http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 25(1): 111-120, 2024
ISSN 2528-9691
DOI: 10.23902/trkjnat.1373797

Systematic Review

A systematic review of antiproliferative and antitumour activity of earthworm extracts

Burcu Yuksel[1*], Asuman Deveci Ozkan[2]

[1] Department of Medical Services and Techniques, Kocaeli Vocational School of Health Services, Kocaeli University, Kocaeli, TÜRKİYE
[2] Department of Medical Biology, Faculty of Medicine, Sakarya University, Sakarya, TÜRKİYE

Cite this article as:

Yuksel B. & Deveci Ozkan A. 2024. A systematic review of antiproliferative and antitumour activity of earthworm extracts. *Trakya Univ J Nat Sci*, 25(1): 111-1120, DOI: 10.23902/trkjnat.1373797

Received: 10 October 2023, Accepted: 17 January 2024, Online First: 11 March 2024, Published: 15 April 2024

Edited by:
Melike Sapmaz Metin

Corresponding Author:
Burcu Yuksel
burcu.yuksel@yahoo.com

ORCID iDs of the authors:
BY. 0000-0001-8691-6447
ADO. 0000-0002-3248-4279

Key words:
Anticancer effect
Antiproliferative
Cancer therapy
Coleomic fluid
Eisenia foetida
Colon cancer

Abstract: Earthworm extracts have become an interesting research area in cancer research due to their ingredients with anticancer and antiproliferative activities. Determining the anticancer effect of an extract on cancer cells may lead to new treatment strategies and alternative medicine to at least reduce the toxic effects of chemotherapeutic agents. In the present study, we aimed to raise awareness for drug development and future anticancer therapeutics by emphasizing the nature of earthworm extracts and the types of cancer they are used in through a systematic review. A systematic search of CINAHL, PubMed, Medline, Scopus and Web of Science databases was conducted to identify publications published between January 2018 and December 2022. We used different arrangements of the keywords 'earthworm', 'earthworm extract' and 'anticancer effect'. We identified original studies written in English depicting in vitro and in vivo cytotoxic effects of earthworm extracts on cancer cells, and included these studies in our review. We found 15 studies matching our search criteria. Among the studies, Eisenia foetida (Savigny) was determined as the most studied earthworm, and colon cancer was the most frequently studied cancer type. The coleomic fluid was found to be the commonly used extract yielding positive results.

Özet: Solucan özleri, kanseri durdurucu ve önleyici çeşitli maddeler içermesi nedeniyle kanser araştırmalarında ilgi çekici bir araştırma alanı haline gelmiştir. Ekstraktın kanser hücreleri üzerindeki antikanser etkisinin belirlenmesi, kemoterapötik ajanların toksik etkilerine karşı yeni tedavi stratejilerine ve alternatif tıp yöntemlerine ilgiyi artırabilir. Bu nedenle bu çalışma, solucan ekstraktlarının doğasını ve kullanıldıkları kanser türlerini sistematik bir inceleme yoluyla vurgulayarak ilaç geliştirme ve gelecekteki antikanser tedavileri için farkındalık yaratmayı amaçlamaktadır. Ocak 2018 ile Aralık 2022 arasında yayınlanan makaleleri belirlemek için CINAHL, PubMed, Medline, Scopus ve Web of Science veritabanlarında sistematik bir arama yapıldı. 'Solucan', 'solucan özü' ve 'antikanser etkisi' anahtar kelimelerinin çeşitli düzenlemelerinden yararlandık. Solucan ekstraktlarının kanser hücreleri üzerindeki in vitro ve in vivo sitotoksik etkisini gösteren İngilizce yazılmış orijinal çalışmaları incelemeye dahil ettik. Arama kriterlerimizle eşleşen 15 çalışma bulduk. Yapılan çalışmalar arasında en çok çalışılan solucan türü Eisenia foetida (Savigny) olurken, en sık çalışılan kanser türü ise kolon kanseri oldu. Koleomik Sıvının antikanser aktivitesini pozitif sonuçlarla test etmek için yaygın olarak kullanılan ekstrakt olduğu bulundu.

Introduction

Cancer, in general, is one of the major causes of human death in global scale (Hanahan 2022, Ozkan *et al.* 2023). Cancer cells are distinguished by a physiological change that causes abnormal cell differentiation and uncontrolled proliferation (Ozkan & Yuksel 2022). Specific challenges faced in fight against cancer are preventing its formation, preventing the growth of the tumour and destroying tumour cells. Metastasis, is a complex multi-step event where the growth of malignant tumours are observed (Kruk *et al.* 2019). The understanding details of biological properties of cancer cells can prevent their proliferation and the formation of metastases. It is therefore important to design studies in order to identify biomolecules that can inhibit the cancer

cell proliferation. Surgery, chemotherapy, radiation and immunotherapy are the current approaches used to reduce the death rate related with cancer cases. However, the search for novel and better therapeutic modalities to halt tumour progression is needed. Because chemotherapeutic treatments cause systemic toxicity and impair life quality (Chabner & Roberts 2005, Yuksel *et al*. 2022), potential chemotherapeutic agents can be selected from natural products with less toxic effects. Extracts obtained from natural products have antioxidant effects with their oxidative radical scavenging feature, an adjunct cancer treatment (Yuksel & Deveci Ozkan 2021).

