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ORIGINAL ARTICLE

In vitro Antiproliferative Effect of Earthworm Coelomic Fluid of *Eudrilus Eugeniae*, *Eisenia foetida*, and *Perionyx Excavatus* on Squamous Cell Carcinoma-9 Cell Line: A Pilot Study

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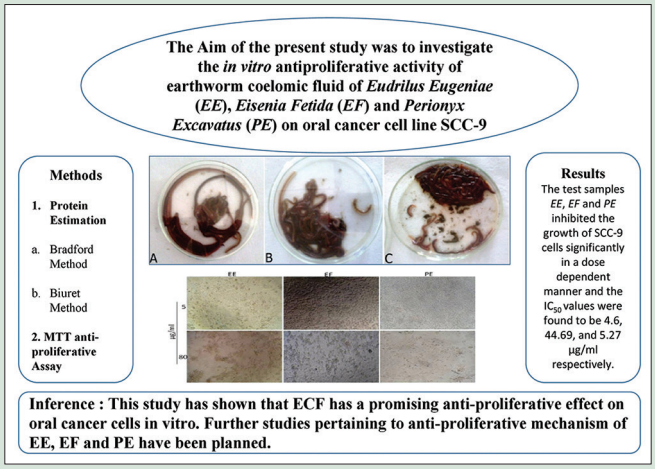
ABSTRACT

Introduction: The earthworm coelomic fluid (ECF) has shown proven antiproliferative effect against breast, liver, gastrointestinal, and brain cancer, but it is least explored in oral cancer. The present *in vitro* study is an attempt to investigate the antiproliferative activity of ECF on oral cancer cell line squamous cell carcinoma (SCC)-9. **Materials and Methods:** ECF was collected from the species *Eudrilus eugeniae* (EE), *Eisenia foetida* (EF), and *Perionyx excavatus* (PE) stored at –80°C. Percentage inhibition of ECF on squamous cell carcinoma-9 cells *in vitro* was recorded at 24 h. Protein estimation was done using Bradford protein assay validated by the biuret method. Cytotoxicity was tested at 2.5, 5, 10, 20, 40, and 80 µg/ml concentrations by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay in SCC-9 cells *in vitro*. GraphPad Prism 7.0 software was used to calculate the inhibitory concentration (IC_{50}). Chi-square test was used to analyze the difference between samples. **Results:** The test samples EE, EF, and PE inhibited the growth of SCC-9 cells significantly in a dose-dependent manner, and the IC_{50} values were found to be 4.6, 44.69, and 5.27 µg/ml, respectively. The antiproliferative effect was found to be variable among the three earthworm species with EE showing the most promising effect followed by PE and EF. **Conclusion:** Establishing the antiproliferative effect of ECF on oral cancer cells could be an initial step toward drug development and future anticancer research. The preliminary investigation has shown that ECF has a promising antiproliferative effect on oral cancer cells *in vitro*.

Key words: Antitumor, cell proliferation, coelomic fluid, mouth neoplasms, *oligochaeta*, squamous cell carcinoma-9 cell line

SUMMARY

- The present pilot study evaluated the *in vitro* antiproliferative effect of earthworm coelomic fluid (ECF) of *Eudrilus eugeniae* (EE), *Eisenia foetida* (EF), and *Perionyx excavatus* (PE) on squamous cell carcinoma-9 cell line. The ECF inhibitory activity was promising at inhibitory concentration values of 4.6, 44.69, and 5.27 µg/ml, respectively. Further studies pertaining to antiproliferative mechanism of EE, EF, and PE have been planned.



