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ORIGINAL ARTICLE

Anti-Proliferative Activity of Earthworm Coelomic Fluid Using Oral Squamous Carcinoma KB 3-1 cells: An *In vitro* Study with Serine Protease Analysis

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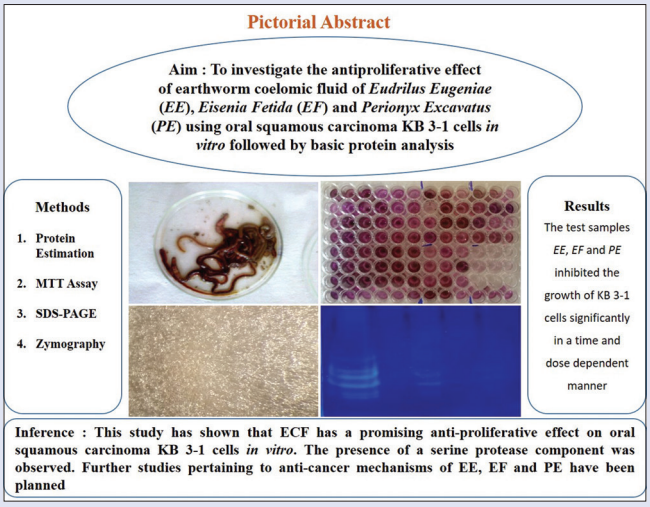
ABSTRACT

Background: Naturally available extracts have gained importance as adjunctive pharmacologic modalities. The earthworm coelomic fluid (ECF) has shown promising *in vitro* cytotoxic effect in hepatocellular, colorectal, and breast cancers. Few *in vitro* studies have been performed pertaining to oral cancer. **Objective:** The objective of this study was to evaluate the time- and dose-dependent antiproliferative effect of ECF of *Eudrilus eugeniae* (EE), *Eisenia fetida* (EF), and *Perionyx excavatus* (PE) on oral cancer cell line KB 3–1. The chemical nature of the ECF was analyzed as well. **Materials and Methods:** The ECF was collected employing the cold shock method. Lowry's protein estimation was used for determining the total protein content. ECF of EE, EF, and PE at concentration of 6.25, 12.5, 25, and 50 µg/mL was tested on KB 3–1 cells *in vitro*. The 3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide antiproliferative assay was used to determine the time- and dose-dependent antiproliferative effect. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate and observe prominent protein bands in the three extracted fluids. The chemical nature of the band was analyzed through an enzyme zymography. **Results:** ECF of PE showed a maximum inhibitory percentage of 94.29% at 50 µg/mL, followed by ECF of EE and EF with values of 89.23% and 79.44%, respectively. ECF of EE showed a superior IC_{50} value of 3.70 µg/mL as compared to EF and PE at 48 h. **Conclusion:** ECF of EE has a promising antiproliferative effect in a time- and dose-dependent manner on oral squamous carcinoma KB 3–1 cells *in vitro*. The serine protease presence in ECF of EE was demonstrated.

Key words: Antiproliferative activity, earthworm coelomic fluid, KB 3–1 cell line, serine protease, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, zymography

SUMMARY

- The present study evaluated the *in vitro* time- and dose-dependent antiproliferative effect of earthworm coelomic fluid (ECF) of *Eudrilus eugeniae* (EE), *Eisenia fetida* (EF), and *Perionyx excavatus* (PE) on oral KB 3–1 cell line. ECF of PE showed a maximum inhibitory percentage of 94.29% at 50 µg/mL, followed by ECF of EE and EF with values of 89.23% and 79.44%, respectively. ECF of EE showed a superior IC_{50} value of 3.70 µg/mL as compared to EF and PE at 48 h. ECF of EE showed a serine protease-like activity.



Abbreviations used: EFE: Earthworm fibrinolytic enzyme; BSA: Bovine serum albumin; DMSO: Dimethyl sulphoxide; ECF: Earthworm coelomic fluid; EE: *Eudrilus eugeniae*; EF: *Eisenia fetida*; PE: *Perionyx excavatus*; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; IC: Inhibitory concentration; MEM: Minimal essential medium; MTT: 3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide; NCCS: National Centre for Cell Science; NCI: National Cancer Institute; OD: Optical density; PBS: Phosphate-buffered saline; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEMED: Tetramethylethylenediamine.