Earthworms, which have important functions in the soil ecosystem and plant growth environment, are attracting the attention of the researchers as a possible source of novel therapeutic candidate compounds due to the availability of various bioactive secondary metabolites (Sadek *et al.* 2022). The steadiness and effectiveness of earthworm metabolites make them an alternative source to unravel novel pharmaceuticals to be used in curative medicine (Wahyuni & Waluyo 2018). Earthworm extracts are one such natural source for metabolites that contains proteases and are reported to have antiproliferative potential (Ozkan *et al*. 2022). The coelomic fluid (CF) found in all earthworms and the cells, termed as coelomocytes, derived from CF include biologically active substances which led researchers to investigate the ways earthworm extracts could be used in cancer treatment. CFs obtained from different earthworm species have been reported to have various therapeutic properties such as fibrinolytic, antibacterial, antiinflammatory, analgesic, anticancer, antiviral, cytotoxic and antioxidant (Endharti *et al.* 2019, Mustafa *et al.* 2022, Sadek *et al*. 2022, Ozkan *et al*. 2022, Hussain *et al.* 2023). Potent bioactive molecules are naturally found mostly in the intestinal fluids of earthworms and then pass into the tissue fluid, where they function in defense reactions and immune responses against the invasion of microorganisms (Gupta & Yadav 2016). These bioactive molecules, with surprising biological properties, in earthworms can be used as anticancer drugs because they have peptide molecules that can constrain the spread of cancer cells (Czerwonka *et al*. 2020). Lysine, serine proteases, G-90 Glycoprotein and earthworm fibrinolytic enzymes are among the cytotoxic components of CF, especially stemming from the coelomocytes of the species *Eisenia foetida* (Savigny), which cause the lysis of target cells (Małota *et al.* 2022). CF and coelomocyte lysates inhibit cell viability by triggering apoptosis in cancer cell lines (Fiołka *et al.* 2019).

Determining the antiproliferative effects of earthworm extracts on cancer cells may lead to new treatment strategies and development of alternative medicine to replace chemotherapeutic agents with toxic effects. Therefore, this study aims to raise awareness for drug development and future anticancer therapeutics by emphasizing the nature of earthworm extracts used and the types of cancer they are used in relation with through a systematic review.

Materials and Methods

Key question

Key questions were identified based on the Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines. The questions are:

- Do earthworm extracts have a significant anticancer effect on cancer cells in tumour tissues?
- Which earthworm species and fluid are most used?
- Which type of cancer has been studied the most with earthworm's fluids?

Study design and search strategy

The study was based on available databases of CINAHL, PubMed, Medline, Scopus, and Web of Science (WoS) internet resources. Our scheme, designed to summarize the results of published studies to identify and analyze the anticancer potential of earthworm extracts, is shown in Fig. 1.

All selected databases were searched to reach the full-length research articles published within the period from January 2018 to December 2022, describing the species of earthworms used, the nature of the earthworm extract and the type of the cancer. In subsequent critical evaluation of the studies, "earthworm", "earthworm extract" and "anticancer effect" was searched using combinations of keywords (Table 1).

Features of articles included in the study.

The publications included in the study are written in English and were all original research articles. The systematic review included *in vitro* and *in vivo* studies focusing on the anticancer effect of earthworm and earthworm extracts.

Table 1. Methodology employed for the systematic review.

Statement of the objective	Method/methodology	Resources used	Keywords
Research articles that analyze and explain the potential anticancer effects of earthworms and the biological activities of earthworms extracts	Collection of articles describing the species of earthworms used, the nature of the earthworm extracts, and the types of cancer studied, followed by a critical evaluation of the studies	CINAHL, PubMed, Medline, Scopus, and WoS	earthworm, earthworm extract and anticancer effect

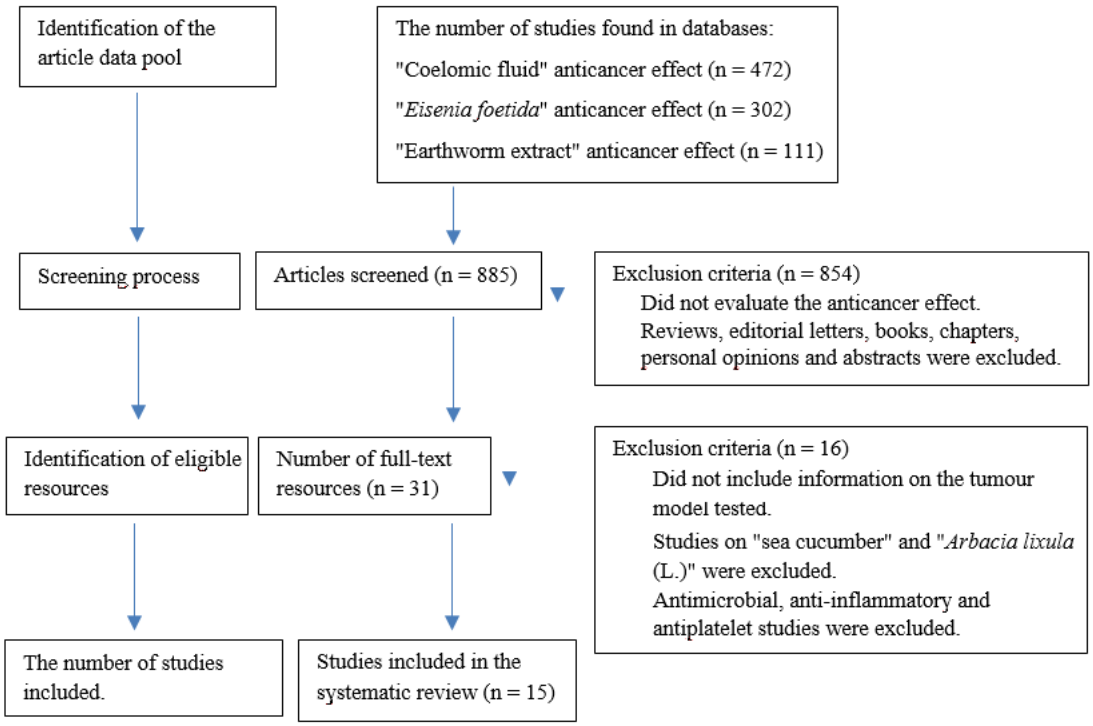

Fig. 1. Study design according to PRISMA guidelines for a systematic review.

Features of articles not included in the study.

1. Articles other than anticancer evaluation of earthworm extracts,
2. Publications such as short articles, editorial articles, letters, book chapters and personal opinions,
3. Studies that do not specify the nature of the earthworm extracts and the species of the earthworms used,
4. Antimicrobial, antiviral, antibacterial and antiinflammatory studies using earthworm extracts,
5. Articles that did not contain information about the treated cancer cell and tumour model,
6. Studies on "sea cucumber" and "*Arbacia lixula*" and
7. Studies in non-cancer cells were excluded from the review.

