

# Anticancer Prospects of Earthworm Extracts: A Systematic Review of *In vitro* and *In vivo* Studies

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## ABSTRACT

In recent times, naturally occurring substances such as earthworm extracts have been used successfully as an antimicrobial and anti-inflammatory agent in wound healing. It has also shown promising antitumor activity in cervical and gastric cancer. The aim of this systematic review is to analyze the anticancer potentials of earthworm extracts. Several databases, including PubMed and Google Scholar, were searched from September 2001 to September 2017 using combinations of the following keywords "Earthworm," "earthworm extract," and "anti-cancer effect." Original studies in English describing cytotoxic effects of earthworm extracts on cancer cells *in vitro* and *in vivo* were included in the study. We excluded letters to the editor, reviews, and unpublished data, antimicrobial and anti-inflammatory studies pertaining to the extracts. There were 23 studies included in the analysis. Eighteen were *in vitro* studies and 4 studies combined *in vitro* and *in vivo* methods. Only one exclusive *in vivo* study was identified. *Eisenia foetida* was the most commonly researched earthworm species. Cervical cancer and hepatocellular carcinoma were the most commonly evaluated cancer types. HeLa cervical cancer cell line was the most commonly used model for cytotoxicity testing. Earthworm extracts showed satisfactory anticancer effect on several types of cancers, especially cervical cancer and hepatocellular carcinoma. The mechanism of apoptosis of cancer cells should be ascertained and the underlying genes and pathways responsible to be determined. This would help to execute long-term randomized controlled trials to assess the clinical efficacy, optimum dosage, and safety in the future.

**Key words:** Anticancer effect, cancer cell biology, cervical cancer, earthworm extract, *Eisenia Foetida*, *Eudrilus eugeniae*, hepatocellular carcinoma, *in vitro*, *in vivo*, proliferation

## 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.<sup>[1]</sup> Potential chemotherapeutic agents can be obtained from natural products.<sup>[2]</sup> As part of the epidemiological transition, cancer incidence is expected to increase in the future, further straining limited health-care resources.<sup>[3]</sup> This encourages the search for new and better therapeutic modalities that can comprehensively alter tumor progression.

By understanding cancer cell biology, it is clear that continued proliferation of cancer cells and metastasis formation represents a complex process of multistep events during the growth of malignant tumors.<sup>[1]</sup> It is under the influence of many systemic and local environmental host factors and of the inherent properties of tumor cells. Recurrence is one of the prime reasons for failure of anticancer therapy.

In recent times, naturally occurring substances such as extracts from earthworms have assumed importance pertaining to their role in preventing the replication and division of cancer cells which is an interesting research concept. Current research should be designed to identify biomolecules that can inhibit cancer cells from proliferating.<sup>[4]</sup>

Establishing the antiproliferative effect of earthworm extract on cancer cells could be an initial step toward drug development and future anticancer promising therapeutics. Therefore, the aim of this study was to determine, through a systematic review, the anticancer potentials of earthworm extracts on cancer cells with emphasis on the nature of extract and cancer models employed.

## METHODOLOGY

### Key question

A key question was constructed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines ([www.prisma-statemnet.org](http://www.prisma-statemnet.org)). The question was "Is there a significant anticancer effect of earthworm extracts on cancer cells?"

### Study design and search strategy

The study was designed as per the PRISMA guidelines to summarize the results of published studies to analyze the anticancer potentials of

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earthworm extracts on cancer cells. An automated detailed literature search of PubMed, Google Scholar, and web sources was carried out as shown in Table 1 using various combinations of corresponding descriptors (MeSH) and free text terms such as earthworm, earthworm extract, anticancer effect, neoplasms, cytotoxic effect, and antiproliferative effect. To restrict the results, the search was limited to studies published in English from September 2001 to September 2017.

## Eligibility criteria

### Inclusion criteria

The articles included in the study were full-length, English language articles that focused on the anticancer effect of earthworm extracts on cancer cells *in vitro* and *in vivo*.

### Exclusion criteria

1. Articles other than anticancer evaluation of earthworm extract cancer were excluded from the study
2. Articles other than original research such as reviews, editorial letters, books, personal opinion, and abstracts were excluded from the study
3. Studies that fail to report the nature of earthworm extract and cytotoxic assay values were also excluded from the study
4. Articles with no information on tumor model tested, antimicrobial and anti-inflammatory studies pertaining to the extracts were also excluded from the study.

## Study selection and data collection process

Two reviewers (DA and RSR) initially screened titles and abstracts of studies based on inclusion and exclusion criteria defined. Full texts of studies found relevant were retrieved and independently reviewed. References of the selected articles were again screened for additional

studies that could have gone unnoticed during electronic search. In case of disagreement, a third reviewer (JA) would be consulted.

The data presented in these studies were carefully extracted and included in evidence tables. The abstracted data included author and year of publication, study type, species of earthworm, nature of extract, type of cancer investigated, tumor models used, method of isolation and protein analysis details if any, and cytotoxicity tests performed. The data are summarized in Table 2.

