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ORIGINAL RESEARCH

Anticancer Activity of Earthworm Powder (*Lumbricus terrestris*) Against MCF-7 and PC-3 Cancer Cell Lines

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Abstract

Background and Aim The importance of earthworm in treatment of various diseases has been proven in ancient literatures. Nowadays, with advances in biotechnology, earthworm is considered a rich natural source of many biomolecules that possesses antioxidant and antitumor activities. The present study aimed to evaluate the antitumor activity of earthworm powder (*Lumbricus terrestris*) against two cell lines, breast cancer cells (MCF-7) and prostate cancer cells (PC-3).

Methods Fully matured earthworms (*L. terrestris*) were collected from soil in Baghdad, Iraq. To assess the cytotoxicity of earthworm powder, the MTT assay was conducted on cancerous (MCF-7 and PC-3 cells) and normal cell line (WRL68 cells) lines.

Results It was revealed that earthworm powder exerts cytotoxic effects against two cancer cell lines. The viability of MCF-7 and PC-3 cells decreased with increasing the concentration. The IC50 values for PC-3 and MCF-7 cell lines were 265.5 and 965.9 μg/ml, respectively, while the earthworm powder exhibited no cytotoxicity against the WRL68 cells. According to the analysis of the results of the multiple cytotoxicity assay (HCS), the treatment of PC-3 cells with 100, 200, and 400 μg/ml of earthworm powder for 24 h at 37 °C led to cell death by changing the permeability of mitochondrial membrane resulting in cytochrome C release and inducing apoptosis.

Conclusion The results of the present study contribute additional evidence for the antitumor activity of earthworm extracts. Therefore, further research should concentrate on isolating and identifying the earthworm's active biomolecules that have antitumor activity by investigating the molecular mechanism, genetics, and pathways responsible for the antitumor activity of these biomolecules.

Keywords Earthworms · Breast cancer cells (MCF-7) · Prostate cancer cells (PC-3) · Antitumor

Introduction

Cancer is a major public health problem and a main cause of morbidity and mortality worldwide [1]. In our country (Iraq), cancer represents the second most common cause of death after cardiovascular disease, and the most common types of cancer are bronchial and lung cancer for men and breast cancer for women [2].

To decrease the mortality rate of cancer, many therapeutic procedures have been used including surgery, chemotherapy, radiation, and immunotherapy.

During the last decade with advances in biotechnology, many studies have been performed to develop therapeutic agents that eliminate cancerous cells with limited damage to the normal cells [3]. Numerous studies have attempted to explain the anticancer activity of earthworm extracts. Whole body extracts of earthworms have the ability to suppress the growth of cancer cells in vitro and to prevent the development of tumors in vivo [4, 5]. It has also been demonstrated that earthworm proteases improve the beneficial effects of both chemotherapy and radiation [6, 7]. Earthworms have a narrow cylindrical shape and belong to the phylum Annelida. They are scientifically classified into twenty-three families, more than 700 genera, and more than 7000 species. Their size ranges from 25.4 to 0.91 m. They live in various tropical

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and warm soils and dig vertical and horizontal tunnels that protect them from low temperatures in winter and from dehydration in summer [8]. In this study, we aimed to evaluate the antitumor activity of earthworm powder (*L. terrestris*) against breast cancer cells (MCF-7) and prostate cancer cells (PC-3).

Materials and Methods

Collection of Earthworms

Fully matured earthworms (*L. terrestris*) were collected from soil in Baghdad, Iraq. The earthworms were firstly washed in warm tap water to remove dust. The earthworms were then classified on the basis of morphological characteristics, such as the number of segments, color, and prostomium and clitellum shape, under a microscope. Next, the earthworms were placed in sterile distilled water for 6–8 h after repeated washes in distilled water. The worms were placed in an incubator at 55 °C for 48 h, and the dead worms were converted into a fine powder by crushing and stored in a cool, dark, and dry place [9].

Cell Line Culture

Breast cancer cells (MCF-7), prostate cancer cells (PC-3), and WRL-68 cells were supplied by the American Type Culture Collection (ATCC) and grown in growth medium containing 10% fetal bovine serum (Thermofisher Scientific, New York, USA), 1% sodium bicarbonate, 10^3 IU of penicillin G, and100 μg ml of G1 streptomycin (Thermofisher Scientific, New York, USA). The cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

To evaluate the inhibitory effect of earthworm powder, an MTT assay (Thermofisher Scientific, New York, USA) was performed according to the manufacturer's advice. Firstly, the cells (1×10^4 to 1×10^6 cells/mlG1) were seeded in 200 μl plates and incubated in a CO2 incubator at 37 °C. After 24 h of incubation, the cells were treated with different concentrations of earthworm powder 12.5, 25, 50, 100, 200, and 400 μg/ml.

