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ORIGINAL ARTICLE

Antitumor Activity of Serine Enzyme and Crude Extracts of The Earthworm *Lumbricus terrestris* Against A549 Human Lung Cancer Cell Line

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ABSTRACT:

Background: Numerous disorders have been successfully treated with earthworms, and this has been documented in ancient texts. These invertebrates are a rich natural source of biological and pharmacological compounds with antioxidant and anticancer properties. The present study aims to isolate and partially purify serine protease from the earthworm and evaluate the cytotoxic and antitumor activities of serine protease and crude extracts of the earthworm.

Methods: Lumbricus terrestris earthworms were collected and processed to prepare crude extracts, isolate, and partially purify them. The anti-tumour activity's serine enzyme was evaluated by MTT assay and Live/Dead Cell Viability Assay using A549 and NHF cell lines.

Results: When the concentration was doubled, it hurt the viability of A549 and NHF cells. The IC50 values for A549cell lines were194.3 and 22.57 µg/ml for crude and serine protease, respectively. Also, the crude extract does not show cytotoxicity against the normal cell line (NHF cells). The IC50 values 391,78.61 mg/ ml, respectively, using Live/Dead Cell Viability Assay, demonstrate that an increased proportion of apoptotic cell deaths in A549 cells after treatment with crude as well as enzyme there is a significant increase in the mean count of dead cells after being treated with enzyme as well as crude the mean count was 172.7 3;127.5 in cells treated with serine enzyme and crude extract, respectively, whereas in the untreated cells, it was 33.1 reflects a significant difference when compared with treated cells with serine enzyme and crud extracts ($P<0.001$).

Conclusion: These findings revealed that Serin enzyme and crude extract of *Lumbricus terrestris* possesses excellent antitumor activity.

KEYWORDS: A549, Antitumor, earthworms, MTT, serine

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INTRODUCTION:

Cancer represents a leading cause of death globally. One in five individuals worldwide develop cancer during their lifetime. The most frequent cancers are breast, lung and colon (Siegel et al.,2022). Although Several cancer treatment methods are accessible, including chemotherapy, radiation therapy, immunotherapy, and surgery. Cancer is still an incurable disorder worldwide due to drug resistance. Moreover, most drugs cannot completely eliminate the cancer cells without causing harm to the rest of the body(Bray,etal.,2018). Through an advanced understanding of the biology of cancer cells, numerous research endeavours have emerged to eliminate cancer cells while minimizing harm to healthy cells effectively. Several biomolecules in the natural product can potentially change the signalling and microenvironment of cancer cells, consequently playing a crucial role in combating cancer cells (Zhang etal.,2022). Numerous studies have been developed in different laboratories to isolate and characterize some biomolecules from earthworms with antitumor activity(Verma etal.,2013; Augustin etal.,2018; Shafi, and Faleh,2019). The earthworm alimentary tract produces a group of proteases with broad substrate specificity. In 1983, a set of fibrinolytic enzymes known as isozymes were discovered in various earthworm species. Subsequently, numerous isozymes have been isolated from various earthworm species, such as *Eisenia fetida* and *Lumbricus rubellus*. For appropriateness, the proteases are termed according to the earthworm species besides the protein function, for example, *Eisenia fetida* protease (Ef P)(Cho etal.,2004). The proteases possess the ability to activate proenzymes. As well as hydrolyze additional proteins, Earthworm proteases have been utilized in several areas like therapy of coagulation disorders, antitumor study, environmental protection and food production (Pan etal.2010; Ding etal,2019). The present work aims to evaluate the anticancer efficacy of the whole extract of the earthworm and isolate and purify serine protease from earthworm to detect the anticancer activity against human lung cancer cell lines.

MATERIAL and METHODS

Collection and processing of earthworms

Healthy and fully mature earthworms were collected from Baghdad, Iraq, after seven days of growing in humus-enriched soil with intermittent water sprays in a controlled laboratory setting. Autolysis began when the mature earthworms were repeatedly washed in sterile distilled water. To induce autolysis, 20 mmol/L of phosphate buffer, pH 7.5, containing 0.02% sodium azide as a bacteriostatic, was heated to 60 degrees Celsius for 3 hours. Worms were stored in the refrigerator at 15 °C for a

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week to induce autolysis. Afterwards, the worms were centrifuged at high speed for 30 minutes (4 °C, 16 000 r/min). The supernatant was stored in sterile tubes away from any potential contamination. The supernatant is filtered via Whattman filter paper to remove any remaining tissue fragments before being purified(Verm and Verma 2013). Purification of a Serine protease isolate the protease from earthworms was purified using the methods described by Nakajima etal., 1993; Vogelstein and Kinzler, 2004; Alotaibi , 2022.