Abbreviations Used: ECF: Earthworm coelomic fluid, EE: *Eudrilus eugeniae*, EF: *Eisenia foetida*, PE: *Perionyx excavatus*, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SCC: Squamous cell carcinoma, BSA: Bovine serum albumin, PBS: Phosphored buffered saline, ATCC: American Type Culture Collection

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INTRODUCTION

The specific problem encountered in combating cancer is the uncontrolled proliferation of cancer cells and metastasis which is a multistep complex event during the growth of malignant tumors. It is influenced by inherent properties of tumor proper, systemic, and local environmental host factors.[1] Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Over 50% of anticancer drugs approved by the United States Food and Drug Administration since 1960 were originated from the natural resources.[2]

The earthworms are complex invertebrates which synthesize a variety of immunoprotective molecules and produce several types of leukocytes. They possess innate immunity, as well as some functions associated with the adaptive immunity (allogeneic tissue rejection).[3-5] These molecules exhibit different activities, such as fibrinolytic, anticoagulative, anticancer, antimicrobial, and thus may be exploited for the treatment of variety of diseases.

Recently, concepts of using naturally available exudates from earthworms to inhibit proliferation of cancer cells have emerged.[6] Few studies on

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the breast, liver, and brain tumors have been employed with limitations, but to the best of our knowledge yet to be explored in oral cancer, this necessitates the need for this study.

The previous studies that explored the antiproliferative potentials of earthworm coelomic fluid (ECF) have evaluated one species at a time; the current study has distinctively compared antiproliferative efficacy of three species of earthworms simultaneously under standard clinical settings on oral cancer cell line squamous cell carcinoma (SCC)-9. Earthworm species such as PE has also been explored in this study which has not been reported earlier.

The aim of the present study is to explore the antiproliferative effect of ECF of three identified species of *Eudrilus eugeniae* (EE), *Eisenia foetida* (EF), and *Perionyx excavatus* (PE) on oral cancer cell line SCC-9.

Since recurrence is one of the prime reasons for the failure of anticancer therapy, this research work is designed to identify biomolecules that have an antiproliferative efficacy on oral cancer cell line SCC-9. The results obtained would pave the way for subsequent exploration in this field of research.

MATERIALS AND METHODS

Collection of coelomic fluid

Ethical approval was obtained from the University ethics committee. Earthworms were procured from a local vermicomposting farm where grouping of species was done before use. A zoologist identified and authenticated the organisms used in this study with the reference features such as EE has reddish-brown color with a greenish tinge, yellowish underside with convex dorsal surface, and flattened ventral side. EF are smaller in size compared to EE and has rusty brown with dark and light alternating stripes of dark brown and light yellow. Distinct bands are present between segments with a rounded tail. PE has bluish anterior region and brownish posterior region with bands between segments and has slightly pointed tail.[7] Several methods of ECF collection exists, namely, mechanical agitation, alternate heat and cold method, warm water method, and electric method. In the present investigation, the cold-shock method was employed for coelomic fluid collection.[8-10]

Protein estimation by modified Bradford method

The concentration of protein was estimated using the modified Bradford assay. Bovine serum albumin (BSA) 4 mg/ml, dissolved in phosphored buffered saline (PBS), was used as standard. Briefly, 10 µL of each protein sample and BSA standards were mixed with 250 µl Bradford reagent (Sigma Aldrich). The absorbance at 595 nm of each sample mixture, that is, proportional to the quantity of solubilized protein was measured using a Tecan plate reader and the values were plotted.[11-13]

Protein estimation by Biuret method (method of validation)

Duplicates of 6 mg/ml of BSA were pipetted. Volume of distilled water was adjusted to 1 ml for the blank. About 2 ml of Biuret reagent mixed and incubated at 37°C for 20 min. Optical density at 550 nm was recorded using spectrophotometer.[14]

A calibration curve was constructed by plotting average optical density reading on "Y" axis against standard protein concentration (in mg) on "X" axis. Value "X" was recorded from the graph corresponding to the optical density reading for the test.

Antiproliferative 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay

The cell line employed in the present study was SCC-9 (Origin: *Homo sapiens* – Tongue tissue) procured from American Type Culture Collection (ATCC). The oral cancer cell lines (SCC-9 cells) were grown in minimal essential medium supplemented with 4.5 g/L glucose, 2 mmol/L L-glutamine, and 5% fetal bovine serum (growth medium) at 37°C in 5% CO_2 incubator.[15,16] SCC-9 were seeded in a 96-well plate at a concentration of 50,000 cells/well and incubated for 24 h at 37°C, 5% CO_2 incubator.