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INTRODUCTION

Oral cancer is one among the most common types of cancers in India. The National Institute of Health and Family Welfare, 2012, states that 86% of the global oral cancer burden is documented in India.[1] Considering the current scenario of oral cancer management, the morbidity and mortality rates still continue to remain high in spite of early diagnosis and management. The statistics is worrisome as the disease-free survival percentage without metastasis is 76% at 5 years; with metastasis to cervical nodes, it drops to 41%; and only 9% when metastasis has occurred below

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the clavicle.[2] These facts challenge the available methods of oral cancer management encouraging the search for new and better modalities such as naturally available extracts (NAEs) that can comprehensively alter tumor progression. Unlike chemotherapy which is a double-edged sword, NAEs are seldom known to exert adverse reactions.

There is little information available on the effect of these extracts on oral cancer cells which are a scarcely researched area in oncology. A recently emerging trend is the use of NAEs from earthworms to inhibit the proliferation of cancer cells.[3] Although it has been explored in breast, liver, and brain tumors with limitations, it is yet to be explored in oral cancer; this necessitates the need for the present study. The use of adjunctive therapies such as NAEs other than those routinely employed for oral cancer management has been inadequately researched. Literature review shows a dearth of cytotoxic and antiproliferative effect studies describing the action of naturally available biomolecules on oral cancer cell lines.[4]

Recently, we have demonstrated the dose-dependent antiproliferative effect of earthworm coelomic fluid (ECF) on oral cancer cell line squamous cell carcinoma (SCC)-9 with noteworthy results.[5] The aim of the present study is to evaluate the time- and dose-dependent antiproliferative effect of ECF of *Eudrilus eugeniae* (EE), *Eisenia fetida* (EF), and *Perionyx excavatus* (PE) on oral cancer cell line KB 3–1. The present study also makes an attempt to investigate the nature of the ECF through basic protein analysis.

MATERIALS AND METHODS

Ethical approval was obtained from the Ethics Committee (No: FDS/EC/2014-16/PhD_03) of M.S. Ramaiah University of Applied Sciences.

Isolation of earthworms

Mature earthworms weighing 400 g (aged 1–1.5 years) were obtained from a local vermicomposting unit located in Bengaluru. The three species were segregated based on their morphological characteristics. EE is reddish brown in color with a greenish tinge, yellowish underside with convex dorsal surface, and flattened ventral side. EF is brownish rusty in color with alternating brownish dark and yellow bands; they are comparatively smaller in size compared to EE. A rounded tail is observed and the segments display prominent bands. PE is smaller than EF and has bluish anterior region and brownish posterior region with bands between segments and has slightly pointed tail.[6]

Coelomic fluid collection – Cold shock method

The earthworms were cleaned and dried on a filter paper. After containing the three species in separate Petri dishes, the cold shock method of fluid collection was employed where the Petri dish was placed over an ice bath for 15 min followed by 5 min of relaxation. The cold shock method was preferred as it is less injurious to the earthworms, simple to perform, and less debris is obtained. This was continued several times to collect the ECF from the three species.

Protein estimation – Lowry's method

The Lowry's protein estimation procedure uses the principle of the Biuret reaction to note the reaction of peptide bonds present in proteins with copper under alkaline conditions. This, in turn, produces copper ions which interact with the Folin reagent. The oxidized aromatic amino acids convert the phosphomolybdotungstic into heteropolymolybdenum blue resulting in a strong blue color. The tyrosine and tryptophan contents play a key role in the development of the blue color. A concentration level of about 0.01 mg of protein/ml can be detected.[7] The components used for Lowry's method are summarized in Table 1.

Table 1: Reagents for Lowry's protein estimation

Components	Concentration
Protein standard BSA- Lowry	1 mg/ml
Folin-Ciocalteu (FC reagent)	1X
Solution A: 10 N NaOH with 2% of Na_2CO_3	-
Solution B: 1% copper sulfate solution	-
Solution C: 2% sodium potassium tartarate	-
1× complex-forming reagent	10:0.1:0.1 of Solution A: Solution B: Solution C

Cell line used and its maintenance

The human mouth cancer cell line KB 3–1 was grown in minimal essential medium (Sigma-Aldrich, USA); it was maintained with 4.5 g/L glucose, 2 mmol/L L-glutamine, 5% fetal bovine serum (Sigma-Aldrich, USA), and 1% penicillin at 37°C in 5% CO_2 incubator. During subculture, cells were detached by trypsinization when they reached 80% confluency (proportion of adherent cells that cover the surface in a flask or Petri dish).

