine for the treatment of many diseases. Among invertebrates, earthworms have been wonderful organisms by virtue of their behavior as a source of food as it naturally contains a good amount of biological molecules such as proteins, minerals, and fatty acids [9–11]. Very few people are aware of earthworm association with medicine despite the availability of literature right from the 14th century. Burma and China claim that earthworms are resources of various bioactive compounds, which are found to be new potentials in the production of new life-saving drugs for cardiovascular and inflammation diseases including cancer [12,13]. The literature revealed that earthworms can synthesize a variety of immune protective biological molecules that exhibit different biological

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activities, such as anticoagulative, antimicrobial, fibrolytic, and antipyretic. They possess an innate immune system to carry out some functions associated with adaptive immunity [14,15] and are exploited for the treatment of a variety of diseases such as antimicrobial and anticancer activities [16].

The concept of using natural products produced out of earthworms such as coelomic fluid (CF) and other important proteins can inhibit the proliferation of cancer cells [17]. With the development of biochemical techniques, the research on the usages of earthworms in pharmaceuticals has been explored and witnessed that earthworms found to have antitumor effects [18]. The antiproliferative potentials of CF of different earthworm species have been evaluated by many workers [19–21], but little work was noticed with respect to antiproliferative/antitumor activities by using different epigeic earthworm species extracts. Therefore, the present study was undertaken to evaluate the antiproliferative activity/efficiency of different epigeic earthworms on MCF-7 cells through 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.

2. MATERIALS AND METHODS

2.1. Collection of Earthworms

Adult epigeic earthworms, such as *Eudrilus eugeniae* (EE), *Eisenia fetida* (EF), and *Perionyx excavates* (PE) were obtained from a stock culture maintained at the Department of Zoology, Karnatak University, Dharwad (Karnataka), India.

2.2. Preparation of Earthworm Powder

The adult earthworms of all three species were initially washed in tap water and fed with a wet blotting paper for 20–24 hours so as to clear up the gut contents. The gut cleaned worms were again washed in distilled water, then were kept in tightly closed petri-plates, and exposed to sunlight for 2–3 days to get brown colored earthworm powder containing its mucus, CF, and other oozed out materials of earthworms.

2.3. *In vitro* Cytotoxicity by MTT Assay

Several assays have been developed for measuring the cell viability and cytotoxicity tests. Cytotoxicity studies broadly involve the metabolic modification of cells including death due to toxic effects of the compounds. An *in vitro* cytotoxicity test eliminates the use of animals, and it is cost effective. Based on the literature review, the MTT assay is widely accepted as a reliable method to examine the proliferation of cells.

An *in vitro* cytotoxicity assay of different epigeic earthworm (*E. eugeniae, E. fetida,* and *P. excavatus*) powders was estimated by using a procedure described by Mosmann [22]. The MCF-7 cells (Michigan Cancer Foundation-Breast cancer cell lines) were grown in Dulbecco's Minimum Essential Medium (DMEM) at 37°C in 5% CO_2 incubator; the cells were trypsinized and aspirated into 15 ml centrifuge tube. The cell pellet was obtained by centrifugation at 300 × g; the cell count was adjusted with DMEM so that 200 µl of suspension contains about 10,000 cells.

To each well of 96-well microtiter plate, 200 µl of cell suspension was added, and the plate was incubated at 37°C in 5% CO_2 incubator for 24 hours. After 24 hours, the spent medium was aspirated. About 200 µl of different sample test concentrations (200, 400, 600, 800, and 1,000 µg/ml from stock) and test chemotherapy drug Cisplatin (15 µg/ml) were added to the respective wells in duplicates. The plates were incubated for 24 hours again, then the plates were removed, and the drug containing media were aspirated. About 200 µl of medium containing 10% MTT reagent was added to each well to get a final concentration of 0.5 mg/ml, and then the plate was incubated for 3 hours. The culture medium was removed completely without disturbing the formed crystals, and then 100 µl of solution, dimethyl sulfoxide (DMSO) was added. The plate was gently shaken in a gyratory shaker to solubilize the colored formazan product. After 30 minutes of incubation, the optical density (OD) was measured by using a microplate reader at a wavelength between 570 nm and 630 nm.