## RESULTS

### Study characteristics

A total of 23 studies<sup>[5-27]</sup> that met eligibility criteria were included in the review [Figure 1]. The majority of studies were *in vitro* studies,<sup>[5-15,17,18,20,21-23]</sup> four studies employed both *in vitro* and *in vivo* methods,<sup>[19,21,24,25]</sup> and only one *in vivo* study was recognized.<sup>[16]</sup>

The majority of studies were conducted in China ( $n = 13$ ),<sup>[6,13,16-22,24-27]</sup> eight studies were performed in India,<sup>[5,7-10,12,14,15]</sup> one study was conducted in Poland,<sup>[11]</sup> and one in Hungary [Figure 2].<sup>[23]</sup>

### Earthworm species

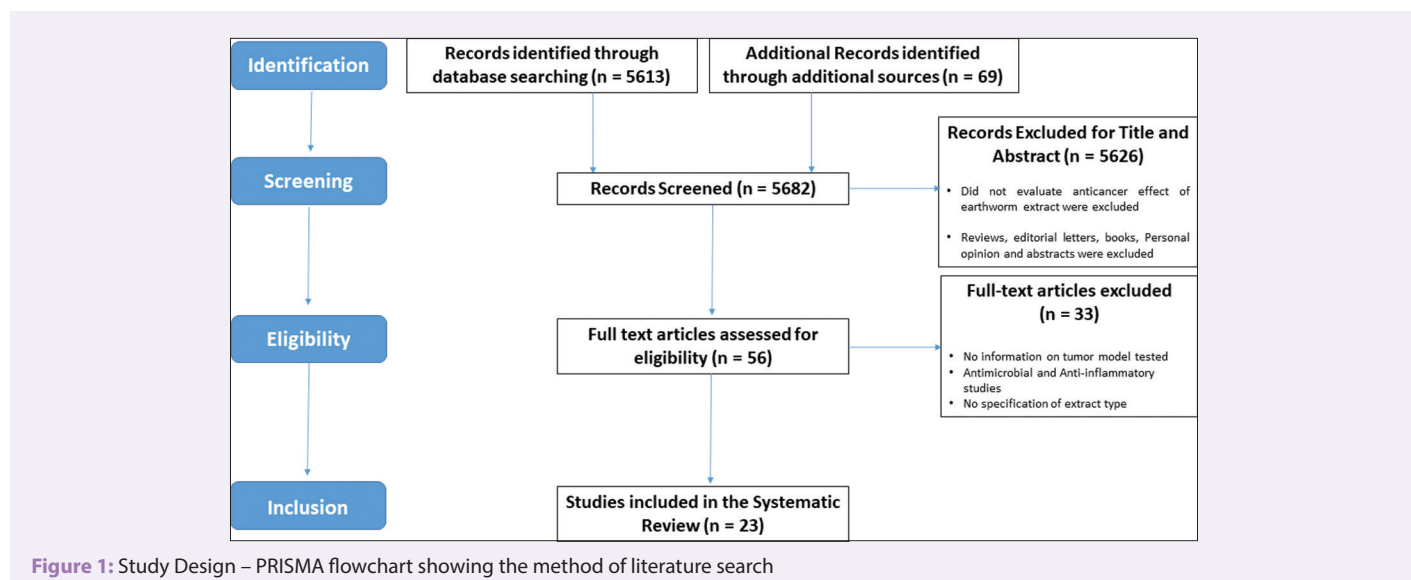
*Eisenia Foetida* was the most commonly researched earthworm species.<sup>[5,7,8,13,19,20,23,25,26]</sup> *Eudrilus eugeniae* was the next commonly researched species.<sup>[5,7,8,12,14,15]</sup> *Perionyx excavatus* was researched in two studies,<sup>[5,8]</sup> while a single study employed *Lampito mauritii* and *Pheretima posthuma* as shown in Table 3.<sup>[9,10]</sup>

### Cancer investigated

Cervical cancer<sup>[12-15,20,21,23,25]</sup> and hepatocellular carcinoma<sup>[16,17,19,20]</sup> were the most commonly employed cancer types for investigation followed by leukemia,<sup>[12,21,22,25,27]</sup> colon cancer,<sup>[7-9,12,14]</sup> lung cancer,<sup>[7,11,14,24]</sup> and breast cancer.<sup>[6,10,11,24]</sup> Three studies investigated gastric carcinoma,<sup>[18,24,25]</sup>

**Table 1:** Methodology employed for the systematic review

Statement of the objective	Method/methodology	Resources utilized	Keywords used
To analyze and critically evaluate research articles that have described the potential anti-cancer effect of earthworm extracts and their mechanisms	Collection of articles followed by critical evaluation of studies describing the specie of earthworm, the nature of extract, tumor models used, and cytotoxicity tests performed	PubMed, Google scholar, and e-journals	“ <i>Oligochaeta</i> ” (MeSH Terms) or “ <i>Oligochaeta</i> ” (All Fields) or “ <i>Lumbricina</i> ” (All Fields) and extract (All Fields) and “anticancer effect” (All Fields) (“neoplasms”) (MeSH Terms)