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

3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a routinely used cell viability test to determine the antiproliferative effect of test compounds *in vitro*. The 96 wells in the tissue culture plate received the trypsinized cells earlier cultured at 37°C in 5% CO_2, at a density of 5×10^3 cells/well. Following a 48-h incubation, the cells were treated with growth media, and ECF of EE, EF, and PE was added in doses of 6.25, 12.5, 25, and 50 µg/mL to achieve a final volume of 100 µL and then cultured. The dose concentration was obtained after laboratory standardization. After 24 h, 5 µL of freshly prepared MTT reagent (0.5 mg/mL in phosphate-buffered saline) was added to each well and incubated at 37°C for 2 h. The growth media was removed and 100 µL of dimethyl sulfoxide was added to solubilize the colored formazan product. The absorbance of the culture plate was determined on an enzyme-linked immunosorbent assay reader at a wavelength of 572 nm. The same was repeated after 48 h to record the optical density (OD) reading. The percentage inhibition caused by ECF of each species on the cell line was calculated using the following formula: (OD of control – OD of sample/OD of control) × 100. The inhibitory concentration (IC_{50}) (drug concentration that is required to reduce half of the cells from the total population) was ascertained using IC_{50} tool kit.

Control drug

Paclitaxel (standard anticancer drug) was taken as the positive control at concentrations of 6.25 and 12.5 µg/mL.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

It is a routinely used technique to separate proteins based on their molecular weight. The separated proteins appear in the form of bands. Using a marker, we can estimate the size of the protein in kDa.

Preparation of 12% resolving gel

Dried glass plates were assembled, and sealing was done using molten agarose. The gel mix (Aristogene Biosciences, India) was prepared and poured between the plates till the level reached 3/4th of the capacity. Immediately, it was overlaid with water to an additional height of 0.5 cm. Gel was allowed to polymerize for 30 min. After polymerization, the excess water was drained completely.[8]

Preparation of 5% stacking gel

Stacking gel mix was prepared and poured onto polymerized resolving gel, and a clean dry comb was carefully inserted without trapping air bubbles [Table 2]. The gel was left for additional 30 min for complete polymerization.

Sample preparation and electrophoresis

Samples were mixed with sample loading buffer and heated at 95°C for 5–10 min. The samples were spun in a microfuge (Aristogene Biosciences, India) for 5 min. The wells were immediately washed with distilled water to remove nonpolymerized acrylamide. Bottom spacer was removed. Care was taken not to introduce any air bubbles between the bottom of the gel and the buffer while filling the electrophoresis apparatus with tris-glycine-sodium dodecyl sulfate (SDS) buffer. The samples were loaded into the bottom of the wells using microliter syringe or micropipette (Eppendorf, India) fitted with long tip [Table 3]. Electrophoresis (Bio-Rad, India) was initiated at 50 V for the first 30 min and then increased to 100 V. The power was turned off when the dye front neared 0.5 cm above the gel bottom. The gel was washed with distilled water for 30 s and transferred to Coomassie blue staining solution and incubated overnight.

Table 2: Components for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolving gel and stacking gel)

Components used
1.5 M Tris pH 8.8
1.0 M Tris pH 6.8
30% acrylamide-bisacrylamide
Ammonium per sulfate
Sample loading buffer
TEMED
Protein standard marker
Gel stainer
Tris-glycine-SDS buffer
Coomassie blue staining solution

SDS: Sodium dodecyl sulfate; TEMED: Tetramethylethylenediamine

Table 3: Loading pattern for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Lane#	Sample	Sample volume (µl)	SLB volume
1	6 Band Marker	10	10 µl SLB for SDS-PAGE
2	Gap	-	-
3	Test sample-1	08	10 µl SLB for SDS-PAGE
4	Gap	-	-
5	Test sample-2	13	10 µl SLB for SDS-PAGE
6	Gap	-	-
7	Test sample-3	05	10 µl SLB for SDS-PAGE
8	Gap	-	-
9	Gap	-	-

SDS PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLB: Sample loading buffer

Screening zymography

Zymography is a procedure performed to visualize the proteolytic activity produced on a Coomassie blue-stained background. A fresh sample (9 mL) of ECF of EE was collected to perform zymography. The protein content of the supernatant was determined before the sample was centrifuged for 10 min at 10,000 rpm.

The supernatant was diluted 1:20 times and the absorbance was checked at 280 nm. The protein concentration was estimated using the following formula: protein concentration = ([1.55 × A280 nm] × dilution factor).