Preparation and Purification of Protein Component of Earthworm Step 1. A total of one kilogram of mature earthworms *Lumbiricus terrestris* washed with sterile distilled water. Autolysis of cleaned earthworms was performed for three hours at 60 °C in 20 mmol/L phosphate buffer pH 7.5 with 0.02% sodium azide as bacteriostatic. To complete the autolysis earthworms, they were stored in a refrigerator for one week at 15 °C. After autolysis, the homogenates were centrifuged at 16 000 r/min for 30 min at four °C. The supernatants were collected into sterile tubes under aseptic conditions. A supernatant of 500 mL volume was filtered several times with Whattman filter paper to remove tissue debris; in this step, crude protein preparation was subjected to purification (Verm and Verma, 2013). The total protein was precipitated from crude using ammonium sulfate and then reconstituted in phosphate buffer saline (pH 7.5). Dialysis was applied to extract the salt from the protein and concentrate the protein by using a dialyzing membrane with molecular weight cut off (MWCO) 20kDa. The next step was the separation of protein using ion exchange chromatography (IEC) on the DEAEcellulose column. Before the column was prepared, the resin was charged with 30 minutes of HCl, 30 minutes with NaOH, and 30 minutes with distilled water. Further, the resin. Approximately 90 fractions were collected and analyzed for protein content and activity. The final step was six fractions with higher protein content subjected to Sephadex G50 beads for column packing under gravity. Packing and equilibration of the column with PBS (pH 7.5). A 5ml of DEAE Cellulose purified and pooled protein faction was loaded into a column and eluted with excess PBS (7.5). The fractions were collected and profiled for protein concentration and activity analysis.

Acridine orange/propidium iodide (AO/PI) assay

A549 and NHF cell lines were planted at a density of 5000 cells per well in a 96-well microtitre plate. Subsequently, The cells were separately treated with the crude and enzyme based on the IC50 values and incubated for 24 hours at 37°C with 5% humidified carbon dioxide. After 10 minutes of incubation, one microliter of acridine orange/propidium iodide (AO/PI) reagent 0.5 mg/ml was

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added to each well. Image J software and fluorescent microscopy were used to measure the fluorescence intensity.

Statistical analysis:

Data are shown as mean standard deviation mean (SDM), and statistical analysis was done with Graph Pad Prism 6 (Graph Pad Software, USA) using a t-test. Differences were counted as being significant at $P \leq 0.05$.

Ethical approval:

The study got ethical approval (BCSMU/0222/00025Z) from the ethical committee at the Mustansiriyah University, Iraq.

RESULTS

Cytotoxic assay

The A549 and NHF cell lines were exposed to 31.2, 62.5, 125, 250, 500, and 1000 µg g/ml of crude extract and serine enzyme, respectively, to evaluate their cytotoxic activity. When the concentration increased by double, it adversely impacted the viability of A549 and NHF cells. The IC50 values for A549cell lines were194.3 and 22.57 µg/ml for crude and serine protease, respectively. Also, the crude extract does not show cytotoxicity against the normal cell line (NHF cells) the IC50 391,78.61 mg/ ml, respectively (Fig. 1) and (Fig. 2).