**Figure 1:** Study Design – PRISMA flowchart showing the method of literature search

**Table 2:** Summary of selected articles

Author and year	Earthworm species	Cancer investigated	Nature of extract	Method of isolation	Protein analysis method	Type of study	Study model	Cytotoxicity tests	Adjunctive investigations
Dominic Augustine, <i>et al.</i> , 2017	<i>E. eugeniae</i> , <i>E. foetida</i> , and <i>P. excavates</i>	Oral cancer	Coelomic fluid	Cold shock method	Modified Bradford protein assay	<i>in vitro</i>	SCC-9 cell line	MTT assay	-
Liu, <i>et al.</i> , 2017	<i>E. foetida</i>	Breast cancer	Fibrinolytic enzyme	Homogenate of frozen <i>E. foetida</i>	DEAE-C ion-exchange chromatography SDS PAGE LCMS	<i>in vitro</i>	MCF-7 cell line	Cell viability using CCK-8	RT PCR-CD44v6 western blot
Vidya N, <i>et al.</i> , 2016	<i>E. eugeniae</i>	Lung cancer and colorectal cancer	Coelomocytes cell culture	Cell culture method	Lowry's method	<i>in vitro</i>	A549 and HCT 116 cell lines	MTT assay	Clonogenic assay
Pushpa Reddy, <i>et al.</i> , 2015	<i>P. excavates</i> , <i>E. eugeniae</i> , and <i>E. foetida</i>	Lung cancer prostate cancer and colorectal cancer	Earthworm paste	Sunlight exposure followed by digestion	Lowry's method	<i>in vitro</i>	MCF-7, PC-3, and HCT-116 cell lines	MTT assay	DNA ladder assay Clonogenic assay cell cycle analysis
Lourdumary, <i>et al.</i> , 2014	<i>L. mauritii</i>	Colorectal cancer	Earthworm powder	Earthworm paste freeze-dried into powder	-	<i>in vitro</i>	HT 29 cell line	MTT assay	Cell cycle analysis Fluorescent staining with AO/EB
MK Verma, <i>et al.</i> , 2013	<i>P. posthuma</i>	Breast cancer	Serine protease isolate	Autolysis performed for 3 h at 60°C in 20 mmol/L phosphate buffer pH 7.5 with 0.02% sodium azide	Lowry's method Caseinolytic plate diffusion assay SDS PAGE DEAE-C chromatography	<i>in vitro</i>	MCF-7 cell line	MTT assay	-
MJ Fiolka, <i>et al.</i> , 2013	<i>D. veneta</i>	Breast ductal carcinoma and endometrioid ovarian cancer	PPC from gut bacteria	Microbial culture	Bradford method	<i>in vitro</i>	T47D and TOV-112D cell line	BrdU labeling kit	-
MS Dinesh, <i>et al.</i> , 2013	<i>E. eugeniae</i>	Cervical cancer, colon cancer, leukemia, and brain cancer	Coelomic fluid	Heat and cold shock method	Lowry's method HPTLC band	<i>in vitro</i>	HeLa, HT-29, WBC malignant tumor line, and brain tumor cell line	MTT assay	-
Zhang Hua, <i>et al.</i> , 2011	<i>E. foetida</i>	Cervical cancer and lung adenocarcinoma	Earthworm protein from coelomic fluid	6 voltage electronic stimulation method	Ultrafiltration, gel chromatography, and ion exchange chromatography SDS PAGE	<i>in vitro</i>	HeLa and LTEP-A2 cell lines	MTT assay	-
Mohamed Jaabir, <i>et al.</i> , 2011	<i>E. eugeniae</i>	Cervical cancer	Cell-free coelomic fluid	5 voltage electronic stimulation method	SDS PAGE	<i>in vitro</i>	SiHa cell line	MTT assay	Fluorescent staining with AO/EB Hoechst 33258 staining DNA ladder assay

Contd...

Table 2: Contd...

Author and year	Earthworm species	Cancer investigated	Nature of extract	Method of isolation	Protein analysis method	Type of study	Study model	Cytotoxicity tests	Adjunctive investigations
Veeramani, <i>et al.</i> , 2010	<i>E. eugeniae</i>	Cervical cancer	Coelomic fluid	Cold facile method	-	<i>in vitro</i>	SiHa cell line	MTT assay and trypan blue viability staining	Fluorescent staining with AO/EB Hoechst 33258 staining DNA ladder assay
Wang Juan, <i>et al.</i> , 2009	-	Hepatocellular carcinoma	Earthworm fibrinolytic enzyme	-	-	<i>in vivo</i>	Xenograft with SMMC-7721 cells developed in nude mice	-	CD44v6 protein by IHC and western blot RT-PCR for CD44v6 mRNA
Chen Hong, <i>et al.</i> , 2008	-	Hepatocellular carcinoma	Earthworm fibrinolytic enzyme	-	-	<i>in vitro</i>	SMMC-7721 cell line	Matrigel-transwell chamber method	Focal adhesion kinase mRNA detection by (RT-PCR) and western blot
Yu Yan-qiu, <i>et al.</i> , 2007	-	Gastric carcinoma	Earthworm fibrinolysin	-	-	<i>in vitro</i>	MGC803 cell line	MTT assay	DNA gel electrophoresis Flow cytometry p53 protein expression by IHC
Chen Hong, <i>et al.</i> , 2007	<i>E. foetida</i>	Hepato-cellular carcinoma	Earthworm fibrinolytic enzyme	Homogenization then extracted by normal saline	Ultrafiltration Gel chromatography Column chromatography	<i>in vitro</i> and <i>in vitro</i>	HLE, Huh7, PLC/PRF/5 and HepG2 cell lines Huh7 cells were developed in male BALB/c AnNCrj-nu nude mice	Cell proliferation assay kit-chemicon	Flow cytometric assay Fluorescent staining with AO/EB MMP-2 detection by Western blot
L.Yanqin, <i>et al.</i> , 2007	<i>E. foetida</i>	Cervical cancer	Coelomic fluid	Earthworms were homogenized with a tissue blender	Ammonium sulfate precipitation and ultrafiltration MALDITOF-MS	<i>in vitro</i>	HeLa cell line	MTT assay	Fluorescent staining with AO/EB DNA ladder assay
HE Dao-wei, <i>et al.</i> , 2005	-	Cervical cancer, esophageal squamous carcinoma, and leukemia	Earthworm extract	-	-	<i>in vitro</i> and <i>in vitro</i>	Eca109, HeLa, and K562 cell lines Nude Mice	MTT assay	Flow cytometric assay
Yuan L, <i>et al.</i> , 2004	-	Chronic myelogenous leukemia, myeloma, acute lymphocytic leukemia, and lymphoma	Earthworm extract	-	-	<i>in vitro</i>	K562, KM3, L6565, and SRS-B2 cell lines	Trypan blue exclusion and light microscope examination	Apoptosis fluorescence assay DNA gel electrophoresis
Engelmann P, <i>et al.</i> , 2004	<i>E. foetida</i>	Cervical cancer, HeLa-derived cancer, pheochromocytoma, and mouse fibroblast	Supernatants of cultured coelomocytes, and lysates from coelomocytes	Derived by mechanical and detergent extraction with 6 voltage electric stimulation	Bradford assay	<i>in vitro</i>	HeLa, HEP-2, PC-12, and PA317	MTT assay and phase contrast microscopic examination	

Contd...

