

Investigating the dual functional regulatory effects of earthworm tissue extract on C2C12 and MCF7 cells

Submitted in partial fulfillment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

By

Mr. MEIKANDAN C

Reg. NO. 42101009



**CENTER FOR MOLECULAR AND NANOMEDICAL SCIENCES
INTERNATIONAL RESEARCH CENTER**

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

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JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **MEIKANDAN C** Reg. No. **42101009** who carried out the project entitled "**Investigating the dual functional regulatory effects of earthworm tissue extract on C2C12 and MCF7 cells**" under the supervision of Dr. Jackson Durairaj from September 2023 to April 2024.

S. Jackson Durairaj
Guide

Dr. JACKSON DURAIRAJ S

Dayanandan A
Head of the Department
Dr. DAYANANDAN A

Submitted for Viva voce Examination held on 18-04-2024

Dayanandan A
Internal Examiner

[Signature]
External Examiner



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DECLARATION

I **MEIKANDAN C** hereby declare that the Project Report entitled "**Investigating the dual functional regulatory effects of earthworm tissue extract on C2C12 and MCF7 cells**" done by me under the guidance of **Dr. Jackson Durairaj S** at Center for Molecular and Nanomedical Sciences, International Research Center, Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of **Master of Science in Medical Biotechnology and Clinical Research**.

Signature of the Candidate

Date: 18-04-2024

Place: Chennai

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ABSTRACT

Cancer remains a global health concern, with millions of lives affected annually. Harnessing natural extracts for therapeutic purposes has gained attention, with earthworm extracts showing promise in combating cancer. Our study investigates the anticancer activity of earthworm extracts, focusing on the gastric region, inspired by the differentiation control mechanism observed in *Hydra*. Notably, our findings reveal a selective cytotoxicity against cancer cells, particularly MCF7, with a significant reduction in cell viability and induction of apoptosis. C2C12 was used as a control somatic cell. Molecular analysis highlights the role of TCTP and p53 in mediating this process, with earthworm extracts suppressing TCTP expression and activating p53-dependent apoptosis pathways. The apoptotic execution protein, Cleaved caspase-3 was much expressed in MCF-7 cells. Interestingly, this effect was specific to cancer cells, sparing C2C12 cells from damage. Immunofluorescence studies further corroborate these findings, illustrating cleaved caspase-3 activation in cancer cells but not in C2C12 cells. Our research underscores the potential of earthworm extracts as a novel therapeutic strategy for breast cancer and calls for further exploration into the intricate molecular mechanisms underlying their anticancer activity.

Keywords: Earthworm gastric region, Body segment extract, Anti-cancer, MCF7, C2C12

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CHAPTER-1

INTRODUCTION

On a global scale, there were approximately 20 million new cases of cancer and 10 million lives lost to this devastating disease. The incidence of cancer is projected to rise by around 60% in the coming decades, placing additional pressure on healthcare systems, individuals, and communities. By 2040, there will be a significant rise in the global burden of cancer, reaching approximately 30 million new cases. This increase will be most prominent in low- and middle-income countries. Cancer, a multifaceted and devastating disease, continues to pose significant challenges to global public health despite advancements in medical science and technology. With approximately 20 million new cases and 10 million deaths annually worldwide, cancer remains a leading cause of mortality and morbidity. The burden of cancer is expected to escalate substantially in the coming decades, particularly in low- and middle-income countries, underscoring the urgent need for innovative therapeutic approaches (PAHO, 2023). Primarily, breast cancer presents substantial obstacles in the field of public health and has a significant global impact, resulting in approximately 2.3 million new cases and 685,000 deaths annually (WHO, 2023). Natural products have long been recognized as valuable resources for the development of potential chemotherapeutic agents. Its complex etiology and heterogeneity pose formidable challenges to effective prevention, diagnosis, and treatment strategies, necessitating continued exploration of novel therapeutic modalities.

Earthworm, *Eudrilus eugeniae* is a clitellum dependent worm and it is very well known for regeneration. In this context, natural products have garnered considerable attention as reservoirs of bioactive compounds with potential chemotherapeutic properties. Among these, earthworms, with their remarkable regenerative abilities and diverse biological activities, have emerged as intriguing subjects of scientific inquiry (Sivasubramaniam, 2021). In addition, it has the ability to generate a range of molecules that offer immunoprotective properties. These molecules possess afibrinolytic, anticoagulative, anticancer, and antimicrobial activity. As a result, they could potentially be utilized in the treatment of various diseases. Earthworms, such as *Eudrilus eugeniae*, are not only renowned for their

role in soil health and nutrient cycling but also for their ability to produce a myriad of bioactive molecules with immunoprotective, anticoagulative, antimicrobial, and anticancer properties (Augustine *et al.*, 2017).