The percent growth inhibition or percent viability of different sample test concentrations and standard chemotherapy drug Cisplatin (15 µg/ml) was calculated. The concentration of test drug needed to inhibit the cell growth by 50% (IC_{50} value) was generated from the dose response curve for the cell line.

3. RESULTS

The cytotoxicity assay was tested on MCF-7 cells by exposing them at various test concentrations (200, 400, 600, 800, and 1,000 µg/ml) of different epigeic earthworm powders and standard antitumor chemotherapy drug Cisplatin (15 µg/ml).

The results of the *in vitro* cytotoxicity studies are shown in Table 1, Figures 1–3, and Plates 1–3. The percent viability and IC_{50} value of MCF-7 cells vary with different test concentrations of all three epigeic earthworm species (Plates 1–3). The IC_{50} value means a particular concentration of test sample required to reduce half of the cells from the total population. In case of *E. eugeniae* and *P. excavatus*, the percent viability decreases with increasing test concentrations, i.e., the percent viability is inversely proportional to the test concentrations. The IC_{50} value was more (1,617.39 µg/m) in *P. excavatus* and outside the test concentrations (1,000 µg/m) followed by *E. eugeniae* (825.67 µg/ml). In case of *E. fetida*, the percent viability is directly proportional to the test concentrations that means it increases with the increasing test concentrations having a minimum IC_{50} value of 113.97 µg/ml (Table 1). The tissue of *E. fetida* seems to be a very good anticancer agent against MCF-7 cells as compared with other two earthworm species, *E. eugeniae* and *P. excavatus*. The IC_{50} value of *E. fetida* was more (113.97 µg/ml) than that of a positive control standard chemotherapy drug Cisplatin (Table 1 and Figs. 1–3).

In this study, the earthworm powders of *E. eugeniae*, *E. fetida*, and *P. excavatus* showed a dose-dependent inhibition of growth or percent viability on MCF-7 cells (Table 1 and Figs. 1–3). The result of the current study demonstrates that the earthworm extracts (*E. fetida*) have potential bioactive compounds involved in strong antitumor activity. The cytotoxicity effect of these earthworm extracts may be related to the components present in the tissues of earthworms such as proteins and peptides. An increase or decrease in cell number resulting in a concomitant change in the formation of formazan indicates the degree of cytotoxicity (Plates 1–3).

Table 1: Results of MTT assay with respect to percent viability and IC_{50} values of untreated, standard drug Cisplatin (Control) and various test concentrations of different epigeic earthworms.

Sl. No.	Test samples and Earthworm species	Concentrations of test samples (µg/ml)	OD at 570–630 nm	Percent viability (%)	IC_{50} values
1	Untreated	0.0	1.12	100	
2	Standard drug Cisplatin (control)	15	0.05	4.59	
		200	1.14	102.41	
		400	0.83	74.46	
3	*E. eugeniae*	600	0.73	65.58	825.67 µg/ml
		800	0.53	47.94	
		1000	0.45	40.75	
		200	0.74	66.11	
		400	1.07	95.84	
4	*E. fetida*	600	1.29	115.66	113.97 µg/ml
		800	1.32	118.25	
		1000	1.49	133.08	
		200	1.33	119.33	
		400	1.16	103.61	
5	*P. excavatus*	600	1.03	92.05	1,617.39 µg/ml[a]
		800	0.98	87.81	
		1,000	0.91	81.25	>1,000 µg/ml

IC_{50} = Inhibition concentration to kill 50% of organisms; OD = Optical density;
[a]*IC_{50} values outside the test concentrations range.