Data collection process

The titles and abstracts of the articles were scanned. Original article type studies providing full text information were examined in more detail. Considering that some relevant articles may have been missed during the database search, the references of the found articles were also investigated. The data presented in the found articles were analyzed and included in the study.

Results

The earthworm species, cancer type, extract, biochemical characterization of protein, the protein composition of the coelomic fluid, antiproliferative potency, in vitro/in vivo assays cell line, adjunctive tests and country information of the articles included in the study are summarized in Table 2.

A total of 15 studies meeting the inclusion criteria were analyzed in this systematic review. As can be seen in Fig. 2, most of these studies were *in vitro*. Moreover, majority of the studies were carried out in Indonesia and Poland (Fig. 2).

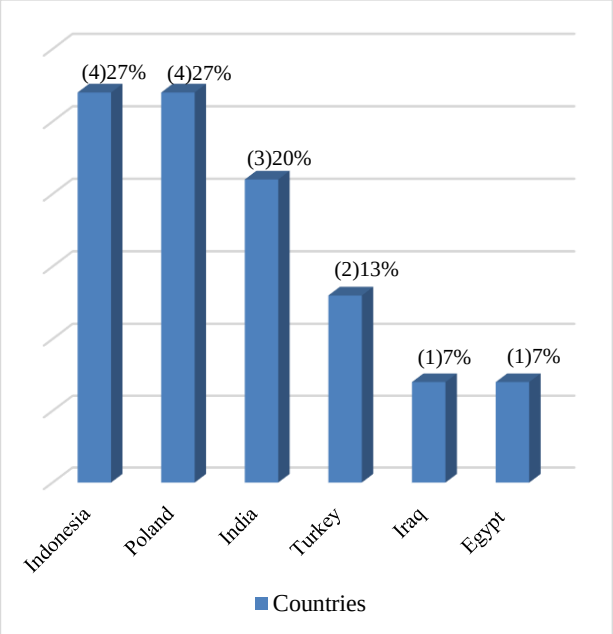

Fig. 2. Distribution of the studies according to the countries.

Table 2. The list of extracted information summary of the selected articles.

Earthworm species	Cancer Type	Extract	Biochemical characterization of protein	Protein composition of coelomic fluid	Antiproliferative potency	Assays	Cell line	Adjunctive tests	Countries	References
Allolobophora caliginosa (*Savigny*)	Hepatoma	Coelomic fluid	HPLC, the Biuret method, Automatic Amino Acid Analyzer	non-essential amino acids constitute 54.70 % (Glutamic acid most content), the essential amino acids constitute 45.30% (Leucine most abundant)	145.99 µg/mL	*in vitro*	HepG2	Eddy's hot plate method, Brewer's yeast-induced pyrexia assay, DPPH assay,phosphomolybdenum assay, the human red blood cell (HRBCs) membrane stabilization technique	Egypt	Sadek *et al*. 2022
Lumbricus terrestris L.	Breast Cancer and Prostate Cancer	Earthworm powder			for PC-3 265.5 µg/mL and MCF-7 965.9 µg/mL	*in vitro*	MCF-7 and PC-3		Iraq	Shafi & Faleh 2019
Eudrilus eugeniae Kinberg	Oral Cancer	Coelomic fluid	MALDITOF & MS-MS Sequencing, SDS-PAGE, Nano-LCMS based Amino Acid Sequencing	158 amino acids	10 and 20 µg/mL 18 ECFP	*in vitro*	SCC-9	RT-PCR and Q-PCR	India	Augustine *et al.* 2022
Eisenia foetida	Prostate Cancer	Coelomic fluid	Bradford method, LC-MS/MS	Lysenin and Lumbrokinase	1 and 10 µg/mL	*in vitro*	PC-3	(RT-PCR) and western blotting, COMET and CBMN analysis, Annexin V Assay, Acridine Orange (AO) Assay	Türkiye	Ozkan *et al.* 2022
Eudrilus eugeniae, Eisenia fetida (Savigny*), Perionyx excavates* Perrier	Oral Cancer	Coelomic fluid	Lowry's method,	serine protease	For EE 4.6 µg/ml, EF 44.69 µg/ml and (PE) 5.27 µg/ml	*in vitro*	KB 3–1	3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, Zymography, SDS-PAGE	India	Augustine *et al.* 2018
Dendrobaena veneta (Michaelsen)	Lung Cancer	Coelomic fluid	Bradford assay		250 µg /mL	*in vitro*	A549	ELISA, Scanning electron microscopy, atomic force microscopy (AFM), fluorescence microscopy, optical microscopy, flowcytometry	Poland	Fiołka *et al.* 2019
Lumbricus rubellus Hoffmeister	Colon Cancer	Coelomic fluid			200 µg/g	*in vivo*	HT-29	Flowcytometry and immunofluorescent	Indonesia	Permana *et al.* 2019

Table 2. The list of extracted information summary of the selected articles (Continued).