The cells were treated with different concentrations of test compounds (2.5, 5, 10, 20, 40, and 80 µg/mL) of coelomic fluid of the three test species, respectively, for 24 h. Colchicine was taken as positive control and saline as negative control.

After 24 h incubation with test samples, 100 µl/well of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent at concentration of 5 mg/10 ml in 1% PBS was added to the respective wells and incubated for 3–4 h. After incubation with MTT reagent, it was discarded by pipetting without disturbing the cells. About 100 µl of dimethyl sulfoxide was added to rapidly solubilize the formazan. The optical density (OD) was measured at 590 nm. The effective lethal concentration required for antiproliferative effect was determined by plotting a graph and obtaining a curve with maximum number of cells killed and concentration of the coelomic fluid used.[17,18] The percentage inhibition was calculated using the formula: (OD of control − OD of sample/OD of control) × 100. The inhibitory concentration or (IC_{50}) (drug concentration that is required to reduce half of the cells from the total population) was ascertained using GraphPad Prism 7 software (San Diego, California). Chi-square test was used to analyze the antiproliferative efficacy between samples.

RESULTS

Collection of coelomic fluid

Cold-shock method of fluid collection was found to be the safest method of coelomic fluid collection. This method of placing worms under the ice was least harmful as seen by the survivability of the worms after each time of collection as shown in Figure 1. About 3.5 ml of ECF was obtained from EE, 3 ml from EF, and PE, respectively. The fluid collected was centrifuged and stored at −80°C. The survivability of the worms was appreciable even after three rounds of fluid collection.

Protein estimation by modified Bradford method

The Modified Bradford protein assay for estimation of total protein concentration was preferred over the Lowry method as it is simpler, faster, and more sensitive. It is subjected to less interference by common reagents and nonprotein components of biological samples.[12] The total protein values obtained for earthworm species EE, EF, and PE were 2.37, 1.94, and 3.41 mg/ml, respectively, as shown in Figure 2 and Table 1.

Protein estimation by Biuret method (method of validation)

The results obtained by the Bradford protein assay method was validated using the biuret protein estimation method, the protein values obtained were similar for the three earthworm species as shown in [Table 2].

Antiproliferative 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay

ECF of EE, EF, and PE at concentrations of 2.5, 5, 10, 20, 40, and 80 µg/ml on SCC-9 cells showed significant dose-dependent inhibition of growth of SCC-9 cells at IC_{50} values of 4.6, 44.69, and 5.27 µg/ml, respectively, as shown in Table 3. GraphPad Prism 7 software was used to determine the IC_{50} values as shown in Figure 3. Positive control drug colchicine exhibited an IC_{50} value of 11.90 µg/ml as shown in Table 4 and Figure 4.

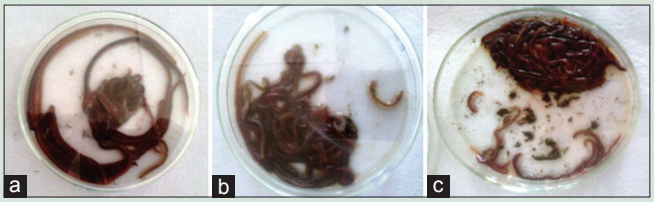

Figure 1: Earthworm species: (a) *Eudrilus eugeniae*, (b) *Eisenia foetida*, and (c) *Perionyx excavatus* during collection of coelomic fluid

Table 1: Protein concentration of samples (Bradford method)

Samples	OD at 595 nm	Concentration in mg/ml
EE-1	1.19	2.37
EF-2	1.05	1.94
PE-3	1.53	3.41