Table 2: Contd...

Author and year	Earthworm species	Cancer investigated	Nature of extract	Method of isolation	Protein analysis method	Type of study	Study model	Cytotoxicity tests	Adjunctive investigations
LI Hong yan, et al., 2004	-	Gastric carcinoma, breast cancer, colon cancer, lung carcinoma, and liver cancer	Earthworm fibrinolytic enzyme	-	-	<i>in vitro</i> and <i>in vitro</i>	BGC823, MCF-7, HCT 8, A549, and Bel7402 cell lines Xenotransplanted nude mice models	MTT assay	-
XIE Jiang bi, et al., 2003	<i>E. foetida</i>	Colon cancer, neuroblastoma, chronic myelogenous leukemia, gastric carcinoma, and cervical cancer	Earthworm extract	-	Acetone sedimentation and gel filtration	<i>in vitro</i> and <i>in vitro</i>	HCT 116, SY5Y, K562, MGC803 and HeLa cell lines Ascites tumor (S180)-bearing mice	-	-
Zhao Rui, et al., 2002	<i>E. foetida</i>	-	Protein kinase	Ethanol precipitation	Purified by Sephadex G 75 Mass spectrometry	<i>in vitro</i>	-	-	Polyacrylamide electrophoresis
Xu Jianmin, et al., 2001	-	Chronic myelogenous leukemia	Earthworm extract	-	-	<i>in vitro</i>	K562 cell line	Trypan blue exclusion and Light microscope examination	Apoptosis fluorescence assay DNA gel electrophoresis

AO/EB=Acridine orange/ethidium bromide, DEAE-C=Diethylaminoethyl cellulose, DNA=Deoxyribonucleic acid, HPTLC=High-performance thin-layer chromatography, IHC=Immunohistochemistry, LCMS=Liquid chromatography mass spectrometry, MALDITOF-MS=Matrix assisted laser desorption ionization-time of flight mass spectrometry, MMP-2=Matrix metalloproteinase, SDS PAGE=Sodium dodecyl sulfate polyacrylamide gel electrophoresis, MTT=3 (4, 5dimethylthiazol2yl) 2, 5diphenyltetrazolium bromide, RNA=Ribonucleic acid, PPC=polysaccharide protein complex, SCC=Squamous cell carcinoma, CCK-8=Cell counting Kit-8, BrdU=Bromodeoxyuridine, RT-PCR=Reverse transcription polymerase chain reaction, WBC=White blood cell, *E. eugeniae*=*Eudrilus eugeniae*, *E. foetida*=*Eisenia foetida*, *P. excavates*=*Perionyx excavates*, *L. mauritii*=*Lampito mauritii*, *P. posthuma*=*Pheretima posthuma*, *D. veneta*=*Dendrobaena veneta*

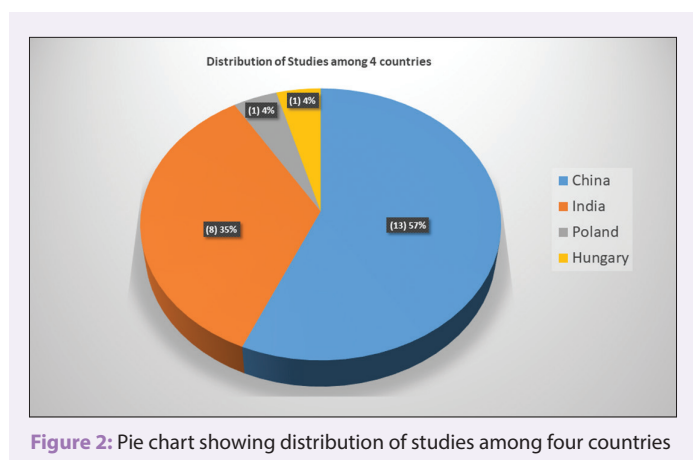


Figure 2: Pie chart showing distribution of studies among four countries

while a single study featured oral cancer cell line squamous cell carcinoma-9 (SCC-9).<sup>[5]</sup>

### Nature of extract

Coelomic fluid<sup>[5,12-15,20]</sup> and earthworm fibrinolytic enzyme (EFE)<sup>[6,16-19,24]</sup> were commonly used to test anticancer activity with favoring results. The results of the individual studies were then summarized. Data on the same were grouped and analyzed. Summarization of individual points of interest across the selected studies was carried out.

## DISCUSSION

The quest to find a suitable solution to the problem of treating cancer and preventing its progression has remained the central dogma of cancer therapeutics. Concepts of using naturally available extracts to inhibit the proliferation of cancer cells have emerged. Chemotherapeutic drugs have got dual effect. Apart from killing cancer cells, certain normal cells get sacrificed in the bargain such as those that line the gastrointestinal tract, bone marrow cells, and hair follicles, thereby causing significant adverse effects.<sup>[28]</sup>

Earthworm extracts are naturally available which would seldom exert adverse effects. The concentration at which natural extracts exert a cytotoxic effect, the stages of cell cycle arrest in cancer cells, the type of cellular damage, and the mechanism through which these extracts exert anticancer effect will be the future expectation from this domain. Over the past few decades, researchers have explored alternate treatment therapies and remedies to prevent cancer progression but have attained with limited success and high failure rates. Recently, concepts of using naturally available extracts to inhibit the division of cancer cells have emerged. The specific topic of “Biomolecules against cancer” is relevant and significant in the present context as it is necessary to identify biomolecules that can inhibit cancer cells. To the best of our knowledge, the present study is the first systematic review to focus on the anticancer prospects of earthworm extracts on cancer cells.