Earthworm species	Cancer Type	Extract	Biochemical characterization of protein	Protein composition of coelomic fluid	Antiproliferative potency	Assays	Cell line	Adjunctive tests	Countries	References
Lumbricus rubellus	Colon Cancer	Coelomic fluid			20 μg/m with 5-FU	*in vitro*	HT-29	Flowcytometry and immunofluorescent	Indonesia	Endharti *et al.* 2019
Eisenia fetida	Colon Cancer	Coelomic fluid			120 mg/mL with setuksimab	*in vivo*		Flowcytometry and immunofluorescent	Indonesia	Permana *et al.* 2020
Lumbricus rubellus	Colon Cancer	Coelomic fluid			20 µg/ml with 5-FU	*in vitro*	HT-29 cells	Flowcytometry, MTT assays	Indonesia	Permana *et al.* 2018
Dendrobaena veneta	Colon Cancer	Coelomic fluid	Bradford method		16,8 and 4 µg/ml (unheated)	*in vitro*	HCT116, A549 and BEAS-2B	MTT assay, fluorescence measurements, flowcytometry, Transmission electron microscopy	Poland	Małota *et al.* 2022
Eudrilus eugeniae, Eisenia foetida, Perionyx excavatus	Oral Cancer	Coelomic fluid	Bradford method	the total protein content of EE, EF and PE as 1.34 mg/ml, 1.53 mg/ml and 1.9 mg/ml	40 80 µg/ml	*in vitro*	SCC-9	Annexin V – FITC/PI assay, comet assay, Lactate dehydrogenase (LDH) assay, Clonogenic assay, Cell cycle analysis	India	Augustine *et al.* 2019
Dendrobaena veneta	Lung Cancer	Coelomic fluid		lysine-related protein 2 (LRP2) and lysine ubiquitin fragments	31.3 and 62.5 µg/ml (venetin 1)	*in vitro*	A549 cancer cells	MTT analysis, Annexin V-FITC/PI assay, flowcytometry, Atomic Force Microscopy, Proteomic analysis, light microscopy, scanning electron microscopy, Transmitted light microscopy	Poland	Rybicka *et al.* 2022
Dendrobaena veneta	Colon Cancer	Coelomic fluid	Bradford method	active protein-carbohydrate fraction (Lysozyme-like peptides)	163 µg/mL (LS180) 38,43 µg/mL (HT-29)	*in vitro*	HT-29 and LS180	Annexin V/PI, Hoechst 33342 staining and active caspase-3 assays, NR-uptake, LDH and May-Grünwald-Giemsa (MGG) assays,MTT and BrdU assays		Czerwonka *et al.* 2020
Eisenia foetida	Breast Cancer	Coelomic fluid	Bradford method, LC-MS/MS	Lysenin and Lumbrokinase	1,5 and 10 µg/mL With NaBu	*in vitro*	MCF-7	Reactive oxygen species (ROS) microplate assay, Imaging of nucleus morphology, RT-PCR	Türkiye	Ozkan *et al.* 2022

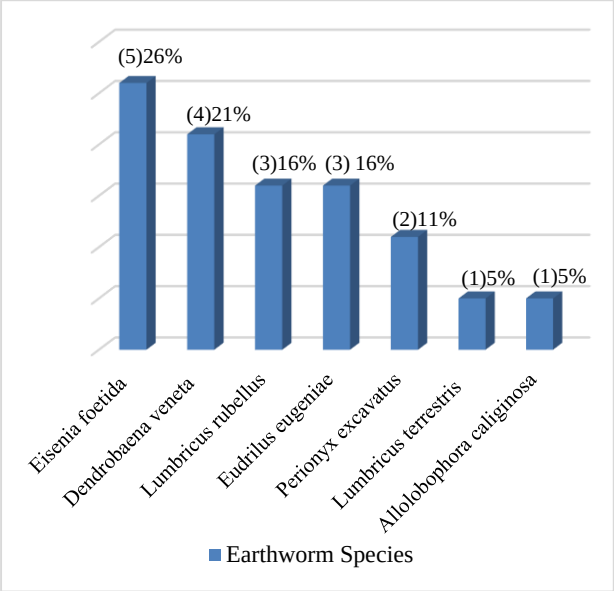

Fig. 3. Distribution of the earthworm species.

Earthworm species

When Fig. 3 is examined, it can be seen that *Eisenia foetida* and *Dendrobaena veneta* were the most commonly studied species while *Lumbricus terrestris* and *Allolobophora caliginosa* were found in only one study.

Cancer types studied

Fig. 4 demonstrates that colon and oral cancer were the most frequently studied cancer types. However, hepatoma cancer was studied in a single study.

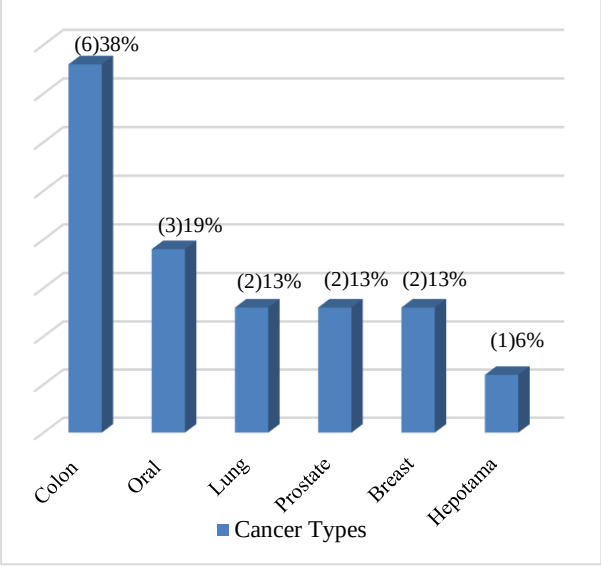

Fig. 4. Distribution of the studies according to the cancer types earthworm species.

The nature of the extract used

Coelomic fluid was determined as the widely used data to test for anticancer activity with positive results, except one study performed with earthworm powder (Shafi & Faleh 2019).

Discussion

The use of natural extracts in search for prevention of proliferation of cancer cells and treating cancer has increased in recent years. In addition, researchers are also investigating potentials of alternative treatment therapies and drugs to prevent cancer progression. Earthworm and earthworm extracts are abundantly present in nature and they show cytotoxic effects against cancer cells, arrest the cell cycle, and elucidate the mechanisms by which these extracts have anticancer effects, and will give researchers promising results in the field of cancer treatment. This review on earthworm extracts with antiproliferative potential, which we can define as sources of biomolecules against cancer, can be a source those involved in biomedical research.