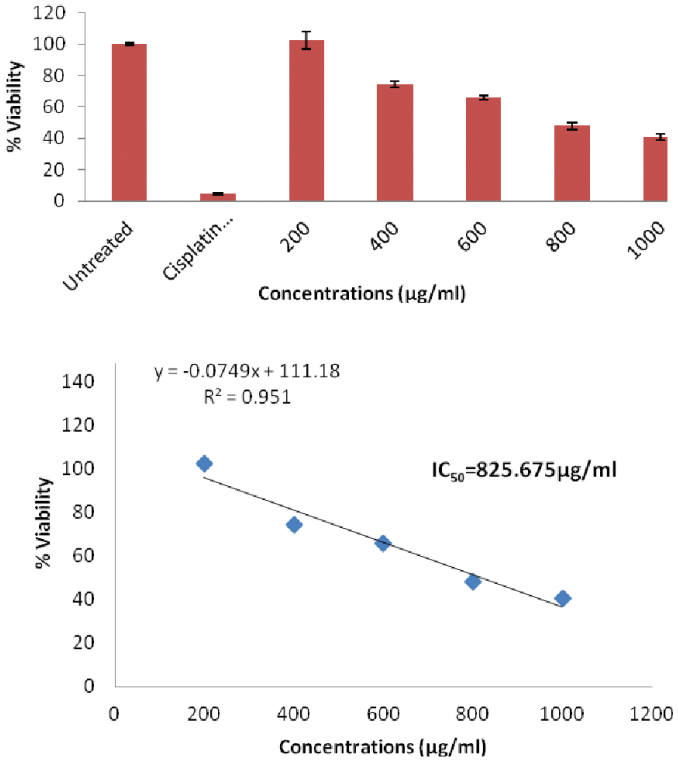

Figure 1: Percent viability and IC50 value of MCF-7 cells against the extracts of earthworm, *E. eugeniae.*

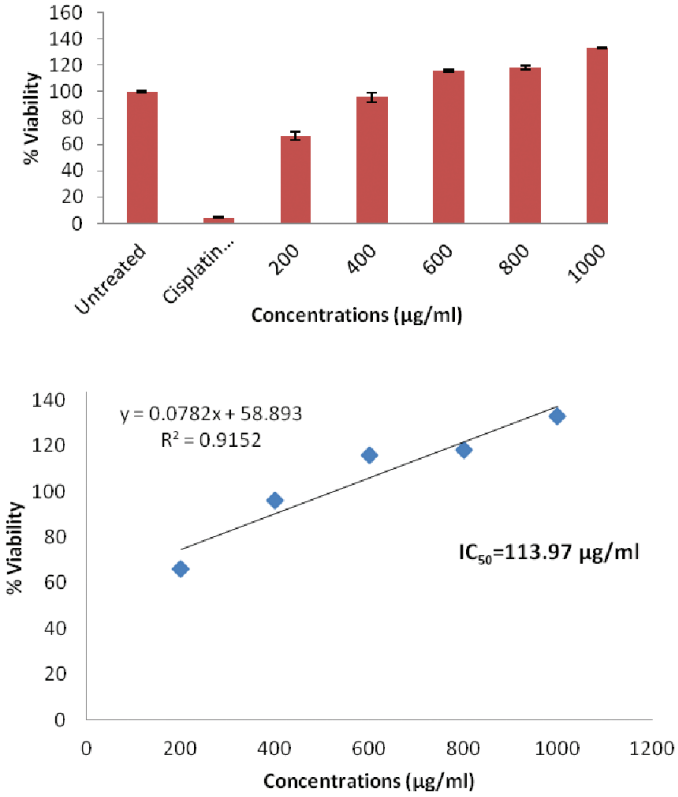

Figure 2: Percent viability and IC_{50} value of MCF-7 cells against the extracts of earthworm, *E. fetida*.