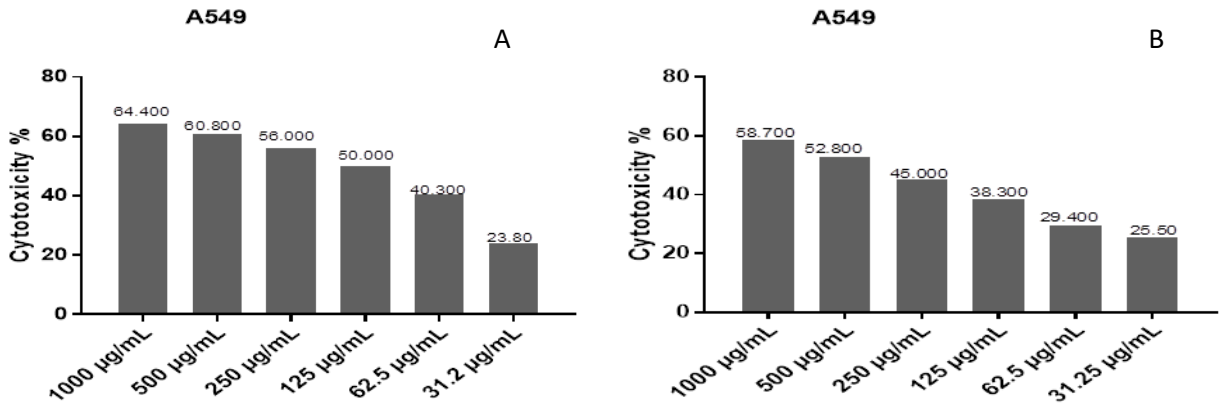

Figure 1. The cytotoxic effect of the various concentrations of crude extract (A) and serine enzyme (B) in A549 cells for 24 h using MMT assay. Values are the mean from three independent experiments.

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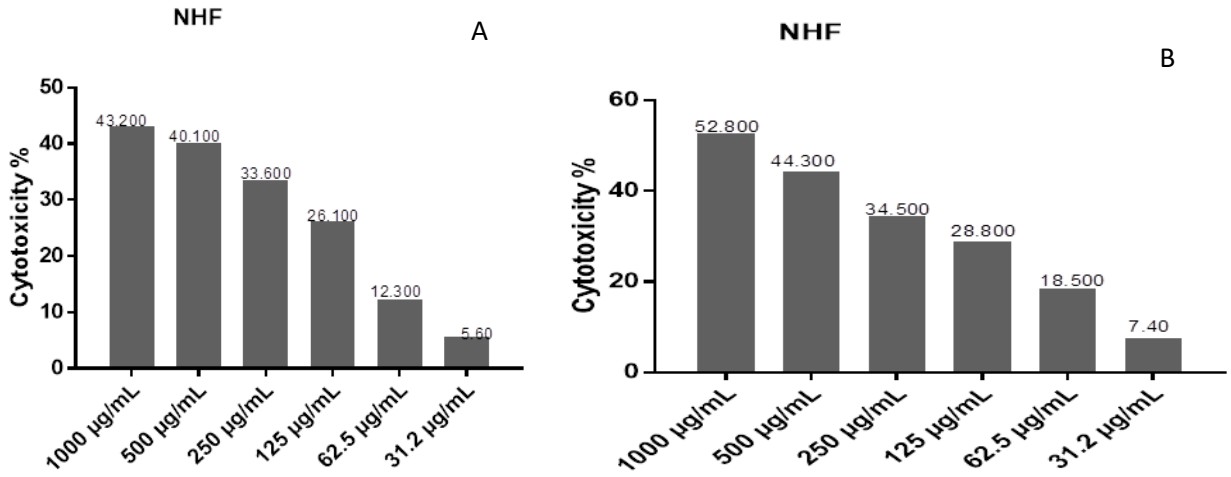

Figure 2. *The cytotoxic effect of the various concentrations of crude extract in NHF cells for 24 h using MMT assay (A: Crude extract; B: Serine enzyme). Values are the mean from three independent experiments.*

Live/Dead Cell Viability Assay

In order to obtain more insight into the type of cell death caused by earthworm extract and serine enzyme, Dual staining, Dual staining was used. Depending on the IC50 values, the cell lines (A549) were treated with 194.3 and 22.57 µg/ml of crude and enzyme. Colour change in acridine orange (AO) and propidium iodide staining determined the percentage of living vs. dead cells. Cells treated with crude or enzyme showed crescent-shaped or granular yellow-green AO nuclear staining, but untreated cells showed bright green fluorescent cells, as shown in Figure 3. This indicates that the treated cells are in the early stages of apoptosis. Also, there are fewer A549 cells in the field of view than untreated cells. As shown in Figure (3), numerous cells are detached from the cell attachment. Only a few cells are still attached, and they are also about to die. The number of living cells decreased considerably compared to control cells (not treated cells). It can be seen from the data in Figure (4) that an increased proportion of apoptotic cell deaths in A549 cells after treatment with crude and enzyme (IC50 concentration of enzyme and crude for 24 hours). Individual cell staining with the DNA-binding dyes acridine orange and propidium iodide revealed the alterations. Active cells exhibit vivid green patches. There is a significant increase in the mean count of dead cells after being treated with enzyme and crude. The mean count was 172.73; and 127.5 in cells treated with serine enzyme and crude extract, respectively, whereas in the untreated cells, it was 33.1, which is significant when compared with treated cells with serine enzyme and crud extracts ($P<0.001$). It is important to mention that When NHF cells were treated with serine enzyme and crude extracts, the mean of viable

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cells was (78.61 and 391) respectively. No significant difference in the number of viable cells was detected when compared with untreated cells(P> 0.05) (figure 5,6).