Doxorubicin was used as a positive control. After 1 day of incubation, the medium was changed and 10 μl of MTT solution was added to each well and incubated at 37 °C. Solubilization solution was loaded to each well after 4 h of incubation, and mixing was performed by pipetting for 5 min to ensure the complete dissolution of all formazan crystals. Thereafter, the absorbance was measured by an ELISA reader (Bio-Rad, Germany) at a wavelength of 575 nm.

Cytotoxicity Assay

The Cellomics Multiparameter Cytotoxicity 3 Kit (Thermo Scientific Cellomics® Multiparameter Cytotoxicity V4, New York, USA) was used to perform multiple cytotoxicity assays. According to the protocol, the cells were grown in a 96-well plate (1×10^6 cells) treated with serial concentrations (100, 200, 400 μg/ml) of earthworm powder and incubated for 24 h. Next, fifty microliters of the cell staining solution was loaded in each well, and the plate was re-incubated at 37 °C for 30 min. The cells were fixed by adding 50 ml of 4% paraformaldehyde to each single well and incubating the plates at room temperature for 20 min. Then, the fixative solution was removed carefully, and 100 ml/well of wash buffer was added to wash the cells. After aspiration of wash buffer, 100 μl of 1X blocking buffer was added to each well, and the plate incubated at room temperature for 15 min. Next, the blocking buffer was removed gently, and 50 μl/well of primary antibody solution was added. After 1 h of incubation at room temperature, the plates were washed three times with 1X washing buffer. Fifty microliters of secondary antibody solution was added, and the plates were re-incubated for 60 min at room temperature. The plates were sealed and evaluated using an ArrayScan HCS analyzer (ThermoScientific, New York, USA) [10].

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to evaluate the differences between groups. Data were expressed as the mean ± standard deviation (SD). The results were statistically analyzed with significance at a P value of < 0.05.

Results

MTT Assay

To assess the cytotoxicity of earthworm powder, the MTT assay was used to detect the in vitro cytotoxic effect on three cell lines (MCF-7, PC-3, WRL 68), as shown in Fig. 1, of exposure for 24 h to different concentrations of the earthworm extract powder (12.5, 25, 50, 100, 200, and 400 μg/ml). The viability of MCF-7 and PC-3 cells decreased with increasing concentration. The IC50 values for PC-3 and MCF-7 cell lines were 265.5 and 965.9 μg/ml, respectively, and the earthworm powder did not show cytotoxicity against the normal cell line (WRL68 cells) (Fig. 1).