Earthworm species

The findings of our review demonstrated that *Eisenia foetida* was the most studied earthworm species, followed by *Dendrobaena veneta*, *Eudrilus eugeniae*, *Lumbricus rubellus*, *Perionyx excavatus*, *Lumbricus terrestris* and *Allolobophora caliginosa*. Augustine *et al.* (2022) used earthworm coelomic fluid (CF) of *Eudrilus eugeniae* to reduce the side effects encountered in oral cancer pharmacotherapy and overcome the limitations of treatment failure. The results of their study identified that the CF of *E. eugeniae* could be added to the list of agents that are naturally available in the fight against oral cancer. Augustine *et al.* (2018) also stated that they studied the anticancer properties of *Perionyx excavatus*, a species found in the Indian peninsula and rarely researched for its beneficial medicinal effect, for the first time. Also Sadek *et al.* (2022) is the only team used *Allolobophora caliginosa* CF (ACCF), an Egyptian earthworm, on hepatoma.

Shafi & Faleh (2019) studied the impact of one of the species of earthworm, i.e., *Lumbricus terrestris*, on breast and prostate cancer. Shafi & Faleh's study is the only earthworm species study found in the literature from 2018-2022, which was the focus time frame of the current review. In contrast to the other similar studies, the researchers used earthworm powder and stated that it was the first study to test the anticancer effect of an earthworm species in Iraq. *Eisenia foetida* is the most studied species, as revealed as least in our database search, which was also systematically reviewed in this study (Augustine *et al.* 2018, Permana *et al.* 2020, Deveci Özkan *et al.* 2022, Ozkan *et al.* 2022). The systematic review of Augustine *et al.* (2018), who included the period from 2001 to 2017 in their study, also stated that *E. foetida* was the most preferred species in anticancer research. *Dendrobaena veneta* was used in the studies conducted by Fiołka *et al.* (2019), Czerwonka *et al.* (2020), Małota *et al.* (2022) and Rybicka *et al.* (2022). In the study of Augustine *et al.* (2018), this species was found in only one out of 23 research articles, while it was identified in four articles in our study. Thus, we can argue that, in recent years, *D. veneta* has been one of the preferred species in anticancer research. *Lumbricus rubellus* was found to be used in

three studies (Permana *et al.* 2018, Endharti *et al.* 2019, Permana *et al*. 2019).

Cancer types and cell lines studied

Sadek *et al.* (2022) reported that the coelomic fluid of the *Allolobophora caliginosa* showed a cytotoxic effect against malignant tumours evaluated by using its antitumour potential against human hepatoma cell line (HepG-2). Shafi & Faleh (2019) evaluated the antitumour activity of *L. terrestris* against breast cancer cells (MCF-7) and prostate cancer cells (PC-3) and reported that earthworm powder had a cytotoxic effect against these two cancer cell lines. They found that earthworm powder applied to cancer cell lines impairs the mitochondrial membrane permeability, increases the release of cytochrome C, and induces apoptosis.

Augustine *et al.* (2022) investigated the antitumour effect of CF of *Eudrilus eugeniae* on oral cancer cell line SCC-9 and determined the binding property of CF with the pro-apoptotic genes Caspase-3 and Caspase-8. As a result, it was stated that the protein obtained from the earthworm source could contribute for developing naturally available drugs to be used for the fight against oral cancer. Özkan *et al.* (2022) applied CF obtained from Eisenia foetida to prostate cancer (PC-3) cells and reported that CF was effective in these cells. Augustine *et al.* (2019) evaluated the time- and dose-contingent antiproliferative impact of CF of *E. eugeniae*, *E. foetida* and *Perionyx excavatus* on the oral cancer cell line KB 3-1 and revealed that the CFs promisingly have antiproliferative effect. Fiołka *et al.* (2019) found that CF of *Dendrobaena veneta* showed cytotoxicity when used in fight A549 lung cancer cells but not in the bronchial epithelial cell line BEAS-2B. This discriminatory impact on tumour cells emerges as important data in drug research effective against lung cancer.

Permana *et al.* (2019) tested CF of *Lumbricus rubellus in vivo* to investigate the antiproliferative activity in colorectal cancer, and found that the mixture of 5-Fluorouracil (5-FU), used in cancer treatment, with CF significantly inhibited the growth of colorectal cancer formed by HT-29 cells in mice. This study is a good example of reducing the adverse effects of chemotherapy drugs using CFs. In the same way, Permana *et al.* (2018), investigated *in vitro* whether *L. rubellus* could decrease or increase the FAK protein level, iCa^{2+} and p21 levels by applying CF and 5-FU combination to HT-29 cell lines. The researchers found that the combination significantly reduced the expression of FAK, iCa^{2+} and p21 in the cells. They also determined that CF might potentially have an anticancer impact in colorectal cancer when combined with 5-FU. In another *in vivo* combination study, Permana *et al.* (2020) investigated the anticancer effect of combining the CF of *E, foetida* and cetuximab on colorectal cancer and found that it significantly reduced the growth of cancer cells. The same researchers combined *Lumbricus rubellus* CF with 5-FU to test its cytotoxic effect on HT-29 cells and determined that the combination decreased the ratio of the G2/M phase of the cell cycle (Permana *et al.* 2018). Małota *et al.* (2022) investigated, for the first time, the effects of *Dendrobaena veneta* CF on the colon cancer cell line HCT116 in two ways, with and without heating CF. They determined that low doses of unheated CF, in particular, caused proliferative and pro-oxidative impact on the tested cell lines. Augustine *et al.* (2019), as a continuation of their work in 2018, examined the cytotoxic effects of CFs of *E. eugeniae*, *E. foetida* and *Perionyx excavatus* on the oral cancer cell line SCC-9, cell cycle analysis, and on cell death mechanism. They observed that the cell cycle stopped in the G2/M phase. It was stated that the cytotoxic effect of CF of *E. eugeniae* was superior, followed by CF of *P. excavatus.* Rybicka *et al.* (2022) investigated the antitumour effects of Venetin-1, a protein-polysaccharide complex obtained from CF of *D. veneta*, against to lung cancer cell line A549 cells and found that the cells died by apoptosis. In another study with CF of *D. veneta*, the active protein-carbohydrate fraction isolated from CF disrupted the cell cycle in human colon adenocarcinoma (HT-29 and LS180) cell lines, inhibited 20S proteasome activity, and procaspase-3 (Czerwonka *et al.* 2020). The same study showed that the isolated active protein-carbohydrate fraction did not affect the viability and morphology of normal human colonic epithelium (CCD 841 CoTr) cells. This can be considered as an indication that CF does not affect normal cells in anticancer studies. Deveci Ozkan *et al.* (2022) sought to remove or lessen the high cytotoxicity of sodium butyrate (NaBu), a chemotherapeutic agent used in the treatment of breast cancer, for both neoplastic and normal cells. The application of CF of *E. foetida* to breast cancer MCF-7 cell lines revealed that the expression levels of Bax and Bcl-2 genes did not change, and the amount of ROS decreased significantly. As a latent therapeutic molecule with rarer side effects that can be practically used in the treatment of cancer, researchers reported CF. All these data and studies show that earthworms and their CF have antiproliferative activity against human cancer cells and a cytotoxic effect inhibiting increased apoptosis. Considering that current cancer treatments have vital side effects and have impact on the life quality of patients, CFs may be an impending beneficial agent with less side effects in cancer treatment. Therefore, researchers seek additional naturally available treatments to reduce tumour proliferation without causing significant adverse reactions.