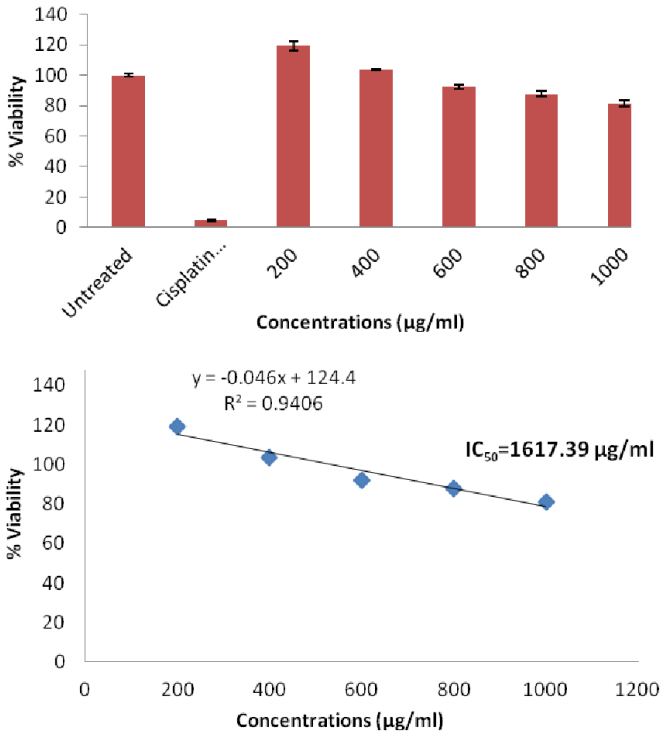

Figure 3: Percent viability and IC_{50} value of MCF-7 cells against the extracts of earthworm, *P. excavatus*.

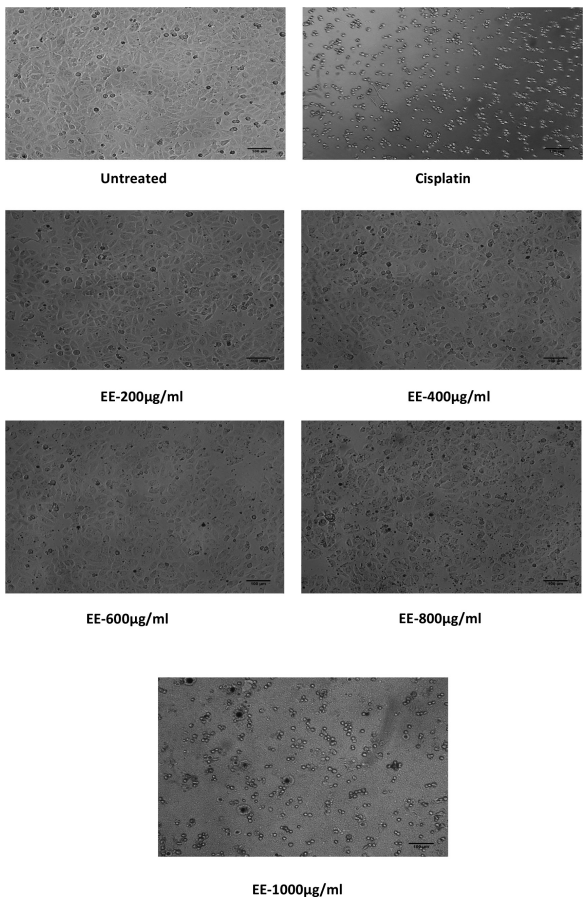

Plate 1: Images of antitumor activities (% viability of MCF-7 Cells) of untreated, standard drug Cisplatin (15 µg/ml) and various test concentrations (200, 400, 600, 800 and 1,000 µg/ml) of *E. eugeniae* (EE).