**Table 3:** Earthworm species associated with the specific type of cancer

Earthworm species	Cytotoxicity against specific cancer
<i>E. foetida</i> <sup>[5,6,8,13,19,20,23,25,26]</sup>	Breast cancer, lung cancer Prostate cancer, colorectal cancer Cervical cancer, oral cancer, and hepatocellular carcinoma
<i>E. foetida</i> <sup>[5,7,8,12,14,15]</sup>	Lung cancer, colorectal cancer, prostate cancer, colon cancer, leukemia, brain cancer, oral cancer, and hepatocellular carcinoma
<i>L. mauritii</i> <sup>[9]</sup>	Colorectal cancer
<i>P. posthuma</i> <sup>[10]</sup>	Breast cancer
<i>P. excavates</i> <sup>[5,8]</sup>	Lung cancer, prostate cancer, colorectal cancer, and oral cancer
<i>D. veneta</i> <sup>[11]</sup>	Breast ductal carcinoma and endometrioid ovarian cancer

*E. eugeniae*=*Eudrilus eugeniae*, *E. foetida*=*Eisenia foetida*, *P. excavates*=*Perionyx excavates*, *L. mauritii*=*Lampito mauritii*, *P. posthuma*=*Pheretima posthuma*, *D. veneta*=*Dendrobaena veneta*

## Method of extraction/preparation

The coelomic fluid is generally secreted by the earthworms for maintaining moisture to help their physiological activities such as respiration and burrowing activities. It consists of watery matrix, the plasma, and a large number of coelomocytes.<sup>[29]</sup> Six studies employed the earthworm coelomic fluid for cytotoxic analysis.<sup>[5,12-15,20]</sup> Coelomic fluid collection by heat and cold shock method was found to be most acceptable by Dinesh *et al.*, 2013, who demonstrated cytotoxic potential of *E. eugeniae* on HeLa cell, colon cancer cells, WBC malignant tumor, and brain tumor cells.<sup>[12]</sup> Augustine *et al.*, 2017, used the cold shock method and obtained 3.5 ml of earthworm coelomic fluid from *E. eugeniae* and 3 ml from *E. fetida* and *P. excavatus* and demonstrated IC<sub>50</sub> values of 4.6, 44.69, and 5.27 µg/ml, respectively, on SCC-9 cell line.<sup>[5]</sup>

## Described below are few other methods of coelomic fluid collection

### Electric method

To clear the gut contents of the earthworms, they are selected and kept in clean water for 3 h. They are thoroughly washed many times in sterile saline solution, and electric current is applied for 10 s. Few techniques have described collection of coelomic fluid by applying electric stimulation or by fluctuating the temperature.<sup>[30,31]</sup>

### Mechanical agitation

This is a procedure where the earthworms are mechanically agitated by placing in a closed Petri dish. The stimulation can also be done with a wooden spatula where the spatula should be gently rubbed over the earthworm's body till its coelomic fluid oozes out.

### Alternate heat and cold method

Here, the earthworms are placed in a Petri dish, placed over ice cubes for 30 s, later the Petri dish is passed over the flame of a Bunsen burner, and the same procedure is repeated constantly till the earthworm's coelomic fluid oozes out.

### Warm water method

In this method, the earthworms are kept immersed under 25 ml of warm water of temperature 45°C, and instead of the coelomic fluid, the whole water is used; hence, volume of collection is calculated as 25 ± 3 ml in 30 min. The body fluid is being collected along with enormous quantity of water which is known as vermiwash.<sup>[32,33]</sup>

### Cold shock method

Here, the earthworms are subjected to cold shock by ice packing, and then the fluid is collected in a dry clean test tube. In cold shock method, the worms are alive and active, though it secretes comparatively larger volume of fluid (1.5 ml) than other methods. The fluid collected is clear brown in color without any debris as seen in the heat and electric shock method.<sup>[34,35]</sup>

### Ethanol extrusion method

In this method, the coelomic fluid is collected through a noninvasive technique. The earthworms are placed in a 15 ml polypropylene tube containing 3 ml of cold extraction medium for 3 min. The extraction medium with 5% ethanol is adjusted to pH 7.3 with 1M NaOH. The volume is increased by adding 12 ml of ice-cold saline and adjusted to pH 7.3 after 3 min.

The cells are recovered by centrifugation at 40°C at 15,000 rpm.<sup>[36]</sup> Zhao *et al.*, 2002, extracted a protein kinase from *E. fetida* using ethanol extraction.<sup>[26]</sup>

### Nature of extract used

Six studies employed the EFE<sup>[6,16-19,24]</sup> to test anticancer activity with appreciable results. Chen *et al.*, 2007, showed that EFE isolated from *E. fetida* exhibits antitumor activity against the human hepatoma cells *in vitro* and *in vivo*. The authors prepared the concentrated protein solution after column chromatography by the use of diethylaminoethyl cellulose (DEAE-C) and eluted by the solution of sodium chloride with a graduated electric conductivity. Five active peaks were obtained. The fibrinolytic active peaks were collected, ultrafiltrated, and freeze-dried. The sample of EFE was white freeze-dried powder with fibrinolytic activity 320 uku. The results indicated that EFE could be used in treatment of hepatoma. The authors estimated the expression of matrix metalloproteinase-2 (MMP-2) which is responsible for invasion and metastasis, and it was found that EFE suppresses MMP-2.<sup>[19]</sup>

Other types of innovative extracts prepared by authors were brought to light through this systematic review, and coelomocytes cell culture of *E. eugeniae* was used by Vidya *et al.*, 2016.<sup>[7]</sup> The results obtained using A549 cell lines with a concentration of 2 mg/ml and showed 90% cytotoxicity. The outcome of the study contributes scientific evidence to utilize coelomocyte culture supernatant as a source to establish anticancer drug treatment.