Assembly of plates and sample preparation

The glass plates were grease freed and assembled using spacers and then the sides of the glass plates were sealed with molten agarose. The resolving and stacking gels were prepared. 1.25, 0.125, and 0.0125 µg of earthworm samples were loaded for zymography. The extracted earthworm sample was mixed with 10 µL zymography sample loading buffer. The stacking gel wells were rinsed with distilled water to ensure the removal of nonpolymerized acrylamide. The samples were loaded into the bottom of the wells using microliter syringe or micropipette fitted with long tip [Table 4]. Electrophoresis was initiated at 50 V for the first 30 min and then increased to 100 V.

Zymography protocol

The gel was rinsed twice with distilled water for 2 min each. The gel was transferred to 50 mL of renaturation solution (Aristogene Biosciences, India) and incubated on rocker for 30 min at room temperature [Table 5]. It was then transferred to 50 mL of ×1 developer solution and incubated at 42°C for 2 h. The final transfer was done to a Petri plate containing 20 mL of ×1 gel stainer and incubated on rocker for 60 min at room temperature. The stainer was removed and 20 mL of ×1 destainer was added. The destainer was replaced until a clear background was obtained. The gel was viewed against a bright background.[9]

RESULTS

Collection of earthworm coelomic fluid

A volume of 3 mL of ECF was collected from EE, EF, and PE species using the cold shock method. Majority of the earthworms were viable after the procedure. The ECF was centrifuged at 20,000 rpm for 15 minutes. The supernatant was collected and stored at −20°C for further use.

Protein estimation – Lowry's method

The total protein concentrates of ECF of EE, EF, and PE were found to be 12.7, 8.16, and 23.9 mg/mL, respectively [Figure 1a]. The fluids were subjected to dosing before the MTT assay so that equal protein concentrations of the fluid are obtained for EE, EF, and PE. The following concentrations were standardized: 6.25, 12.5, 25, and 50 µg/mL.

Table 4: Zymography loading pattern

Lane#	Sample	Sample volume (µl)	SLB volume
1	Protein molecular weight marker	30 µl	10 µl SLB for SDS-PAGE
2	Gap	-	-
3	1.25 µg extract	1 µl from 1:100 dilution	10 µl zymo SLB for SDS-PAGE
4	Gap	-	-
5	0.125 µg extract	10 µl from 1:10,000 dilution	10 µl zymo SLB for SDS-PAGE
6	Gap	-	-
7	0.0125 µg extract	1 µl from 1:10,000 dilution	10 µl zymo SLB for SDS-PAGE

SDS PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLB: Sample loading buffer

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

Percentage inhibition after 24 h

Following 24 h incubation of KB 3–1 cell line with ECF of EE, EF, and PE at increasing concentration of 6.25, 12.5, 25, and 50 µg/mL, a satisfactory percentage inhibition was observed [Figure 1b]. ECF of PE showed a maximum inhibitory percentage of 94.22% at 50 µg/mL, followed by ECF of EE and EF with values of 80.32% and 74.78%, respectively. ECF of EE showed a superior IC_{50} value of 5.32 µg/mL as compared to EF and PE, which showed IC_{50} values of 8.88 µg/mL and 11.15 µg/mL, respectively [Figure 2].

Percentage inhibition after 48 h

Following 48 h incubation of KB 3–1 cell line with ECF of EE, EF, and PE at increasing concentration of 6.25, 12.5, 25, and 50 µg/mL, a satisfactory percentage inhibition was observed [Figure 1b]. ECF of PE showed a maximum inhibitory percentage of 94.29% at 50 µg/mL, followed by ECF of EE and EF with values of 89.23% and 79.44%, respectively. ECF of EE showed a superior IC_{50} value of 3.70 µg/mL as compared to EF and PE, which showed IC_{50} values of 9.34 µg/mL and 11.51 µg/mL, respectively [Figure 2].

Control drug

Paclitaxel showed a high inhibition percentage of 82.43% and 95.34% at concentrations of 6.25 and 12.5 µg/mL following 24 h incubation, respectively. After 48 h, the inhibition percentage was higher at 96.58% and 97.26% for the same concentrations [Figure 1b]. Photomicrographs of KB 3–1 cells after treatment with ECF showed a reduction in the number of cells with clear spaces after 24 h incubation, compared to the monolayer of cells before treatment. Significant reduction in the number of cells with clear spaces was evident after 48 h incubation [Figure 3a-c].

Table 5: Components for zymography gel development

Components used	Preparation
Renaturation solution, 50 ml	1 ml of TritonX-100 in 49 ml of distilled water
Developer solution, 50 ml	10 mg of naphthyl acetate in 1 ml of acetone, dissolved in 50 ml of sodium phosphate buffer pH 7.2 containing 20 mg of Fast Blue RR

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Multiple hairline bands were observed in samples of EE, EF, and PE. However, a characteristic thick band was appreciated in the 18–20 kDa region for sample 1 (EE) [Figure 4a].