OD: Optical density; EE: *Eudrilus Eugeniae*; EF: *Eisenia fetida*; PE: *Perionyx excavatus*

Table 2: Protein concentration of samples (Biuret method)

Samples	OD at 595 nm	Concentration in mg/ml
EE-1	0.116	2.40
EF-2	0.106	1.90
PE-3	0.136	3.41

OD: Optical density; EE: *Eudrilus eugeniae*; EF: *Eisenia foetida*; PE: *Perionyx excavatus*

Table 3: Results of 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide assay-Coelomic fluid of the three species and the percentage inhibition obtained

Earthworm species	Concentration (µg/ml)	Absorbance 590 nm	Percentage inhibition	IC_{50}
Control	0.0	0.562	0.00	
EE-1	2.5	0.478	14.93	4.60 µg/ml
	5	0.401	28.63	
	10	0.254	54.80	
	20	0.199	64.58	
	40	0.157	72.06	
	80	0.109	80.60	
EF-2	2.5	0.556	1.00	44.69 µg/ml
	5	0.520	7.46	
	10	0.437	22.16	
	20	0.404	28.17	
	40	0.215	61.70	
	80	0.128	77.26	
PE-3	2.5	0.457	18.67	5.27 µg/ml
	5	0.399	28.99	
	10	0.299	46.79	
	20	0.256	54.44	
	40	0.201	64.23	
	80	0.188	66.54	

IC_{50}: Inhibitory concentration; EE: *Eudrilus eugeniae*; EF: *Eisenia foetida*; PE: *Perionyx excavatus*

Chi-square test showed difference in efficacy of antiproliferative effect between samples. EE and PE showed highly significant difference compared to EF. The difference in efficacy of antiproliferative effect between EE and PE was insignificant [Table 5].

DISCUSSION

Oral cancer is the sixth most common cancer in males and the twelfth most common in females. In developing countries, such as India, it is the most common cancer. Approximately, 94% of all oral malignancies

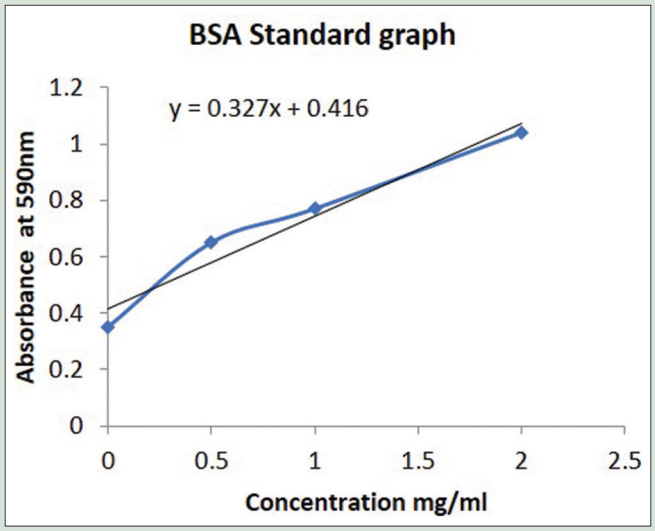

Figure 2: Standard graph obtained for protein estimation by Bradford method

are squamous cell carcinoma.[19] Over the past few decades, researchers have explored alternate therapies and remedies to prevent its progression but have succumbed to low success rates. Chemotherapy plays as a double-edged sword; apart from killing cancer cells it also kills certain adult cells that divide more rapidly, such as gastrointestinal lining, bone marrow cells, and hair follicles, thereby causing significant adverse effects. Targeted therapy of oral cancer is promising following identification of anticancer biomolecules.[20] Natural ways to prevent cancer recurrence is currently the latest trend in cancer therapeutics.