Earthworm paste was prepared by Reddy *et al.*, 2015, to test the anticancer effect of *P. excavatus*, *E. eugeniae*, and *E. fetida* on MCF-7, PC-3, and HCT-116 cell line.<sup>[8]</sup> 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 worms forming a brown-colored earthworm paste.<sup>[37]</sup> Lourdummy and Ramesh, 2014, employed earthworm powder of *Lampito mauritii* on HT-29 cell line and showed a tremendous percentage of inhibition for HT-29 cells recorded up to 82.25% at 320 µg/ml in 48 h. Treated HT-29 cells at 80 µg/ml concentration showed 47.67% cell cycle arrested at G2/M phase. This result concluded that the sample arrested cell cycle at G2/M phase and at the same time these treated HT-29 cells underwent apoptosis.<sup>[9]</sup>

Cell-free coelomic fluid of *E. eugeniae* was tested for anticancer activity by Mohamed Jaabir *et al.*, 2011. An increase of apoptotic rate from 68.91% to 79.32% was observed with the increase in the incubation period from 48 to 72 h suggesting that induction of apoptosis in SiHa cells *in vitro* by the coelomic fluid was time and dose dependent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the coelomic fluid revealed the presence of a 42 KDa protein identified

earlier to be a tumorigenic factor analogous to the vertebrate cytokine tumor necrosis factor.<sup>[14]</sup>

Engelmann *et al.*, 2004, isolated supernatants of cultured coelomocytes and lysates from coelomocytes of *E. fetida* by mechanical and detergent extraction with 6 voltage electric stimulation and showed that these extracts significantly decreased the ratio of living cells compared to controls in a dose-dependent manner in HeLa, HEP-2, PC-12, and PA317 cell lines.<sup>[23]</sup>

Serine protease isolate of *Pheretima posthuma* was used by Verma *et al.*, 2013, on MCF-7 cell line and suggested that the 15 kDa fraction has potent cytotoxic activity. The protease was prepared by autolysis of the earthworm after repeated washes with sterile distilled water. Caseinolytic plate diffusion assay, SDS PAGE, and DEAE-C chromatography were used for purification and isolation.<sup>[10]</sup>

In a unique study by Fiolka *et al.*, 2013, a polysaccharide-protein complex (PPC) isolated from metabolites of gut bacteria *Raoultella ornithinolytica* from *Dendrobaena veneta* earthworms exhibited activity against breast ductal carcinoma (T47D cell line) and in the endometrioid ovarian cancer line (TOV-112D) *in vitro*.<sup>[7]</sup> The complex was prepared by collecting the fraction of molecular weight above 100 kDa and lyophilizing. Next, it was solubilized in 5 mL of 25 mM Tris-HCl, pH 9.0, buffer (about 5 mg) and applied to a column of an anion exchanger DEAE-Sepharose Fast Flow equilibrated with 25 mM of Tris-HCl, pH 9.0, buffer.

Lowry's method was the most routinely employed method for protein estimation<sup>[7,8,10,12]</sup> followed by Bradford protein assay.<sup>[5,11,24]</sup> Other advanced protein analyses performed included caseinolytic plate diffusion assay, SDS PAGE, DEAE-C ion-exchange chromatography, liquid chromatography-mass spectrometry (MS), high-performance thin-layer chromatography band, matrix-assisted laser desorption ionization-time of flight MS, and acetone sedimentation and gel filtration.

## Cancer study models employed

Majority of the studies have used cell lines for cytotoxicity research. Cancer cell lines are adequate models for cancer research that present a more complex genetic constitution and typically present extensive chromosomal rearrangements, mutation of oncogenes, and allelic imbalance. Cancer cell progression up to metastasis can be studied through evaluating phenotypic and genotypic features which evolve through multiple divisions.<sup>[38]</sup>

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

Four studies employed *in vivo* models apart from the existing *in vitro* investigations such as nude mice model to test the earthworm extract used by He *et al.*, 2005.<sup>[21]</sup> Li Hong yan *et al.*, 2004, used xenotransplanted nude mice models to test the EFE.<sup>[24]</sup>

Huh7 cells xenografted in nude mice were employed by Chen *et al.*, 2007, to test EFE of *E. fetida* against hepatocellular carcinoma.<sup>[19]</sup> Ascites tumor (S180)-bearing mice was employed by Xie Jiang bi *et al.*, 2003, to test the earthworm extract of *E. fetida*. While a study conducted by Wang *et al.*, 2009, tested EFE against xenograft with SMMC-7721 cells developed in nude mice.<sup>[16]</sup>

The use of cell lines, which are invaluable experimental models for cancer studies, simplifies the task of genetic manipulation and molecular characterization. Studies using cell lines have revealed signaling pathways in cancer and have been used to test and develop drugs and therapies in the past.<sup>[38]</sup>

## Cancers and cancer cell lines evaluated

Cervical cancer<sup>[12-15,20,21,23,25]</sup> and hepatocellular carcinoma<sup>[16,17,19,20]</sup> were the most commonly employed cancer types for investigation, followed by leukemia,<sup>[12,21,22,25,27]</sup> colon cancer,<sup>[7-9,12,14]</sup> lung cancer,<sup>[7,11,14,24]</sup> and breast cancer.<sup>[6,10,11,24]</sup> Three studies investigated gastric carcinoma,<sup>[18,24,25]</sup> while a single study featured oral cancer cell line SCC-9.<sup>[5]</sup>

MCF-7 was the commonly used breast cancer cell line.<sup>[6,8,10,24]</sup> Liu *et al.*, 2017, employed MCF-7 to test the cytotoxic effect of EFE of *E. fetida*. The cells began to undergo apoptosis after 24 h. Focal adhesion kinase (FAK) and CD44v6 expression was measured using reverse transcription polymerase chain reaction (RT-PCR) and Western blot. Downregulation was observed in a concentration-dependent manner (20–80 µg/ml), resulting in the suppression of MCF-7 cells' adhesion. The authors concluded that EFE displayed an antitumor effect on MCF-7 cells *in vitro*, revealing the therapeutic potential of *E. fetida*.<sup>[6]</sup>