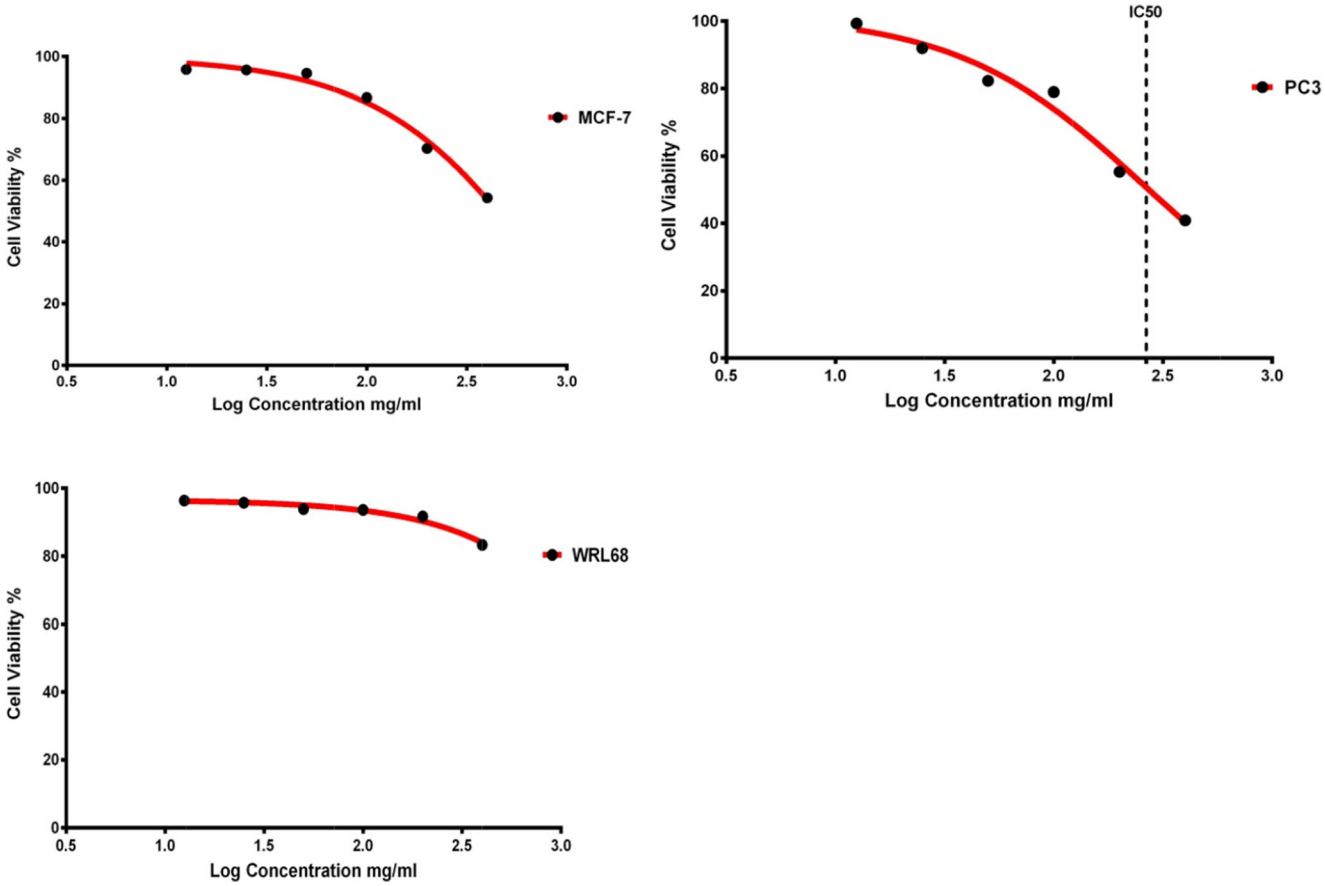

Fig. 1 The breast cancer cell (MCF-7), prostate cancer cell (PC-3), and WRL68 viability (%) levels measured by the MTT assay after 24 h of treatment with serial concentrations of earthworm powder (12.5, 25, 50, 100, 200, and 400 μg/ml)

Multiple Cytotoxicity Assay (HCS)

Six independent factors involved in apoptosis processes, including cell viability, cell membrane permeability, alteration in the size and shape of the nucleus, changes in mitochondrial membrane potential, and cytochrome C release, were measured in the PC-3 cells treated for 24 h using the Multiparameter Cytotoxicity 3 Kit. The viable cell count was decreased significantly with increasing concentrations of earthworm powder as compared with that of control cells (untreated cells). The decrease in cell count (percentage inhibition rate) was 1, 9.17, and 29.19% for cells treated with 100, 200, and 400 μg/ml earthworm powder. Additionally, the percentage of inhibition was increased significantly ($P < 0.05$) at higher concentrations (200 and 400 μg/ml) as depicted in Table 1 and Fig. 2.

Cell Membrane Permeability

The loss of cell membrane integrity is a common feature associated with marked cytotoxicity. This parameter was used to evaluate the cell earthworm powder interaction. A significant increase was observed in the cell membrane permeability (609 ± 18.17) of cells treated with the highest

Table 1 Count of viable PC-3 cells evaluated on the ArrayScan HCS reader after exposure to serial concentrations of earthworm powder (L. terrestris)

Treatment	Viable cell count (mean ± S.D)	Inhibition (%)
Untreated cells (control)	2180 ± 20.51	0%
Doxorubicin 20 mM	954 ± 16.97	56.23
400 (μg/ml)	1544 ± 33.94	29.17
200 (μg/ml)	1980 ± 24.75	9.17
100 (μg/ml)	2158 ± 4.24	1