Naturally available extracts have been sought after in this regard as an adjunctive therapeutic modality.[21] Current research in the head and neck cancer mainly focuses to understand the molecular mechanisms of oral cancer development and progression to target the biomarkers and facilitate the development of new treatment strategies.[22] Studies with cell lines can serve as an initial screen for agents that might regulate drug resistance and to establish whether the differences exist in the different drug-resistant sublines.[23,24]

In the present study, we used tongue cancer cell line SCC-9 from ATCC to perform the cytotoxic study. Veeramani studied the characterization of coelomic fluid of EE and demonstrated that the cold shock method is a reliable technique for collection of ECF.[25] In this study, cold-shock method of fluid collection was employed to collect 3.5 ml from species EE, 3 ml from EF, and 3 ml from PE, respectively. In the cold-shock method, the earthworms secrete comparatively larger volume of fluid (1.5 ml) than other methods. The fluid collected is clear brown without any debris.

The Bradford protein assay employs the principle of Coomassie Blue G250 dye binding to protein.[11] The Biuret test which uses complexation of copper ions to functional groups in the protein's peptide bonds was employed to validate the protein analysis results obtained from the modified Bradford protein assay.[12]

This accurate protein estimation test has been employed in few studies like the one performed by Merzouk *et al.* to estimate the total proteins in Leech saliva extract.[26] Traditionally, *in vitro* determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye.

Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters, and others which rely on dyes and cellular activity.

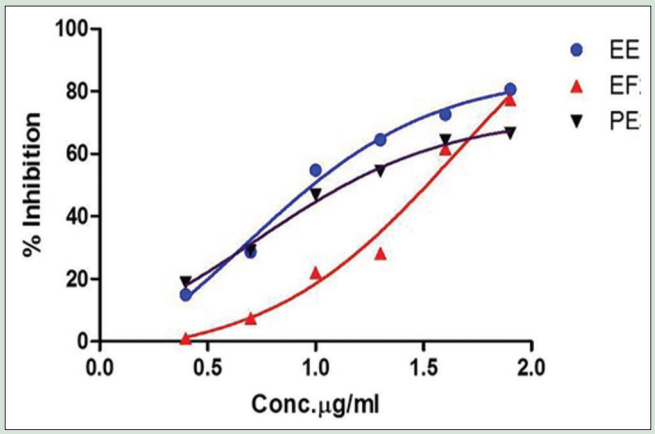

Figure 3: Inhibitory effect of coelomic fluids of three earthworm species on squamous cell carcinoma-9 cell proliferation

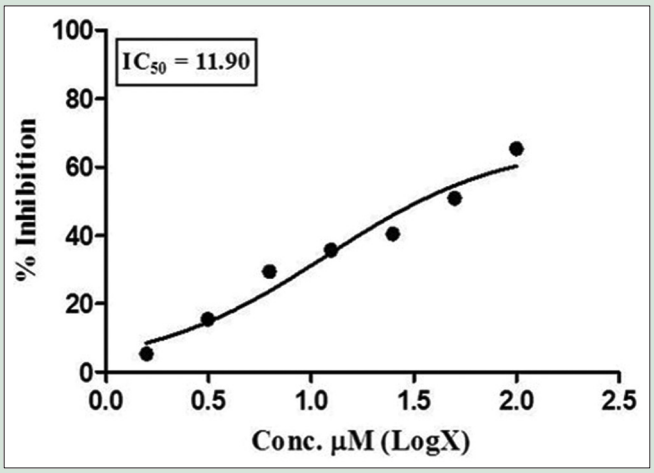

Figure 4: Effect of colchicine on squamous cell carcinoma-9 cells and IC_{50} value

Table 4: Percentage inhibition of colchicine (positive standard) on squamous cell carcinoma-9 cells

Compound name	Concentration µM	OD at 540 nm	Percentage inhibition	IC_{50}
Colchicine	Control	0.5926	0.00	11.9
	1.57	0.5609	5.35	
	3.125	0.5012	15.42	
	6.25	0.4181	29.45	
	12.5	0.3811	35.69	
	25	0.3531	40.42	
	50	0.2911	50.88	
	100	0.2051	65.39	

OD: Optical density; IC_{50}: Inhibitory concentration

Table 5: Results of Chi-square analysis

Test sample	χ^2	*P*<0.05 - significant	Inference
EE versus EF	28.031	0.00003589	Significant
EF versus PE	33.526	0.00000296	Significant
EE versus PE	1.543	0.90805492	Not significant

EE: *Eudrilus eugeniae*; EF: *Eisenia foetida*; PE: *Perionyx excavatus*

The MTT assay is a means of measuring the activity of living cells through mitochondrial dehydrogenases.[27,28] The resulting purple solution is spectrophotometrically measured.[29,30] An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test.