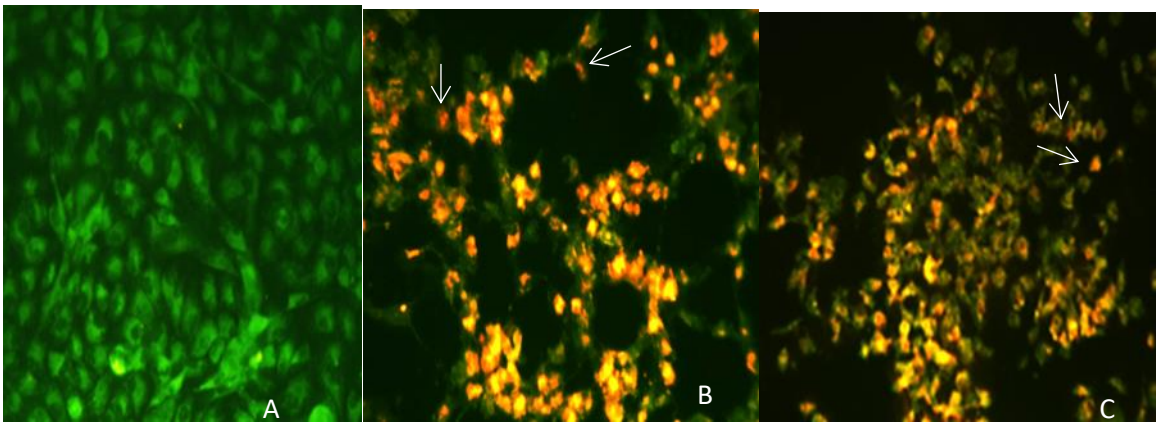

Figure3. *Apoptotic effect with Acridine orange/propidium iodide assay. Induction of apoptotic effect upon incubation of(A549cells)with Serine enzyme and crude extracts describing the early and late stages of apoptosis as orange and red fluorescence microscopy images, respectively. (A) Control (untreated cells); (B) Serine enzyme-treated cells; (C) crude extract-treated cells.*

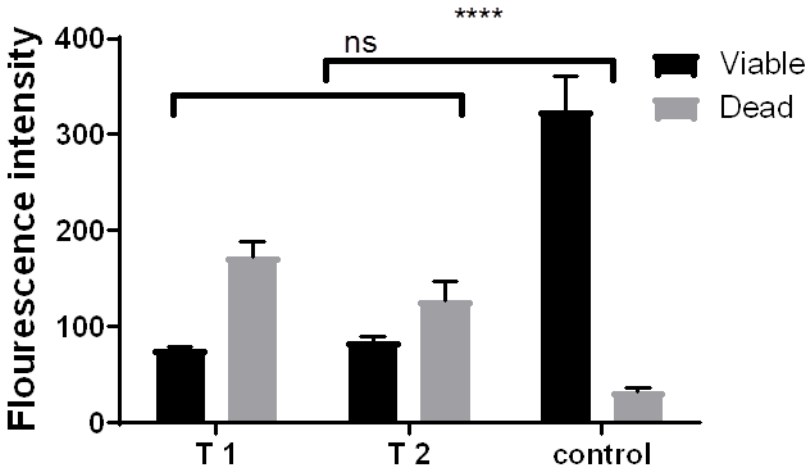

Figure 4:*Effect of apoptosis on A549 cells following a 24-hour incubation with the enzyme (T1) crude extract 2 (T2). Acridine orange/propidium iodide assay percentage of viable and dead A549 cells. The results are the mean of at least three independent tests + standard deviation P<0.0001*