Screening zymography

The total protein concentration in the fresh sample of ECF of EE was calculated using the following formula: protein concentration = (1.55 × A280 nm) × dilution factor = (1.55 × 3.784) × 20 = 117.3 mg/mL.

The gel was completely stained due to the presence of gelatin, except the band region of serine protease enzyme that had digested gelatin and produced a negative staining. While comparing the bands with standard molecular weight marker, the protein had an average molecular weight in the range of 18–20 kDa [Figure 4b].

DISCUSSION

Apart from their use to improve soil fertility, the benefits of earthworms in the medical field have been greatly studied over the past decades. The pharmacological significance of the molecules in the intestinal and tissue fluid of the earthworms is due to its habitat encountering various microorganisms such as bacteria, fungi, and viruses. Molecules from earthworm extracts with cytotoxic effect have also been demonstrated *in vitro* and *in vivo* studies.[10]

Over the past few years, research on earthworm extracts has emerged. Liu *et al.*, 2017, demonstrated the anticancer potential of earthworm fibrinolytic enzyme (EFE) of EF on breast cancer cell line MCF-7. Significant apoptosis of cells was observed resulting in the suppression of MCF-7 cells.[11] Coelomocyte cell culture of EE was used by Vidya *et al.*, 2016, on A549 and HCT 116 cell lines *in vitro* to evaluate the antitumor property. Significant results were obtained using the MTT assay and clonogenic assays.[12] Reddy *et al.*, 2015, used the earthworm pastes of PE, EE, and EF on MCF-7, PC3, and HCT 116 cell lines, respectively.[13] The earthworm pastes of the three individual species showed potent anticancer activity. Maximum efficacy was observed in the paste of PE. Chen *et al.*, 2007, stated that the EFE of EF has evoked a great interest due to its antitumor activity against the human hepatoma cells *in vitro* and *in vivo*. The results indicated that EFE could possibly be used in the treatment of hepatoma.[14]

Verma *et al.*, 2013, used a serine protease isolate of *Pheretima posthuma* on MCF-7 cell line and suggested that the 15 kDa fraction has potent cytotoxic activity. The extract was prepared by earthworm autolysis followed by repeated washes in distilled water.

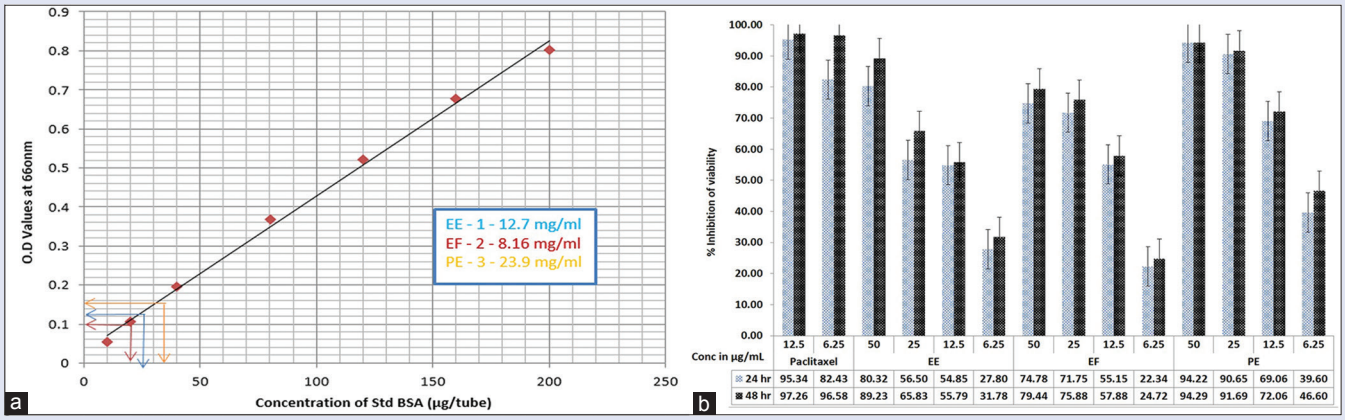

Figure 1: (a) Graph for protein estimation of earthworm coelomic fluid of *Eudrilus eugeniae*, *Eisenia fetida,* and *Perionyx excavatus* by Lowry's method. (b) Bar graph depicting percentage inhibition with dose- and time-dependent antiproliferative effect for three earthworm species. (Control drug – paclitaxel)