Earthworm extracts are readily available in nature and rarely have negative consequences. Future research in this field should focus on the concentration at which natural extracts have a cytotoxic effect, the stages of cell cycle arrest in cancer cells, the kind of cellular damage, and the mechanism by which these extracts have an anticancer effect. Researchers have been looking at alternative therapy approaches and cures to stop the spread of cancer for the past few decades, but their efforts have met with little success and a high failure rate. There have been suggestions lately about employing naturally occurring extracts to stop cancer cells from proliferating (Augustine *et al.*, 2018).

The exploration of earthworm extracts as potential anticancer agents represents a promising avenue in cancer research. Previous studies have demonstrated the cytotoxic effects of earthworm-derived compounds on various cancer cell lines, including breast cancer (MCF7), lung cancer (A549), prostate cancer (PC-3), and others. Earthworm extracts and their excreting fluids known for anti-cancer activity. Example, earthworm powder has been found to possess the potential to inhibit the growth of certain cancer cells. The concentration had a negative impact on the viability of MCF-7 and PC-3 cells. The IC₅₀ values for PC-3 and MCF-7 cell lines were 265.5 and 965.9 µg/ml, respectively. In contrast, the earthworm powder did not exhibit any cytotoxic effects on the WRL68 cells (Shafi and Faleh, 2019). However, the precise molecular mechanisms underlying their anticancer activity remain incompletely understood, presenting a compelling area for further investigation. One intriguing aspect of earthworm biology that warrants exploration is the selective cytotoxicity of earthworm extracts towards cancer cells while sparing healthy somatic cells. Understanding the molecular basis of this selectivity could provide valuable insights into the development of targeted anticancer therapies with minimal off-target effects.

In this study, we delve deeper into the anticancer properties of earthworm extracts, focusing specifically on a segment within the gastric region of *Eudrilus eugeniae*. Inspired by the interstitial stem cell dynamics observed in organisms like

Hydra vulgaris, we hypothesize that the gastric epithelial cells of earthworms may exert regulatory effects on cancer cells, akin to their role in controlling stem cell differentiation. The topical findings from flow cytometry analysis with Annexin V confirmed the proapoptotic impact of the earthworm crude coelomic fluid on A549 lung cancer cells (Fiołka *et al.*, 2019). By elucidating the molecular mechanisms underlying the anticancer activity of earthworm extracts, we aim to contribute to the burgeoning field of natural product-based cancer therapeutics. Recent studies have shown that the nanoparticle which is produced from the earthworm coelomic fluid has the potential to combat cancer cells in various types of cancer including HeLa, A549, Pa17, PC-12, or Hep-2 cancer cell lines (Rybicka *et al.*, 2022).

There have been multiple reports suggesting that extracts from earthworm bodies, as well as their crude and coelomic fluid components, may possess anti-cancer properties. However, the exact molecular mechanism responsible for this activity remains unclear.

For this study, we have selected a specific segment in the gastric region of an earthworm. The gastric region of an earthworm is composed of various tissue layers, such as epithelial layers, circular muscle layer, and longitudinal layer. However, it does not contain any distinct organs. The inner wall of the intestine is folded inward, forming a structure known as the typhlosole. This fold is responsible for housing blood vessels and enhancing the surface area of the intestine for absorption. The inner epithelium is composed of columnar cells and glandular cells. The selection of the gastric region is influenced by insights gained from studying the interstitial stem cell lineage of *Hydra vulgaris*, which is controlled by gastric epithelial cells. The production of epithelial peptides by gastric epithelial cells has a significant impact on the differentiation of stem cells. The key point is these epithelial cells releasing peptides only regulates the stem cell differentiation and not affecting the somatic cells.

We have inspired from this particular mechanism in this study have selected gastric tissue extracts on MCF7 (Cancer cell line) and C2C12 (Somatic cell line) to investigate the potential molecular mechanism behind the anticancer activity. In this study, we present the findings of our research on the potential anti-cancer activity of the gastric region of earthworms. We also examine the effects of this activity on

protein levels in cancer cells, while observing that it does not impact the protein pattern of somatic cells.

CHAPTER-2

LITERATURE SURVEY

2.1 NATURE OF THE EARTHWORM *EUDRILUS EUGENIAE*

Earthworms are creatures that live in soil and play a role in the breakdown of organic matter and soil formation. Earthworms of the *Eudrilus eugeniae* species are frequently utilized in tropical and sub-tropical regions for vermicomposting (Somniam and Suwanwaree, 2009). Earthworm *Eudrilus eugeniae* is indigenous species to Africa but it has a bred in USA, Canada, Europe and Asia. It is also called as an Africa nightcrawler, to be used as fish bait.