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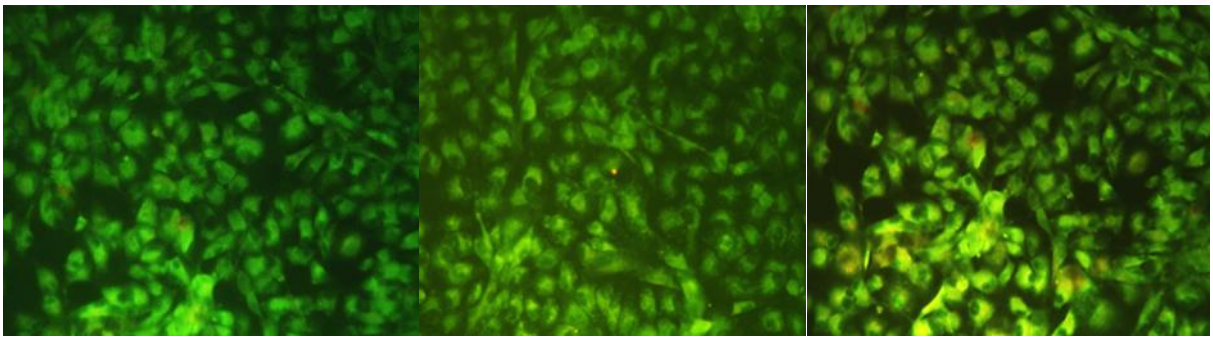

Figure 5. *Apoptotic effect with Acridine orange/propidium iodide assay Induction of apoptotic effect upon incubation of(NHFcells)with serine enzyme and crude stating the early and late apoptosis depicted by orange and red colour respectively, which was visualized by fluorescence microscope. Active cells exhibit vivid green patches (A) Control (untreated cells); (B) cells treated with serine enzyme; (C) cells treated with crude extract.*

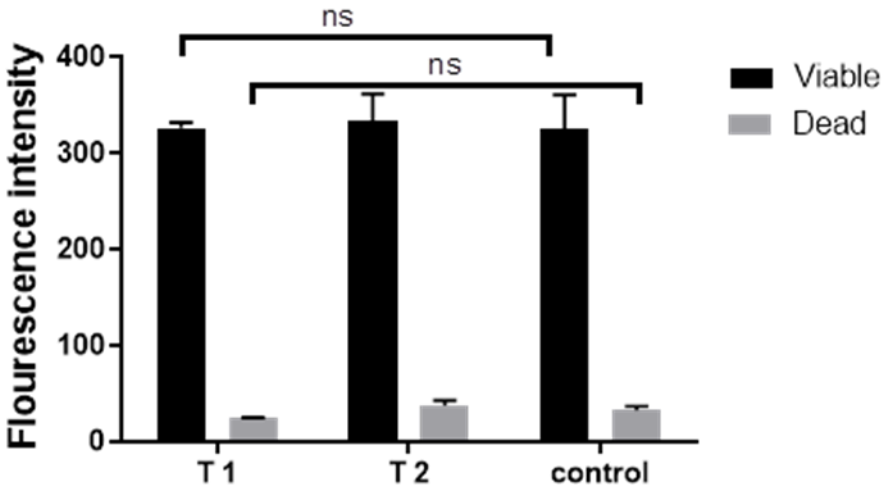

Figure 6:Apoptotic effect on NHF cells after 24-h incubation with the enzyme (treated T1) crude extract (treated T2). The results represent the mean of at least three independent experiments _ standard deviation).

DISCUSSION

The current study demonstrates that the crude extract and enzyme extracted from (*Lumbricus terrestris*) have a cytotoxic effect against the A549 cells. The results of the current study match those observed in earlier studies. More than 20 publications concerning antitumor properties of earthworm-derived materials were analyzed for various cancers, including breast cancer, cervical cancer, lung cancer, oral cancer, colon cancer, and others (Augustin *et al.,*2018). The antitumor