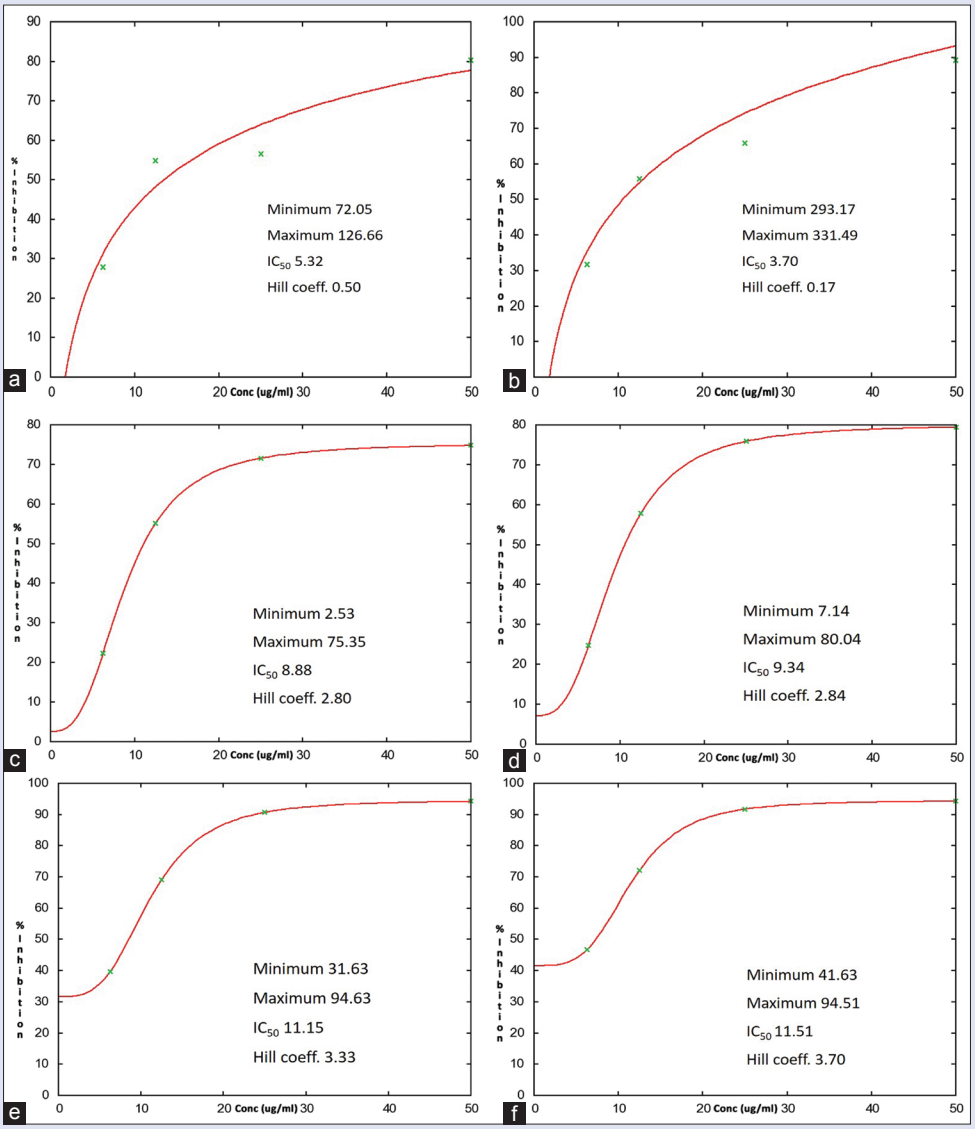

Figure 2: Dose response curve. (a) Dose response curve of *Eudrilus eugeniae* on KB 3-1 cells at 24 h (IC_{50} – 5.32 µg/mL). (b) Dose response curve of *Eudrilus eugeniae* on KB 3-1 cells at 48 h (IC_{50} – 3.70 µg/mL). (c) Dose response curve of *Eisenia fetida* on KB 3-1 cells at 24 h (IC_{50} – 8.88 µg/mL). (d) Dose response curve of *Eisenia fetida* on KB 3-1 cells at 48 h (IC_{50} – 9.34 µg/mL). (e) Dose response curve of *Perionyx excavatus* on KB 3-1 cells at 24 h (IC_{50} – 11.15 µg/mL). (f) Dose response curve of *Perionyx excavatus* on KB 3-1 cells at 48 h (IC_{50} – 11.51 µg/mL)