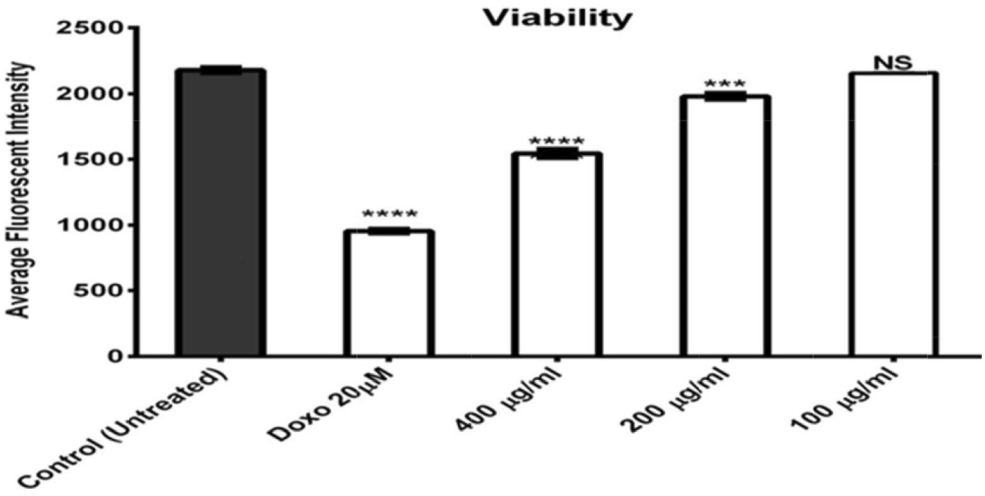

Fig. 2 Reduction in PC-3 cell count after 24 h exposure to serial concentrations of earthworm powder at 37 °C and analyzed on the ArrayScan HCS reader. The differences between the means of the control (untreated cells) and the other groups were evaluated for significance

concentration (400 μg/ml) compared with that of untreated cells (516.4 ± 2.40) (Fig. 3 and Table 2).

Mitochondrial Membrane Permeability

The effect of earthworm powder on mitochondrial membrane permeability was assessed in PC-3 cells treated with a serial of concentrations of earthworm powder, as shown in Table 2. Moreover, as exhibited in Fig. 4, PC3 cells treated at 400 μg/ml undergone a significant increase in mitochondrial membrane permeability fig. 4.

Nuclear Intensity

The treatment of PC-3 cells with a series of concentrations of earthworm powder for 24 h led to the nuclear morphological changes (Fig. 5). These changes were measured by staining the cells with Hoechst 33342 dye. The results revealed that the nuclear intensity was decreased with increasing the concentrations of earthworm powder as shown in Fig. 5 and Table 2. The nuclear intensity was 329.4 ± 0.98, 311.4 ± 2.05, and 295 ± 5.44 for the three concentrations 100, 200, and 400 μg/ml, respectively. The two concentrations 200 and 400 μg/ml exerted significant differences in nuclear intensity when compared with the untreated cells (control) (332.7 ± 8.91).

Cytochrome C

The treatment of PC3 cells with earthworm powder initiated the migration of cytochrome c from the mitochondria into the cytosol, as shown in Fig. 6 and Table 2. We detected an increase in mean count of cytochrome c intensity in treated cells. The mean count was 200.7 ± 0.14 in cells treated with 400 μg/ml, whereas in the untreated cells it was 175.4 ± 6.85 which was a significant increase in cytochrome C release intensity.