2.2 PROJECTING CANCER MORTALITY IN INDIA: URGENT NEED FOR ACTION

Cancer is indeed a leading cause of death globally, with significant impacts on individuals and healthcare systems. One of the main causes of death worldwide is cancer. In the India, more than a million new instances of cancer were reported in 2023, and more than 14,00,000 people lost their lives to the disease (Smith and Mallath, 2019). The unchecked spread of cancer cells and metastasis, a multi-step, intricate process that occurs throughout the development of malignant tumors, present unique challenges in the fight against cancer. It is impacted by systemic, local environmental host factors, and intrinsic characteristics of the tumor itself (Lu, Weaver and Werb, 2012). The unchecked spread of cancer cells and metastasis, a multi-step, intricate process that occurs throughout the development of malignant tumors, present unique challenges in the fight against cancer. It is impacted by systemic, local environmental host factors, and intrinsic characteristics of the tumor itself. Over time, significant attempts have been made to produce novel anti-cancer medications. Nonetheless, a number of cancer types still have 5-year survival rates that are less than 20%, including pancreatic and lung cancer. Furthermore, individuals who received radiation and chemotherapy experience side effects like nausea, hair loss, muscle soreness, and even an increased chance of getting cancer again because the therapy also damages healthy, functioning cells in addition to cancerous ones (Popoviæ *et al.*, 2001).

Efforts to gauge cancer mortality in India have been limited due to data scarcity. A recent attempt projected cancer deaths from 2011 to 2026, estimating 0.44 million deaths in 2011, rising to 0.70 million by 2026. Male deaths increased from 0.23 to 0.38 million, while female deaths rose from 0.20 to 0.32 million. Breast, cervical, and ovarian cancers were major causes for women, while lung, esophageal, prostate, and stomach cancers led among men. This highlights the urgent need for robust action to reduce risk factors, strengthen screening, and improve treatment facilities (DSouza, Murthy and Aras, 2013). The fundamental tenet of cancer treatments has continued to be the search for an appropriate solution to the challenge of treating cancer and stopping its progression. There are ideas of employing naturally occurring extracts to stop the growth of cancer cells. Chemotherapeutic medications have two effects. In addition to eliminating cancer cells, some healthy cells are also sacrificed, including those lining the gastrointestinal system, bone marrow cells, and hair follicles, which has serious negative effects (Udensi and Tchounwou, 2014).

2.3 TRADITIONAL MEDICINE FROM EARTHWORM

Traditional medicine has a long history of using earthworms as a therapy for disease. Burmese people utilized the ashes from burnt earthworms to treat fever; Laotians used earthworms to treat smallpox; and the ancient Chinese employed earthworms to treat blood clots, hemiplegia, and convulsions brought on by fever (Fu *et al.*, 2014).

Naturally occurring materials, including extracts from earthworms, have gained attention recently for their potential to stop cancer cells from proliferating and dividing, which is an intriguing area of research. Finding biomolecules that can stop cancer cells from growing should be the goal of current research. In determining the earthworm extract's ability to inhibit the growth of cancer cells may be a first step toward the creation of drugs and other potentially effective anticancer treatments. Thus, this study's objective was to ascertain, by a comprehensive evaluation, the anticancer potential of earthworm extracts on cancer cells, with a focus on the extract's composition and the cancer models used (Bhavana and Lakshmi, 2014). Using the MTT assay, researchers observed that the caelomic fluid induces cell death in a concentration-dependent manner. This suggests the presence of

bioactive molecules with potential applications in cancer treatment (Dinesh *et al.*, 2013).

Six studies employed the earthworm fibrinolytic enzyme [6,16-19,24] to test anticancer activity with appreciable results. MCF-7 cell growth and migration can be significantly inhibited by earthworm fibrinolytic enzyme by *Eisania foetida* in a dose- and time-dependent manner. Furthermore, 24 hours after being exposed to 40 µg/ml of earthworm fibrinolytic enzyme, MCF-7 cells started to undergo apoptosis. The expression of CD44v6 and focal adhesion kinase, as determined by Western blot and reverse transcription polymerase chain reaction, was down-regulated in a concentration-dependent way (20-80 µg/ml), which led to the suppression of adhesion of MCF-7 cells (Liu *et al.*, 2017). The study investigated the inhibitory effects of earthworm fibrinolytic enzyme (EFE) on xenografted hepatocellular carcinoma (HCC) tumors in nude mice. EFE demonstrated significant tumor growth inhibition and synergistic antitumor activity when combined with 5-fluorouracil (5-Fu). It also suppressed the expression of CD44v6, a protein associated with cancer progression. EFE showed no adverse effects on the hematopoietic system (Yan-Qiu *et al.*, 2007; Wang *et al.*, 2009). Both in vitro and in vivo, EFE had strong anti-tumor action in hepatoma cells. This may be because EFE inhibited MMP-2 production and caused apoptosis in hepatoma cells. This implies that EFE may have a part in the management of hepatoma (Hong *et al.*, 2007).