Biochemical composition of earthworm extracts used

Detection of the biochemical structure of active biomolecules with antitumour activity is important for drug development for future cancer research. As revealed in the systematic review we prepared, some researchers conducted preliminary experimental studies to understand the nature of the substances they studied. For example, Sadek *et al.* (2022) investigated the active ingredients, total protein content and amino acid profile of ACCF using HPLC, the Biuret method and an amino acid analyzer. They identified several active phenolic and flavonoid compounds and significant amounts of essential and non-essential amino acids in ACCF. Their results

revealed that the percentage of total bioactive components in gallic acid in ACCF represented the highest concentration at 69.85%, and the presence of p-hydroxybenzoic acid, o-coumaric acid, benzoic acid, cinnamic acid, p-coumaric acid and ferulic acid. As a result of HPLC studies, they identified rutin, catechol, catechin and quercetin flavonoids. They found the total protein content of ACCF to be 496.8 mg/dl. While 54.70% of this protein content is composed of non-essential amino acids (glutamic acid, alanine, and aspartic acid), 45.30% is composed of essential amino acids (leucine, lysine, and isoleucine). Augustine *et al.* (2022) used energy-based methods such as Molecular Dynamics Simulation, Protein-Protein Docking and Prime module-Schrodinger to identify 158 amino acids in the molecular structure of the a18 kDa (18-ECFP) protein in *E. eugeniae* CF. Their results also revealed that it was upregulated by determining the 18-ECFP binding affinity with the pro-apoptotic genes Caspase-3 and Caspase-8.

Augustine *et al.* (2018) investigated a characteristic thick band in the 18-20 kDa region in *E. eugeniae*. The presence of serine protease was detected in this band due to SDS-PAGE and zymography studies. Rybicka *et al.* (2022) found that the main component of the protein-polysaccharide complex Venetin-1, which they obtained from the CF of *D. veneta*, was lysine-related protein 2 (LRP2) and lysine. The SageELF (Electrophoretic Lateral Fractionation) system was used to detect other components of discriminating Venetin-1. They also stated that the ubiquitin fragments identified in the fractions could be a multiprotein complex. Another study isolated the active protein-carbohydrate (AF) fraction from the ECF of *D. veneta* and applied it to cancer cells (Czerwonka *et al*. 2020). The authors stated that AF compounds might be lysozyme-like peptides responsible for the anticancer activity. Finally, Deveci Ozkan *et al.* (2022) examined the protein composition of *E foetida* CF using LC-MS/MS and found that the presence of lysine and lumbrokinase showed toxic properties in cancer.

Antiproliferative potency

As shown in this systematic review, the researcher used a variety of methods to determine the antiproliferative properties of the extracts on tumour cells. Some researchers used only CFs, while others used compounds isolated from CFs. We also observed that there were some researchers who used a combination of CFs with existing chemotherapy drugs and CFs. In the study of Sadek *et al.* (2022), in vitro antitumour potential of ACCF was evaluated using the Sulphorhodamine-B (SRB) assay against the human hepatoma cell line (HepG-2), and the proliferation of HepG2 was found to be inhibited by ACCF with an IC50 value of 145.99 µg/ml. Shafi and Faleh (2019) identified IC50 values of 265.5 and 965.9 µg/ml for PC-3 and MCF-7 cell lines of *Lumbricus terrestris,* respectively. However, in their study, worm dust did not show cytotoxicity against normal cells (WRL68). Augustine *et al.* (2022) treated SCC-9 cells with 18-ECFP protein concentrations of 10 µg/mL and 20 µg/mL to determine their anticancer potential.

In another study, Ozkan *et al.* (2022) examined the antiproliferative effect of CF obtained from *E. foetida* at 1 and 10 µg/mL in AR-insensitive and sensitive PC-3 cells. Moreover, Augustine *et al.* (2018) identified CFs of *E. eugeniae*, *E. foetida* and *P. excavatus* using IC50 GraphPad Prism 7.0 software on SCC-9. IC50 values were 4.6, 44.69 and 5.27 µg/ml, respectively. The results reported in Fiołka *et al.* (2019) demonstrated that maximum cytotoxicity against A549 cells at a concentration of 250 µg/mL CF heated at 70°C for 10 minutes. It showed no cytotoxicity against CF cell lines heated at 90°C for 10 minutes. Permana *et al.* (2019) reported that CF was most effective at a dose of 200 µg/g when applied to cells in combination with 5-Fluorouracil (5-FU) and CF. Endharti *et al.* (2019) applied the combination of CF and 5-FU to HT-29 cells at 5, 10, or 20 µg/ml CF concentrations. In another combination study, tumour cells were treated with CF of *E. foetida* and cetuximab at 30, 60, and 120 mg/kg BW doses (Permana *et al.* 2020). In another combined application study, positive results were obtained when HT-29 cells were treated with 5-FU (5 µg/ml) and 5 µg/ml, 10 µg/ml or 20 µg/ml CF (Permana *et al.* 2018). Małota *et al.* (2022) showed lower concentrations of unheated CF (16, 8 and 4 µg/ml) caused proliferative and pro-oxidative effects. Augustine *et al.* (2019) used 40µg/ml and 80µg/ml CFs for cytotoxic studies. Rybicka *et al.* (2022) reported that Venetin-1 obtained from CF was effective against lung cancer cells at 31.3 and 62.5 µg/ml doses. Czerwonka *et al.* (2020) reported that the IC50 values of the active protein-carbohydrate fraction (AF) isolated from CF used in human colon adenocarcinoma cell lines were reported as 163 µg/mL (LS180) and 38.43 µg/mL (HT-29). In another study which examined the antiproliferative potency, Deveci Ozkan *et al.* (2022) reported that NaBu with CF concentrations of 1, 5 and 10 µg/mL showed cytotoxic effects in MCF-7 cells.