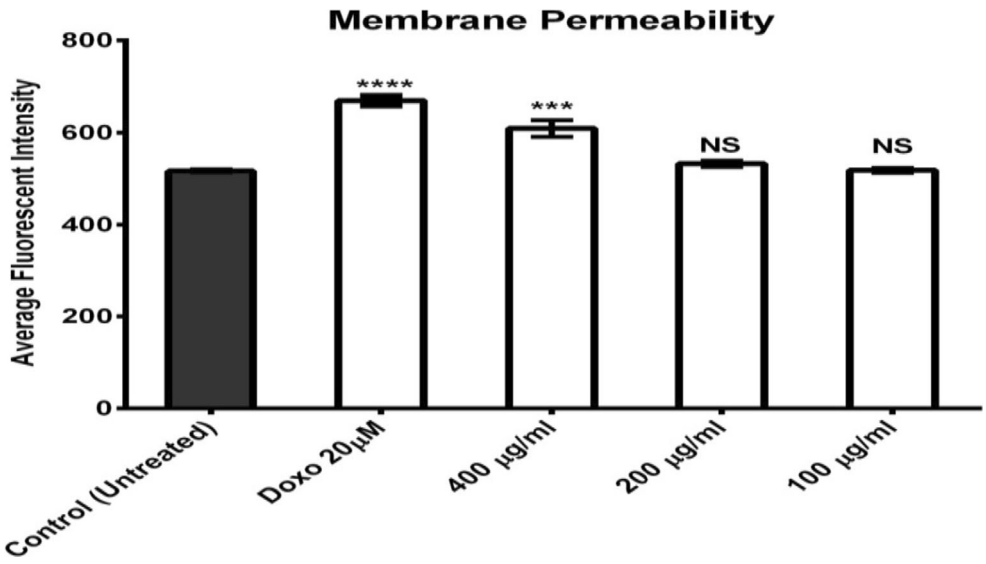

Fig. 3 Effect of earthworm powder treatment on the membrane permeability (MP) of PC-3 cells treated with 100, 200, and 400 μg/ml of earthworm powder for 24 h at 37 °C. The differences between the means of the control (untreated cells) and the other groups were evaluated for significance. Quadruple asterisks denote significant differences at $P < .0001$

Table 2 Effect of earthworm powder treatment on nuclear intensity, cell membrane permeability, mitochondrial membrane permeability, and cytochrome c in PC-3 cells

Treatment (mg/ml	Nuclear intensity	Cell membrane permeability	Mitochondrial membrane permeability (mean ± S.D)	Cytochrome C (mean ± S.D)
Untreated cells (control)	332.7 ± 8.91	516.4 ± 2.40	469.7 ± 4.31	175.4 ± 6.85
Doxorubicin 20 mM	233.1 ± 2.97	669.7 ± 11.53	318 ± 11.03	231 ± 7.56
400	295 ± 5.44	609 ± 18.17	369 ± 5.02	200.7 ± 0.14
200	311.4 ± 2.05	532.1 ± 5.51	448.7 ± 8.20	190.7 ± 2.05
100	329.4 ± 0.98	517.8 ± 3.96	464.7 ± 6.50	179.9 ± 3.74

Discussion

The results of the present study suggested that earthworm extracts exert a higher toxicity to cancer cells than to normal cells. Moreover, earthworm powder (*L. terrestris*) had a stronger inhibitory effect on PC-3 cells, and the IC50 value was greater than that on MCF-7 cells (IC50 value of 965.9 μg/ml). For this reason, the PC-3 cell line was chosen for further assays. Majority of the studies have applied cell lines for cytotoxicity research. Cervical cancer and hepatocellular carcinoma cell lines have been mostly used in earthworm powder anticancer evaluations [5]. However, owing to the paucity of studies on the *L. terrestris* anticancerous trains, they have been included here.

The findings observed in this study mirror those of previous studies that have examined the antitumor effect of earthworm extracts against different tumor cell lines. Although many studies have been carried out in different laboratories to demonstrate that some biomolecules compounds in earthworms have antitumor activity, each laboratory used a different method for preparing earthworm extracts [5, 11, 12, 13]. Therefore, several questions remain unanswered about which biomolecules or proteins that possess antitumor activity and involved in the mechanism of the antitumor effects of earthworm extracts [14, 15, 16, 17].

According to the analysis of the results of the present study, the treatment of PC-3 cells with earthworm powder for 24 h led to cell death by changing the permeability of mitochondrial membrane resulting in cytochrome release and inducing apoptosis. Cytochrome C translocation from the mitochondrial intermembrane space into the cytosol is an early and apoptosis-specific event. When cytochrome C is released together with ATP, it binds with APaf-11 apoptotic protease-activating factor-1. These events subsequently lead to the activation of caspase-9 and the initiation of the apoptotic protease cascade [14].

These data support previous findings in which the earthworm extracts directly inhibit the proliferation of tumor cell lines and induce apoptosis in tumor cell lines. It was concluded that preparations from three species of earthworm (*Eudrilus eugeniae*, *Perionyx excavatus*, and *Eisenia fetida*) showed anticancer activity against the PC-3, MCF-7, and HCT-116 cell lines [18].

Yang et al. (2007) stated that fibrinolysin extracted from earthworms inhibits the proliferation of human gastric carcinoma (MGC803) by inducing apoptosis and decreasing the expression of the P53 gene [19]. Hong et al.