In 2013, Fiolka *et al.* employed a breast ductal carcinoma cell line T47D along with endometrioid ovarian cancer line TOV-112D. The cytotoxicity was determined at 20% and 15% against the T47D line and TOV-112D line, respectively. The PPC exerted a cytopathic effect against the T47D cell line, whereas in the TOV-112D line, it caused a reduction in the cell number. The PPC promoted tumor cell death through apoptosis and necrosis.<sup>[11]</sup>

HeLa cell line was the most commonly employed cell line to assess cytotoxicity.<sup>[12,13,20,21,23,24]</sup> In 2007, a study by Yanqin *et al.* proved that the cytotoxicity of coelomic fluid on HeLa cell line in a concentration-dependent manner after 48 h treatment as observed by the 3-(4, 5dimethylthiazol2yl)-2, 5diphenyltetrazolium bromide (MTT) assay. This coelomic fluid (1 mg/ml) showed toxic effects on HeLa cells with an inhibition rate of 84.22%, leading to cell lysis. The inhibition rates at concentration of 0.1 and 0.01 mg/ml treatments were 10.24% and 2.99%, respectively. The acridine orange/ethidium bromide (AO/EB) staining of 0.1 mg/ml and 0.01 mg/ml concentration showed apoptotic rates of 79.1% and 22.2%. The agarose gel electrophoresis of DNA revealed a smear pattern at the concentrations of 0.1 mg/ml and 0.01 mg/ml of coelomic fluid.

The coelomic fluid at a concentration of 0.1 mg/ml promoted apoptosis in HeLa cells in a time-dependent manner. This work suggests that some of the coelomic fluid components might be helpful for pharmaceutical applications in the cancer therapy.<sup>[20]</sup>

HEP-2 cell line which is a HeLa contaminant was used by Engelmann *et al.*, 2004, to test cell-free coelomic fluid, supernatants of short-term cultured coelomocytes, and lysates from coelomocytes along with other cell lines such as HeLa, PC-12, and PA317. The earthworm coelomic fluid significantly decreased the ratio of living cells compared to controls in a dose-dependent manner.<sup>[23]</sup>

SiHa cell lines were used by Mohamed Jaabir *et al.*, 2011, and Veeramani *et al.*, 2010.<sup>[14,15]</sup> Both studies evaluated the effect of coelomic fluid of *E. eugeniae* on cervical cancer cells by MTT assay followed by fluorescent staining with AO/EB, Hoechst 33258 staining, and DNA ladder assay. Results showed that the coelomic fluid had a dose-dependent effect on SiHa cells.

Colorectal cancer HCT cell line was employed in several studies.<sup>[7-9,24,25]</sup>

In 2015, Reddy *et al.* used HCT-116 cell line with PC-3 and MCF-7 line and proposed that the earthworm paste of *P. excavatus* showed anticancer activity at IC<sub>50</sub> values of 87.45, 104.8, and 239.1 µg/ml against these cell lines. *E. eugeniae* and *E. fetida* also showed IC<sub>50</sub> values of 320.9 µg/ml, 321 µg/ml and 14.18 µg/ml, 25.95 µg/ml in MCF-7 and HCT-116 cancer cells, respectively, but no activity against PC-3 cells. DNA ladder assay, clonogenic assay, and cell cycle analysis were also performed. The authors stated that earthworm paste at concentration 320 µg/ml showed

dose-dependent cytotoxicity with a maximum cell cycle arrest of 25.85%, 27.88%, and 30.03% for *P. excavatus*, *E. eugeniae*, and *E. fetida* at G2/M phase, respectively, and promoted apoptosis. The authors concluded that further research on the principal active components of earthworm pastes of the three mentioned species would lead to the development of novel drugs to treat human cancer.<sup>[8]</sup>

K562 was the leukemic cell line preferred by many researchers.<sup>[12,21,22,25,27]</sup> Jianmin *et al.*, 2001, stated that, at concentrations of 10–20 mg/L, the earthworm extract could facilitate the proliferation of K562 cells, at concentrations of 50 mg/L and above the cell numbers declined slightly. The morphology of apoptosis, characteristic DNA ladder on DNA gel electrophoresis appeared, and apoptotic cells were accounted for 30.3% by fluorescence assay.<sup>[27]</sup>

A plethora of cell lines were used for hepatocellular carcinoma. The SMMC-7721 cancer cells were evaluated *in vitro* by Chen *et al.*, 2008. EFE (2, 4, and 6 uku/ml) could inhibit the adhesion of SMMC-7721 to Matrigel and the ability of cell migration and chemotaxis of SMMC-7721 with statistical significance. The mechanism may be associated with the downregulation of FAK, suggesting that EFE has a potential value in preventing metastasis of hepatocellular carcinoma.<sup>[17]</sup> SMMC-7721 was evaluated *in vivo* by Wang *et al.*, 2009, who developed xenograft with SMMC-7721 cells in nude mice and evaluated the EFE. It was found that EFE could inhibit the expression of CD44v6 protein by immunohistochemistry and Western blot, the inhibitory rates were 47.16% and 28.37%, respectively. On validation, RT-PCR also showed that EFE could inhibit the expression of CD44 v6 mRNA with an inhibitory rate of 16.44%. The EFE inhibitory effect on hepatoma xenografted tumor and synergistic antitumor activity with 5-Fu was well appreciated.