Conclusion

Given that current cancer treatments have significant side effects and impact life quality of patients, researchers are looking for additional naturally available treatments that can reduce tumour proliferation without causing adverse reactions. To this end, combining traditional chemotherapies with new technologies is a promising strategy. The studies analyzed in this systematic review and the data within these studies demonstrated that earthworms and their CF have antiproliferative activity against human cancer cells and a cytotoxic effect that increases apoptosis. Our review highlights that earthworm, and its extracts can be an adjunct pharmacological agent in cancer management. It provides a solid basis for further research on isolating and identifying active biomolecules in earthworms exhibiting antitumour activity.

Based on the implications of this systematic review, we argue that researchers should focus on isolating and

identifying active biomolecules of earthworms with antitumour activity by examining the molecular mechanism and genetic pathways accountable for the antitumour activity of these biomolecules. ECFs may be a likely helpful showing rarer side effects in cancer treatment in the future. As a result of the studies we reviewed, it was concluded that ECFs have a cytotoxic effect on cancer cells, causing cell cycle arrest and triggering apoptosis. Thus, further research to elucidate the anticancer effect mechanisms caused by these extracts will give researchers promising results in cancer treatment.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: B.Y., A.D.O., Design: B.Y., A.D.O., Execution: B.Y., A.D.O., Data analysis/interpretation: B.Y., Writing: B.Y., Critical review: A.D.O.

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The authors declared that this study has received no financial support.

References

1. Augustine, D., Rao, R.S., Jayaraman, A. & Murthy K.C. 2018. Antiproliferative activity of earthworm coelomic fluid using oral squamous carcinoma KB 3-1 cells: an in vitro study with serine protease analysis. *Pharmacognosy Magazine*, 14(59): 528. http://dx.doi.org/10.4103/pm.pm_412_18

2. Augustine. D., Rao. R.S., Anbu, J. & Murthy, K.C. 2019. In vitro cytotoxic and apoptotic induction effect of earthworm coelomic fluid of *Eudrilus eugeniae*, *Eisenia foetida*, and *Perionyx excavatus* on human oral squamous cell carcinoma-9 cell line. *Toxicology Reports*, 6: 347-357. https://doi.org/10.1016/j.toxrep.2019.04.005

3. Augustine, D., Rao, R.S., Gns, H.S., Anbu, J. & Saraswathy, G.R. 2022. Anticancer Potential of *Eudrilus Eugeniae* Coelomic Fluid Protein on SCC-9 Cells-In Vitro Gene Expression entwined with Cutting-Edge In-Silico Strategy, *Research Square*. https://doi.org/10.21203/rs.3.rs-439242/v1

4. Chabner, B.A. & Roberts, T.G. 2005 Chemotherapy and the war on cancer. *Nature Reviews Cancer*, 5(1): 65-72. https://doi.org/10.1038/nrc1529

5. Czerwonka, A., Fiołka, M.J., Jędrzejewska, K., Jankowska, E., Zając, A. & Rzeski, W. 2020. Pro-apoptotic action of protein-carbohydrate fraction isolated from coelomic fluid of the earthworm Dendrobaena veneta against human colon adenocarcinoma cells. *Biomedicine & Pharmacotherapy*, 126: 110035. https://doi.org/10.1016/j.biopha.2020.110035

6. Deveci Özkan, A., Alimudin, J., Kilciler, Y., Yuksel, B., Aksoy, O. & Betts, Z. 2022. In vitro chemo-protective effect of Eisenia foetida coelomic fluid against histone deacetylase inhibitor-induced oxidative toxicity in breast cancer cells. *International Journal of Environmental Health Research*, 1-10. https://doi.org/10.1080/09603123.2022.2120970

7. Endharti, A.T., Purnamasari, Y., Primasari, R., Poeranto, S. & Permana, S. 2019. The coelomic fluid of Lumbricus rubellus synergistically enhances the cytotoxic effect of 5-Fluorouracil through modulation of focal adhesion kinase and P21 in the HT-29 cancer cell line. *The Scientific World Journal*, 2019: 5632859. https://doi.org/10.1155/2019/5632859

8. Fiołka, M.J. Rzymowska, J., Bilska, S., Lewtak, K., Dmoszyńska-Graniczka, M., Grzywnowicz, K. & Urbanik-Sypniewska, T. 2019. Antitumor activity and apoptotic action of coelomic fluid from the earthworm Dendrobaena veneta against A549 human lung cancer cells. *Apmis*, 127(6): 435-448. https://doi.org/10.1111/apm.12941

9. Gupta, S. & Yadav, S. 2016. *Immuno-Defense* Strategy in Earthworms: A Review Article. *International Journal of Current Microbiology and Applied Sciences*. 5: 1022-1035 http://dx.doi.org/10.20546/ijcmas.2016.504.117.