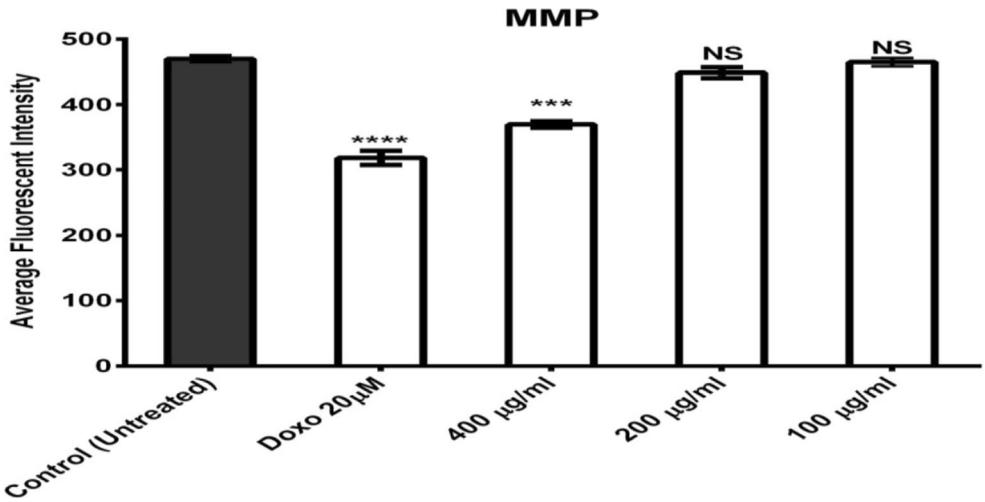

Fig. 4 Effect of earthworm powder treatment on the mitochondrial membrane permeability (MMP) of PC-3 cells treated with 100, 200, and 400 μg/ml of earthworm powder for 24 h at 37 °C. The differences between the means of the control (untreated cells) and the other groups were evaluated for significance. Quadruple asterisks denote significant differences values at $P < .0001$

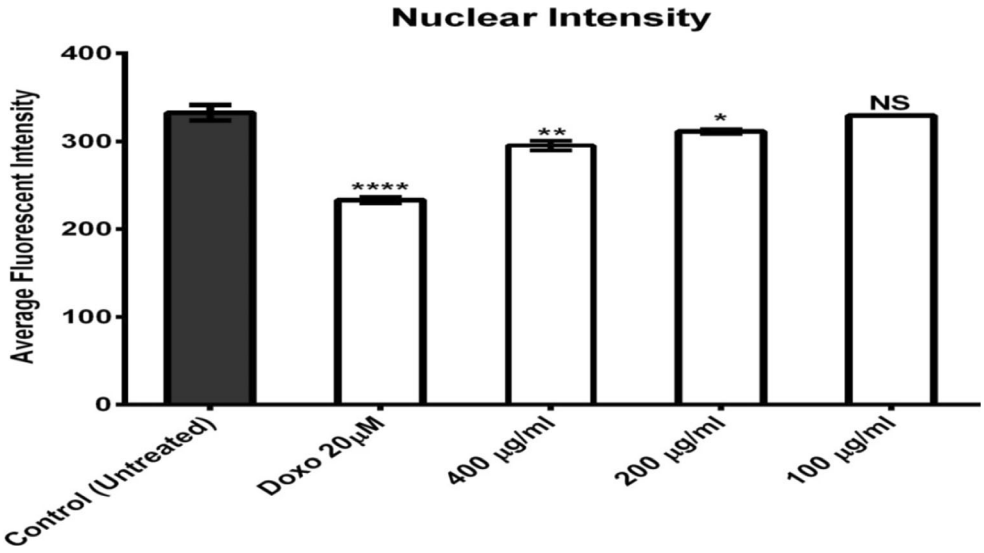

Fig. 5 Effect of earthworm powder treatment on the nuclear intensity of PC-3 cells treated with 100, 200, and 400 μg/ml of earthworm powder for 24 h at 37 °C. The mean differences between the control (untreated cells) and the other groups were evaluated for significance

(2007) demonstrated that fibrinolytic enzyme (EFE) extracted from Eisenia foetida can inhibit hepatoma cell proliferation in vitro and in vivo by inducing apoptosis pathway and decreasing the expression of matrix metalloproteinase 2 (MMP-2) [4].