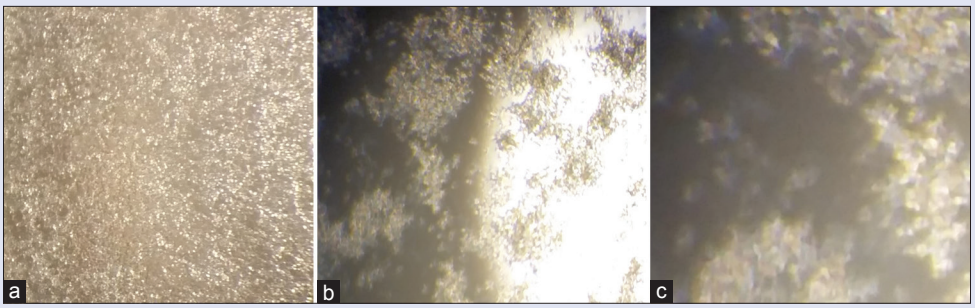

Figure 3: (a) Inverted microscope image of KB 3-1 cells in monolayer prior to treatment with earthworm coelomic fluid of *Eudrilus eugeniae*. (b) Reduction in the number of cells with clear spaces is evident after 24 h incubation. (c) Significant reduction in the number of cells with clear spaces is evident after 48 h incubation

Caseinolytic activity was shown using a plate diffusion assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and diethylaminoethyl cellulose chromatography were used for isolation and purification of the extract.[15]

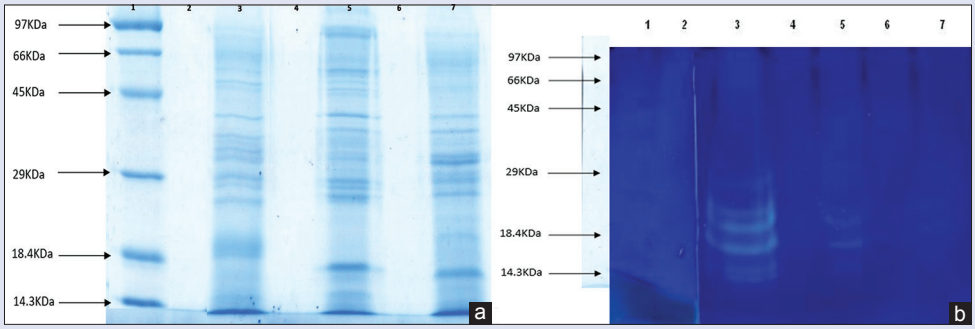

Figure 4: (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained gel showing presence of thick band in lane 3 (*Eudrilus eugeniae*) at18–20 kDA region. (Lane 1 – Standard marker, Lane 2 – Blank, Lane 3 – EE, Lane 4 – Blank, Lane 5 – EF, Lane 6 – Blank, Lane 7 – PE). (b) Screening zymography demonstrating blue gel staining except in the band region of serine protease (average molecular weight of the range 18–20 kDa), which would have digested gelatin

Lowry's method of protein estimation was used to determine the total protein concentration of the test samples EE, EF, and PE. Lowry's method uses the principle of acid hydrolysis that is an accurate technique to determine protein concentrations. Amino acid analysis usually follows the acid hydrolysis. In other methods, the amino acid content of the protein is sensitive to testing due to which it is challenging to get absolute concentrations. The sensitivity of Lowry's protein estimation procedure is moderate from one protein to another; this makes it a widely accepted procedure to follow. It is a reliable method to estimate the total proteins in crude extracts and proteins cocktails; it is also a supplementary method which can be used in the place of other absolute protein determination techniques.[16]

Testing in cell lines is the most commonly used modality for drug screening as it provides an inexhaustible supply of cells to be tested on. The cells *in vitro* represent different stages of tumor development. Molecular characterization of cell lines can serve as gene discovery tools. Gene expression of cells can be assessed after exposure to a potential anticancer agent, and this can also reveal signaling pathways that can be exploited in targeted therapy of cancer.[17] Earlier cell lines had disadvantages as they required high doses of the test compound to initiate transformation. The new generation of cell lines has overcome these disadvantages and is useful in biomarker discovery and targeted therapy.[18,19]

KB 3–1 cell line was employed from NCCS (Pune, India) in the present study, which is a human mouth cancer cell line.[20] Viability of the cells signifies the capability of their existence, survival, and development. Various experiments had been carried out with cells in the culture medium rather than using animal models. This is particularly done keeping in mind the determination of safety and cytotoxicity of several compounds such as the food additives, anticancer drugs, cosmetics, and pharmaceuticals. *In vitro* testing for safety evaluation and cytotoxicity eliminates the use of animals and it is cost effective. Cytotoxicity studies broadly involve the metabolic modifications of the cells, including the death of cells due to toxic effects of the compounds. The metabolic alterations for cosmetics and allergic responses are more significant. There are many assays in the laboratory which are developed for measuring the cell viability and cytotoxicity.[21]