In the present study, ECF of EE, EF, and PE was used at concentrations of 2.5, 5, 10, 20, 40, and 80 µg/ml on SCC-9 cells *in vitro* and evaluated for antiproliferative efficacy by MTT assay. Colchicine, a standard anticancer drug was used as a positive control.

The test samples EE, EF, and PE treatment showed significant dose-dependent inhibition of growth of SCC9 cells at IC_{50} values of 4.6, 44.69, and 5.27 µg/ml, respectively. Positive control drug colchicine exhibited an IC_{50} value of 11.90 µg/ml. These results suggest that all three test samples have antiproliferative effect against SCC-9 cells [Figure 5].

In the earlier studies, ECF was employed to demonstrate antiproliferative activity on other types of cancers. XIE Jiang Bi *et al.* in 2003 studied the *in vitro* antitumor activity of the earthworm EF on HCT 116, SY5Y, K562, MGc803, and HeLa cell lines and 50% of growth inhibition was observed at 60-110 mg/L of dose. The authors also reported on *in vivo* results of the prolonged lifespan of ascites tumor (S180) bearing mice.[31] HE Dao-wei in 2005 performed an *in vitro* study to evaluate the inhibitory effects of earthworm extract on the cellular growth of Eca-109. The results demonstrated a dosage of (900,450 mg/L) had prominent inhibitive effects on Eca-109 cells.[32]

The antitumor activity of EFE (earthworm fibrinolytic enzyme), isolated from EF, on human hepatoma cells *in vitro* and *in vivo* was evaluated by Chen *et al.* in 2007. A dose-dependent *in vitro* inhibition was observed. The growth of tumor in nude mice was significantly suppressed in EFE group compared to the control group.[33] The cytotoxic and apoptotic activity of the EF coelomic fluid was evaluated *in vitro* by Yanqin *et al.* in 2007. A concentration of 1 mg/ml exhibited inhibitory effects on HeLa cells with an inhibition rate of 84.22%.[34]

Mohamed Jaabir *et al.* in 2011 tested anticancer activity of the coelomic fluid of the earthworm EE in SiHa cells *in vitro*. At higher concentrations of 80 µl/ml, the cell death observed was 68% and at 100 µl/ml, the cell death was 89%. The IC_{50} concentration was determined to be 50 µl/ml.[35] Dinesh *et al.* in 2013 evaluated the cytotoxic effect of coelomic fluid from EE on HeLa cells, colon cancer cells, leukemic cells, and brain tumor cells *in vitro* and found a dose-dependent inhibitory effect.[36]

Antitumor activity of serine protease from the Indian earthworm *Pheretima posthuma* on MCF-7 cells was determined by Verma *et al.* 2013. An inhibition of 38.5% at concentration of 276.04 µg/ml and 263.14 µg/ml was observed.[37] *In vitro* anticancer activity of the earthworm powder (EWP) obtained from *Lampito mauritii* in HT-29 cells was evaluated by Lourdumary and Ramesh in 2014. At low dilution rates (10 µg/ml), the viability was unaffected; however, at higher concentration (320 µg/ml) 82% growth inhibition or cytotoxicity was observed.[38]

Only meager studies on the effect of ECF of species EE, EF, and PE on cancer cell lines have been performed in India, and to the best of our knowledge, globally, none have collectively evaluated ECF of three species. There is limited information available on the effect of ECF on oral cancer cells which is scarcely researched area in oncology and appears to have not been attempted.