10. Hanahan, D. 2022. Hallmarks of cancer: new dimensions. *Cancer discovery*, 12(1): 31-46. https://doi.org/10.1158/2159-8290.CD-21-1059

11. Hussain, M., Liaqat, I., Ali, N.M., Arshad, N., Hanif, U., Sajjad, S., Sardar, A.A., Awan, U.F., Khan, F.S. & Slahuddin. 2023. Antibacterial and Bacteriostatic Potential of Coelomic Fluid and Body Paste of *Pheretima Posthuma* (Vaillant, 1868) (Clitellata, Megascolecidae) against Ampicillin Resistant Clinical Bacterial Isolates. *Brazilian Journal of Biology*, 2023: 83, http://doi.org/10.1590/1519-6984.247016

12. Kruk, J., Aboul-Enein, H.Y., Kładna, A. & Bowser, J.E. 2019. Oxidative stress in biological systems and its relationship with pathophysiological functions: the effect of physical activity on cellular redox homeostasis. *Free radical research*, 53(5): 497-521. https://doi.org/10.1080/10715762.2019.1612059

13. Małota, K., Urbisz, A. & Skonieczna, M. 2022. Dose-dependent proliferative and pro-oxidative effects of earthworm coelomic fluid on the HCT116 colon cancer cell line. *Research Square*, https://doi.org/10.21203/rs.3.rs-2258914/v1

14. Mustafa, R.G., Saiqa, A., Domínguez, J., Jamil, M., Manzoor, S., Wazir, S., Shaheen, B., Parveen, A., Khan, R., Ali, S., Ali, N.M. & Jalal, F. 2022. Therapeutic Values of Earthworm Species Extract from Azad Kashmir as Anticoagulant, Antibacterial, and Antioxidant Agents. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 2022: 1-20. http://doi.org/10.1155/2022/6949117.

15. Ozkan, A.D. & Yuksel, B. 2022. POLY I: c-ınduced TLR3 activation on oxidative stress levels in PC-3 (hormone insensıtıve) and LNCAP (hormonesensıtıve) as prostate cancer cells. *Journal of Health Sciences of Kocaeli University*, 8: 18-24. http://doi.org/10.30934/kusbed.915511

16. Ozkan, A.D., Eskiler, G.G., Sarihan, M., Kazan, N., Aksoy, O., Yuksel, B. & Betts, Z. 2022. Anticancer properties of Eisenia foetida proteins in prostate cancer cells in vitro.

International Journal of Peptide Research and Therapeutics, 28(4): 1-11. https://doi.org/10.1007/s10989-022-10428-8

17. Ozkan, A.D., Betts, Z., Yuksel, B., Alimudin, J., Aydin, D. & Guzel, E. 2023. Assessing in vitro anticancer efficacy of sulfonated water-soluble phthalocyanine on breast and prostate cancer cells. *Journal of Porphyrins and Phthalocyanines*, 27(01n04), 493-500. https://doi.org/10.1142/S1088424623500256
18. Permana, S., Sholihah, Z., Iskandar, A., Susanti, H. & Endharti, A.T. 2018. Cytotoxic effects and antiproliferative cancer activity of coelomic fluid from Lumbricus rubellus promotes apoptosis and reduces G2/M phase progression in HT-29 cells. *Journal of Applied Pharmaceutical Science*, 8(11): 028-034. https://doi.org/10.7324/JAPS.2018.81104
19. Permana, S., Hadi, R.P., Norahmawati, E. & Endharti, A.T. 2019. Coelomic fluid of Lumbricus rubellus enhances the anti-prolioniferative effect of 5-fluorouracil by modulating focal adhesion kinase express and IL-1β of colorectal cancer in mice. *Journal of Applied Pharmaceutical Science*, 9(8):041-046 https://doi.org/10.7324/JAPS.2019.90806
20. Permana, S., Putri Fityanti, R., Norahmawati, E., Iskandar, A,, Anggraini Mulyadi, E.D. &Tri Endharti, A. 2020. Coelomic Fluid of Eisenia fetida Ameliorates Cetuximab to Reduce K-Ras and Vimentin Expression through Promoting RUNX3 in an AOM/DSS-Induced Colitis Associated Colon Cancer. *Evidence-Based Complementary and Alternative Medicine*, 2020: 9418520. https://doi.org/10.1155/2020/9418520
21. Rybicka, M., Czaplewska, P., Rzymowska, J., Sofińska-Chmiel, W., Wójcik-Mieszawska, S., Lewtak, K. & Fiołka, M.J. 2022. Novel Venetin-1 nanoparticle from earthworm coelomic fluid as a promising agent for the treatment of non-small cell lung cancer. *Scientific Reports*, 12(1): 1-21. https://doi.org/10.1038/s41598-022-21665-8
22. Sadek, S.A., Sayed, M.G., Fahmy, S.R. & Soliman, A.M. 2022. A Coelomic Fluid of Allolobophora caliginosa as Novel Prospects for Medicinal Antioxidants, Anti-inflammatory, Antiproliferative, Analgesics, and Antipyretics. *Biointerface Research. Appliend in Chemisrty*. 13: 209. https://doi.org/10.33263/BRIAC133.209
23. Shafi, F.A.A. & Faleh, N. 2019. Anticancer activity of earthworm powder (*Lumbricus terrestris*) against MCF-7 and PC-3 cancer cell lines. *Journal of Gastrointestinal Cancer*, 50(4): 919-925. https://doi.org/10.1007/s12029-019-00268-z
24. Wahyuni, D. & Waluyo, J. 2018). Acute Toxicity of Earthworm (*Pheretima Javanica* K.) Powder on Renal Histopathological Description of Rat (*Rattus norvegicus* B.). *Bioedukasi*, 15: 1-6. https://doi.org/10.19184/bioedu.v15i2.6930
25. Yuksel, B. & Deveci Ozkan, A. 2021. The Role of Citrus Nobiletin on Oxidative Stress Levels and Superoxide Dismutase Activities in Metastatic Castration-Resistant Prostate Cancer. *Commagene Journal of Biology*, 5(1): 84-89. https://doi.org/10.31594/commagene.895415
26. Yuksel, B., Ozkan, A.D., Aydın, D. & Betts, Z. 2022. Evaluation of the antioxidative and genotoxic effects of sodium butyrate on breast cancer cells. *Saudi Journal of Biological Sciences*, 29(3): 1394-1401. https://doi.org/10.1016/j.sjbs.2021.12.061