In 1995, Suzuki and Cooper demonstrated that coelomocytes from coelomic fluid of earthworms (*L. terrestris*) have the ability to destroy tumor cells in vitro. Researchers from Japan at Meiji University found that the daily administration of lombricine by injection or as a dietary supplement inhibited the proliferation of mammary tumors in SHN mice. In 1986, Wang et al. concluded that the earthworm crude extract had the ability to inhibit the occurrence and development of tumor in vivo and to kill cancer cells directly in vitro [5].

According to some studies, earthworm proteases have potential roles in the treatment of some kind of tumors [17, 20]. Engelmann et al. (2004) concluded that lytic factors isolated from coelomocytes of earthworms have cytotoxic effects on mammalian HeLa, HEp-2, PC-12, and PA317 cells [21].

Over the past century, there has been a dramatic increase in research on the use of earthworm extracts as antitumor agents. To the best of our knowledge, the present study is the first conducted in Iraq to test the anticancer effect of earthworm powder (*L. terrestris*) on the PC-3 cell line. A total of twenty-three studies, most of them conducted in China, have employed *E. foetida*. The next most commonly researched species was *E. eugeniae*. Two studies employed *Perionyx excavates*, while *L. mauritii* and *Pheretima posthuma* have been researched in only a single study [12].

In conclusion, the findings of the present study contribute to provide additional evidence for the antitumor activity of earthworm extracts. Therefore, further research should concentrate on isolation and identification of the active

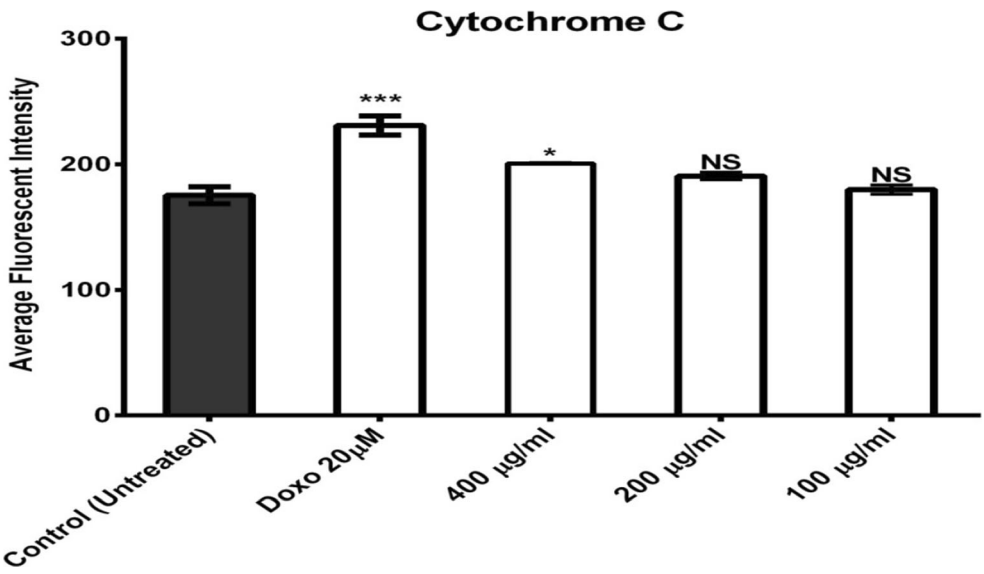

Fig. 6 Effect of earthworm powder treatment on the cytochrome C release of PC-3 cells treated with 100, 200, and 400 μg/ml of earthworm powder for 24 h at 37 °C. The mean differences between the control (untreated cells) and other groups were evaluated for significance

biomolecules in earthworms exerting promising antitumor activity and should investigate the molecular mechanism, gene, and pathways responsible for the antitumor activity of these compounds.

The potential limitations of our study include lack of in vivo study on mouse model, cell receptor docking analysis, gene expression in cancer cells, and assessment of alterations in cancer cell lines toward achieving exact targets for *L. terrestris*. Testing in cancer cell lines has remained the initial step for drug testing for many years. Additionally, this study needs further follow up to determine and separate the anticancerous compounds and their exact impacts levels inside powder to introduce potential molecules.

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Compliance with Ethical Standards

This study was ethically approved by the College of Science, Mustansiriyah University, Baghdad, Iraq.

Conflict of Interest The authors declare that they have no conflict of interest.

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