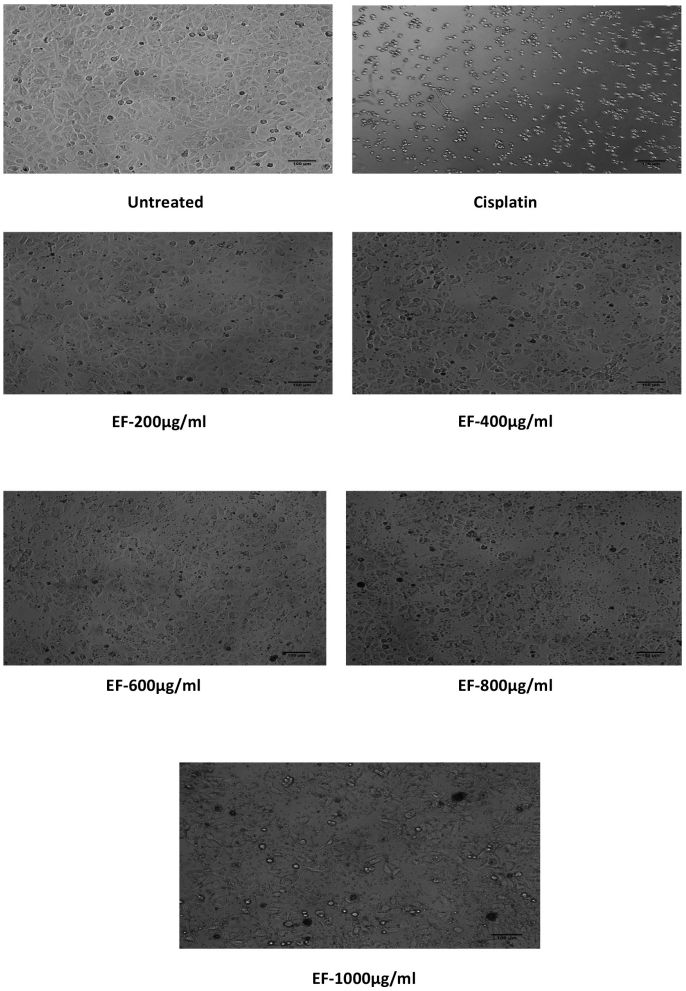

Plate 2: Images of antitumor activities (% viability of MCF-7 Cells) of untreated, standard drug Cisplatin (15 µg/ml) and various test concentrations (200, 400, 600, 800 and 1,000 µg/ml) of *E. fetida* (EF).

4. DISCUSSION

Many researchers found that antitumor activities were found in either CF or extracts of different species of earthworms, such as earthworm coelomic fluid (ECF) which was employed to demonstrate antiproliferation activity on different types of cancers. In addition to this, ECF exhibits other biological activities such as bacteriostatic, proteolytic, cytolytic, and mitogenic activities [16]. The whole earthworm, *Pheretima posthuma*, tissue has active ingredients involved in fibrolytic activity and considerable cytotoxic and antitumor activities [19]. Earthworm fibrinolytic enzyme (EFE) could also inhibit the proliferation of several cancer cell line *in vitro* conditions such as gastric cell line – —SCG 7901, esophagus cancer cell line – —Eca-109, cervical cancer cell line – —HeLa, leukemia cell line – —IC562. It suggests that antitumor spectrum of EFE was relatively wide and broad [23,24]. Antitumor activity of serine protease extracted from the Indian earthworm, *P. posthuma,* on MCF-7 cells was also determined by Verma et al. [19]. They observed the inhibition rate of 38.50% at a concentration of 276.04 and 263.14 µg/ml.