The tests for measurement of cell viability and cytotoxicity detect the live or dead cells at the time of assay; they are mainly of short term. When the cells are subjected to toxicity mostly, i.e., irradiated, exposed to drugs, the effects are not instantaneous but may be observed either after several hours or days. The assays are based on the survival of cells such as retention of regenerative capacity and reproductive integrity.[22] The present study employed the MTT assay to ascertain the viability of cells *in vitro* following incubation with the test samples. The potential test drug is incubated with the growing cells in the log phase; following this, the cells are allowed to multiply with 2–3-fold population doubling time. The MTT dye reaction with the formazan-colored product enables identification of the surviving fraction of cells by spectrophotometry.

In the present study after a 24 h incubation of KB 3–1 cell line with ECF of EE, EF, and PE at increasing concentration of 6.25, 12.5, 25, and 50 μg/mL, ECF of PE showed a maximum inhibitory percentage of 94.22% at 50 μg/mL, followed by ECF of EE and EF with values of 80.32% and 74.78%, respectively. ECF of EE showed a superior IC_{50} value of 5.32 μg/mL as compared to EF and PE. Using the same concentrations after 48 h and exposure, ECF of PE showed a maximum inhibitory percentage of 94.29% at 50 μg/mL, followed by ECF of EE and EF with values of 89.23% and 79.44%, respectively. ECF of EE showed a superior IC_{50} value of 3.70 μg/mL as compared to EF and PE. The current study used paclitaxel as the positive standard anticancer drug. Paclitaxel was initially discovered as taxol, a natural product from the bark of the Pacific yew tree in 1962.[23]

Subsequent experimental studies were performed to deduce preliminarily the chemical nature of the coelomic fluid; hence, we performed SDS-PAGE and enzyme zymography. SDS-PAGE is a technique that applies an electrical field to a gel matrix containing proteins resulting in separation of proteins based on their size. Analyzing proteins is more sophisticated compared to DNA analysis as proteins can be both positively and negatively charged.[24] SDS possesses a hydrophobic end (dodecyl) and a hydrophilic end (sulfate group). The protein core is concentrated with hydrophobic bonds. The hydrophobic end of SDS interacts with the side chain amino acid end terminals of the protein core through hydrophobic interactions. The samples EE, EF, and PE were analyzed for separated protein bands; a characteristic thick band was appreciated in the 18–20 kDa region for sample EE. To determine the protease nature of the band, a zymography was performed for sample EE.

Zymography is an electrophoretic technique based on SDS-PAGE analysis employing a substrate such as albumin, casein, gelatin, or hemoglobin, which is copolymerized with the polyacrylamide matrix. The SDS-PAGE is used to prepare the proteins under nonreducing conditions. The proteins are separated by their respective molecular mass in the standard SDS-PAGE procedure with copolymerization by a protein substrate. Clear horizontal bands against a bright blue Coomassie background are indicative of proteolytic activity.[25] A fresh sample of ECF of EE was collected to perform zymography. Following the loading of sample, the band region of serine protease enzyme produced a negative staining seen as white bands, having an average molecular weight in the range of 18–20

kDa. Further, isolation and purification of the serine protease of ECF of EE have been planned along with testing of its individual anticancer effect compared to the crude extracts of EE, EF, and PE.

There has been an increasing demand to test natural products over the past few years. This has resulted in the evaluation of an array of natural products belonging to a family of polyphenolics, alkaloids, glycosides, etc. The National Cancer Institute of the United States of America has extracted 114,000 compounds from 35,000 primary samples and has evaluated them against a plethora of tumor systems.[26] The ECF is one such NAE having promising antiproliferative potential that requires further exploration.

CONCLUSION

The current study emphasizes the use of ECF as an adjunctive pharmacological strategy in the management of cancer. ECF of EE showed a superior IC_{50} value of 3.70 μg/mL as compared to EF and PE at 48 h. The serine protease nature of ECF of EE was arrived at. Purification of the serine protease of ECF of EE has been initiated and further experiments have been planned. However, experiments at the molecular level such as cell cycle analysis and gene expression studies are the need of the hour to understand the mechanism of ECF action on cancer cells.

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Conflicts of interest

There are no conflicts of interest.

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