Chen *et al.*, 2007, employed the maximum number of hepatoma cell lines, namely, HLE, Huh7, PLC/PRF/5, and HepG2. Furthermore, the authors tested an *in vivo* mice model (Male BALB/c AnNCrj-nu nude mice, 5 weeks old). The IC<sub>50</sub> values for HLE, Huh7, PLC/PCF/5, and HepG2 were 2.11, 5.87, 25.29, and 17.30 uku/ml, respectively. The growth of tumor xenograft of Huh7 cells in nude mice was significantly inhibited *in vivo*, with subsequent administration of EFE orally for 4 weeks. The tumor inhibitory rates were satisfactory. The fluorescent staining with AO/EB revealed that the apoptotic morphology in the EFE-treated groups compared to untreated groups. Posttreatment with EFE assessed by Western blot assay showed that the secretions of MMP-2 were significantly inhibited in HLE and Huh7 cells.<sup>[19]</sup>

The Bel7402 cell line was used by LI Hong yan *et al.*, 2004, along with BGC823, MCF-7, HCT 8, and A549 to investigate the anticancer potential of EFE. Xenotransplanted nude mice models with human cancer and experimental implanted tumor mice model were used to evaluate its antitumor activity. No inhibition was observed in BGC823, MCF7, HCT 8, A549, and Bel7402 cancer cell proliferation at 50 µg/L. EFE inhibited human xenograft in nude mice with human gastric cancer BGC823 and breast cancer when administered at 200–1000 mg/kg for 15–17 days in a dose-dependent manner.<sup>[24]</sup>

The study of Jiang Bi *et al.*, 2003, used five cell lines in *in vitro* (HCT 116, SY5Y, K562, MGc803, and HeLa) and an *in vivo* model (Ascites tumor [S180]-bearing mice) to evaluate the anticancer effect of *E. fetida* extract. It was found that a 50% growth inhibition was observed in different human tumor cell strains (HCT 116, SY5Y, K562, MGc803, and HeLa) at a concentration between 60 and 110 mg/L. The inhibition of normal cell strains such as HEK293 and COS 7 was much weaker than those of human tumor cell strains tested. It was observed that *E. fetida* extract at doses such as 28 mg/kg and 36 mg/kg showed prolonged lifespan of ascites tumor (S180)-bearing mice by 135.3% and 123.5%, respectively. Meanwhile, treatment with *E. fetida* extract was reported to be less toxic than with that of cyclophosphamide treatment (76.5%).<sup>[25]</sup>

## Cytotoxicity tests

Cytotoxicity studies broadly involve the metabolic modifications of the cells including the death of cells due to toxic effects of the compounds. Several assays have been developed for measuring the cell viability and cytotoxicity. *In vitro* testing for safety evaluation and cytotoxicity eliminates the use of animals and it is cost-effective.<sup>[39]</sup>

Based on the literature review conducted, the MTT assay was the preferred investigation for cytotoxicity testing.<sup>[5,7-10,12-15,18,20,21,23]</sup> MTT-based cytotoxicity assay is performed in the following stages:

- i. Incubation of the monolayer cultures with fluctuating drug concentrations in the microtiter plates
- ii. The treatment of plates with MTT and then the removal of the medium and MTT
- iii. The measurement of the MTT-formazan in an enzyme-linked immunosorbent assay plate reader
- iv. A sigmoid curve is obtained when the absorbance of control or test wells of the microplate is plotted against the cytotoxic drug concentration.

Measurements of metabolic responses of cells are the basis of the metabolic assay. These tests are done after exposure of the cells to cytotoxic drugs by either immediately incubating or incubation after 2–3 population doublings. The commonly used metabolic measurements are protein synthesis besides the assay of dehydrogenase enzymes.<sup>[39]</sup>

## Adjunctive investigations

The abnormalities in cancer result from mutations in the proteins' coding for specific genes that regulate cell division. Genes that repair these proteins can also undergo mutation and become defective. Consequently, the mutations increase and accumulate. These mutations produce defects in the daughter cells or the progeny. Some of these mutated cells undergo apoptosis and die, whereas the others survive and become immortal, thus they develop features of limitless replication potential and transform into full-fledged cancer.

Advanced investigations are often required in anticancer research as several phenomena occur at the molecular level. The literature reviewed showed that cell cycle analysis, flow cytometric assay, RT-PCR, and Western blot were performed in few studies only.<sup>[6,8,9,16-18]</sup> Experimental studies at the genomic and proteomic level are the need of the hour to determine the exact molecular mechanisms, genes, and pathways responsible for the anticancer activities of earthworm extracts. Natural ways to prevent cancer recurrence is now the latest trend in cancer therapeutics. Naturally available extracts have been sought after in this regard as an adjunctive therapeutic modality.<sup>[40-45]</sup>

Least researched cancers such as oral cancer should also be evaluated in future studies as oral cancer comprises a significant disease burden in India being the most common type of cancer. Globally, it is the 6<sup>th</sup> most common cancer in males and the 12<sup>th</sup> most common cancer in females. Approximately 94% of all oral malignancies are squamous cell carcinoma.<sup>[46]</sup>

Following critical appraisal of literature, it is clear that earthworm extracts have a satisfactory anticancer effect on cancer cells. However, their mechanisms of action and specific pathways on cancer cells are largely unknown. The potential research gaps or issues that need further research, and the dearth of studies to prevent proliferation is a noted feature; extensive research is required to further investigate anticancer effect of these molecules.

## CONCLUSION AND FUTURE AVENUES

The present review has summarized the current status of the anticancer effects of earthworm extracts. Potential benefits by addressing these



gaps would be the development of earthworm extracts as a favorable pharmacological agent. The earthworm extracts can be developed into an adjunctive anticancer drug. Following these results, the antimetastatic effect of earthworm extracts can also be evaluated. Based on the discussions and review, it is reasonable to conclude that there is enough scope to investigate “anticancer activity of earthworm extracts on cancer cells.” The current study highlights the importance of earthworm extracts as a pharmacological strategy, suggesting their role in the therapeutics of cancer as well as their possible use as co-adjuvants in modern management therapies.

However, further experimental studies at the molecular level with cell receptor docking analysis and gene expression results are required to ascertain the pathways and genes responsible for the anticancer effect and thereby scientists can exploit the beneficial aspects of the earthworm extracts.

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## Conflicts of interest

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