The cytotoxic and apoptic activities of the CF of *E. fetida* were evaluated *in vitro* by Yanqin et al. [25]. Mohamed Jaabir et al. [26] tested *in vitro* anticancer activity of CF of the earthworm,

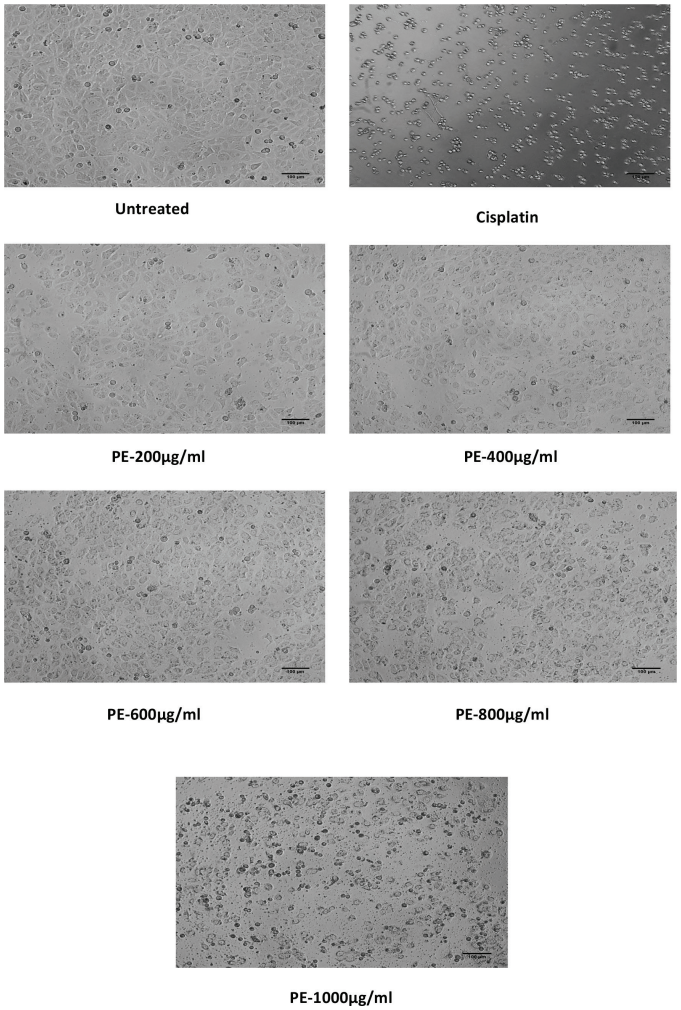

Plate 3: Images of antitumor activities (% viability of MCF-7 Cells) of untreated, standard drug Cisplatin (15 µg/ml) and various test concentrations (200, 400, 600, 800 and 1,000 µg/ml) of *P. excavatus* (PE).

E. eugeniae, on Sitta cells, and they witnessed 68% of cell death at a higher concentration of 80 µg/ml and 89% cell death at 100 µl/ml with an IC_{50} value of 50 µl/ml. Dinesh et al. [27] evaluated the *in vitro* cytotoxic effect of CF of the earthworm, *E. eugeniae* on HeLa cells, colon cancer cells, leukemic cells, and brain tumor cells and found dose-dependent inhibitory effect. Therefore, the current study demonstrates that whole *E. fetida* worm extract has active constituents involved in strong cytotoxic and antitumor activities, but the actual chemical nature and composition of the constituents responsible for these effects are to be explored at molecular levels.

5. CONCLUSION

Based on the results obtained in this study, it can be concluded that the antitumor spectrum of the earthworm, *E. fetida* (113.97 µg/ml), is comparatively more than that of *E. eugeniae* (825.67 µg/ml) and least in *P. excavatus* (1,617.39 µg/ml). The uniqueness of the present study is that here we have covered antiproliferative potentials of three different epigeic earthworm species together and determined successfully. The limitations of this study include the usage of a single cancer cell line (MCF-7 cells) to explore antiproliferative potentials. Therefore, such research on these aspects could be useful for the development of novel therapeutic agents against different types of cancers. Further, experimental studies at molecular levels are required to ascertain the pathways and genes responsible for the anticancer effects, and thereby, we can exploit beneficial aspects of many earthworm species in this respect.

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CONFLICT OF INTEREST

Authors declared that they do not have any conflict of interest.

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