Anticancer properties of earthworms species such as PE which was yet to be explored on any type of cancer has shown to have promising antiproliferative effect on oral cancer cells SSC-9 (IC_{50}-5.27 µg/ml) along with earthworm species EE and EF that showed an IC_{50} values of 4.6 µg/ml and 44.69 µg/ml respectively.

Testing in cancer cell lines has remained the initial step for drug testing for many years. It is thereby considered the first step in assessing several complex therapeutic preparations before its use in large scale *in vivo*.

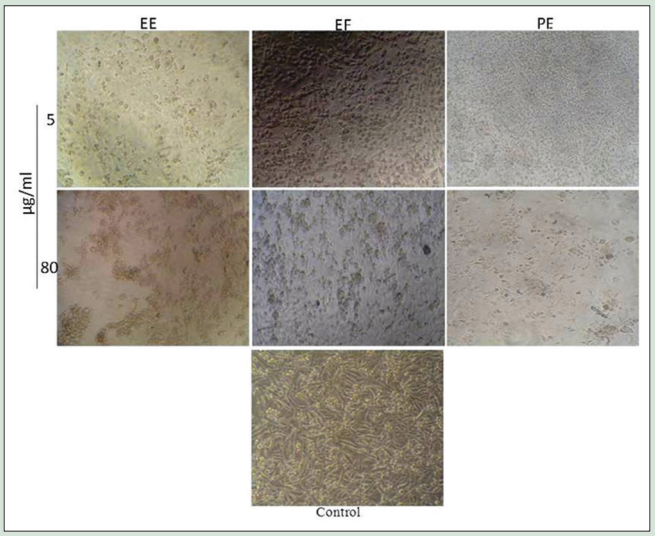

Figure 5: Antiproliferative effect of earthworm coelomic fluid of three species on squamous cell carcinoma-9 cell lines

Cytotoxicity evaluation in cancer cell lines has been advantageous and is expected to provide results which extrapolate with the original tumor.[39]

There has been an increasing interest to research natural products available in nature, which can combat cancer and its side effects, and prevent them from occurring and increase the lifespan and quality of life of patients. The present study has shown ECF of EE, EF, and PE has an appreciable antiproliferative effect on oral cancer cell line SCC-9, with *EE* showing the best effect followed by PE and EF. The antiproliferative effect was variable among the three species.

Targeted therapies developed from cell lines *in vitro* may be translated *in vivo* directed against the primary tumor at the cellular level of tumor development, and thus, this therapy may find its way in the treatment of early-stage head and neck cancer.[24,40] There is certainly scope to translate these findings in clinical settings.

CONCLUSION

The results obtained in this study revealed that ECF of EE, EF, and PE has antiproliferative potential on SCC-9 cells with following IC_{50} values 4.6, 5.27 and 44.69 µg/ml for EE, PE, and EF, respectively, and could be useful for the development of novel therapeutic agent against oral cancer with negligible side effects. The limitations of this study include use of a single cancer cell line to explore the antiproliferative potential. However, the uniqueness of the present study is that the antiproliferative potential of 3 earthworm species has been compared together. EE and EF have known antiproliferative effect on other cancer cell lines, we have demonstrated on oral cancer cell lines. The antiproliferative potential of earthworm species PE has not been explored thus far.

It can be concluded that the ECF of EE and PE are more efficacious than EF comparatively based on the IC_{50} determined. The scope and future avenues are to ascertain the specific bioactive molecules responsible for this antiproliferative activity, perform higher anticancer experiments, and determine ECF mechanism of action on cancer cells. These experiments are ongoing in our laboratory. These bioactive molecules need to be screened against different cell lines apart from the selected cell line used to ensure the wide range of their antiproliferative action. Antiproliferative effect of ECF of different species of earthworm obtained from this study is promising and necessitates performance of advanced anticancer studies on oral cancer cell lines.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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