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activity of earthworm-derived materials could be generally categorized into four theories. First, biomolecules extracted from earthworms could initiate apoptosis in mammalian tumour cell(Mácsik etal.,2015). Secondly, earthworm-derived material can inhibit the growth and proliferation of tumour cells (Augustin *et al.,*2018; Permana et al.,2018). The third mechanism might be via the improvement of body immunologic function(Mao *eta*l.,2006). Lastly, earthworm fibrinolytic enzymes and other active materials suppress tumour development or migration, possibly via matrix metalloproteinases 9(MMP9) inhibition and decrease of the Micro-vessel density at the side of tumors(Wang etal,2016; Yang etal,2008). The selective action of the crude extract against (A549) cells but not against normal cells (NHF) was reported earlier by Fiołka et al.(2019), conclude that the short heated coleme fluid of the (Dendrobaena veneta) showed cytotoxicity towards the A549 lung cancer cells but not against the normal cells (the bronchial epithelial cell line BEAS-2B). Also, analysis of results demonstrates that the colem flude initiates the apoptosis pathway. In 2007, Chen *et al*. (2007) concluded that EFE isolated from E. fetida had effective anticancer activity on human hepatoma cells in vivo and in vitro, the author detected a decrease in the expression levels of matrix metalloproteinase 2 (MMP 2), which mediated the invasion and metastasis.Shafi and Faleh (2019) assessed the effect of earthworm powder on PC-3 cells by MTT assay followed by Apoptosis fluorescence assay. There are scarcely researched areas in the anticancer activity of extracts prepared from Lumbricus terrestris; to the best of our knowledge, the most frequently researched earthworm species was *Eisenia Foetida*, and the next was *Eudrilus eugeniae* species. Nakajima et al.,1993; Cho etal.,2004; Pan et al.,2010; Cooperet al.,2012; Mácsik etal.,2015). While the Perionyx excavates were included in two studies whereas single research used *Lampito mauritii* and Pheretima posthuman(Mao,etal. 2006).Verma *et* al. (2013) employed serine protease purified from Pheretima posthuma and found that the 15 kDa fraction exhibits strong cytotoxic activity against MCF 7 cell line. After numerous washes with sterile distilled water, the earthworm was autolyzed to produce the protease. Purification and isolation were accomplished using caseinolytic plate diffusion assay, SDS PAGE, and DEAE C chromatography. Many types of novel extracts created by authors were identified. Vidya et al. (2016) utilized the coelomocytes cell culture of *E. eugeniae*. At a 2 mg/ml dosage, 90% cytotoxicity was observed against the A549 cell line. The results of this study provide supporting evidence for the potential use of anticancer drugs derived from coelomocyte culture supernatant. Earlier studies revealed the potential of the earthworm crude extract to kill cancer cells *in vitro* directly and to inhibit tumor growth *in vivo* (Zhang etal.,1987; Zeng etal.,1995). Earthworm proteases have also been demonstrated to enhance the therapeutic benefits of radiation therapy and chemotherapy(- zhang et

al.,1992). In addition, the glycolipoprotein combination (G-90), extracted from E. fetida, can lysis fibrin (Grdisa etal.,2001). These findings revealed that enzyme and crude Lumbricus terrestris possess excellent antitumor activity. These results match those observed in earlier studies. The findings of Earthworm extracts have been shown to have anticancer action, and this work adds to that body of evidence. Therefore, future research should focus on discovering and isolating proteins with anticancer activity from the earthworm by investigating the mechanism responsible for this activity. Although many studies demonstrate the antitumor activity of crude or biomolecules extracted from earthworms. The anticancer activity of extracts prepared from Lumbricus terrestris must be better investigated. Therefore, the anticancer effect of crude extract and active biomolecules prepared from Lumbricus terrestris on diverse cancer cells should be investigated. This investigation focussed on the effect of crude and serine enzymes on cells. To confirm the anticancer effects, such as anti-metastasis and apoptotic effects, in vivo animal testing will be our next step. We anticipated that our research would contribute to developing new cancer treatments.

CONCLUSION:

Serine enzyme and crude extract of *Lumbricus terrestris* have been shown to have a significant antitumor activity against human lung cancer cell line (A549). The finding demonstrate that these extract can effectively induce cytotoxic effects and apoptosis in cancer cells while exhibiting minimal toxicity to normal cells. This research underscores the potential of earthworm-derived compounds as a promising avenue for developing novel anticancer therapies. Future investigations should further explore the mechanisms underlying these effects and the broader applicability of these extracts in cancer treatment.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

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Authors contributions : [first author]: Contributed to the data collection process and participated in data analysis and interpretation. [Author 2] played a role in drafting and revising the manuscript for important intellectual content, Secured funding for the research and supervised the study's progress. study's conceptualization, design, and methodology. Additionally, [Author 3] provided critical

feedback and revisions during manuscript development.

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