In the study conducted by Reddy *et al.*, earthworm paste derived from three different species, namely *Pheretima excavatus*, *Eudrilus eugeniae*, and *Eisenia fetida*, was utilized to assess its anticancer effects on three distinct cancer cell lines: MCF-7 (breast cancer), PC-3 (prostate cancer), and HCT-116 (colorectal cancer). The paste preparation involved processing earthworms from these species into a form suitable for testing. The researchers then evaluated the efficacy of the earthworm paste against the respective cancer cell lines to determine its potential as an anticancer agent (Reddy, Kumar and Saidullah, 2015). Using earthworm powder from *Lampito mauritii*, Lourdummy and Ramesh (2014) demonstrated a remarkable percentage of inhibition for HT-29 cells, reaching up to 82.25% at 320 µg/ml in 48 hours. HT-29 cells treated at 80 µg/ml concentration exhibited 47.67% cell cycle arrest at the G2/M phase. The findings indicated that the sample's cell cycle was stopped at the G2/M phase, and that these treated HT-29 cells also

experienced apoptosis during this period (Lourdumary and Ramesh, 2014). Mohamed Jaabir *et al.* (2011) evaluated the anticancer activity of *E. eugeniae*'s cell-free coelomic fluid. When the incubation period was extended from 48 to 72 hours, an increase in the apoptotic rate from 68.91% to 79.32% was seen, indicating that the coelomic fluid's induction of apoptosis in SiHacells was dose- and time-dependent. Analysis of the coelomic fluid using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of a 42 KDa protein that had previously been found to be a tumorolytic factor similar to the vertebrate cytokinetumor necrosis factor (Jaabir *et al.*, 2011).

In 2004, Engelmann *et al.* extracted supernatants of cultured coelomocytes and lysates from coelomocytes of *E. fetida* using mechanical and detergent extraction combined with six voltage electric stimulation. Their findings demonstrated that these extracts, in a dose-dependent manner, significantly reduced the ratio of living cells in HeLa, HEP-2, PC-12, and PA317 cell lines when compared to controls (Engelmann *et al.*, 2004). Verma *et al.* (2013) employed a serine protease isolate from *Pheretima posthuma* on the MCF-7 cell line, indicating that the 15 kDa fraction exhibited strong cytotoxic activity. The earthworm was autolyzed to produce the protease following several washings in sterile distilled water. SDS PAGE, DEAE-C chromatography, and the caseinolytic plate diffusion assay were utilized for the purification and isolation processes (Verma *et al.*, 2013). A polysaccharide-protein complex (PPC) that was isolated from metabolites of the gut bacteria *Raoultella ornithinolytica* from *Dendrobaena veneta* earthworms demonstrated activity against the T47D cell line, a cell line used to study breast ductal carcinoma, and the TOV-112D endometrioid ovarian cancer line in vitro, according to a novel study by Fiołka *et al.* (Fiołka *et al.*, 2013).

2.4 CANCER STUDY MODELS EMPLOYED

Cell lines have been employed in the majority of studies to investigate cytotoxicity. For cancer research, cancer cell lines are suitable models because they have a more complex genetic makeup and frequently exhibit substantial chromosomal rearrangements, oncogene mutations, and allelic imbalance. It is possible to study the course of cancer cells up to metastasis by examining the phenotypic and genotypic characteristics that change across several divisions (Raju

et al., 2017). For many years, the first stage in medication testing has been testing on cancer cell lines. Thus, it is regarded as the initial stage of evaluating a number of intricate medicinal preparations prior to their large-scale *in vivo* application. In addition to the current *in vitro* investigations, four studies used *in vivo* models, such as a model of naked mice, to examine the earthworm extract that used (He, Chen and Ye, 2005). Li Hong Yan *et al.* (2004) tested the EFE using models of xenotransplanted nude mice (Li *et al.*, 2004). The process of genetic modification and molecular characterization is made easier by the use of cell lines, which are essential experimental models for cancer research. Cell line studies have been utilized in the past to test and develop medications and therapies, and they have also uncovered signaling pathways in cancer (Riss *et al.*, 2016). Mice were given varying doses of EE orally for a period of 25 days after receiving an ascitic fluid inoculation made from mice injected with S180 sarcoma tumor cells. Tumor samples were examined for cell apoptosis and size. Furthermore, we discovered that when the quantity of EE given increased, the tumor's weight and size steadily shrank. Compared to the untreated group, the EE-treated group had more apoptotic cells and a decreased level of lactate dehydrogenase (LDH), a biomarker of tumor invasiveness (Deng *et al.*, 2019).

2.5 CYTOTOXICITY TESTS

Cell death brought on by a compound's harmful effects is one aspect of cytotoxicity research that encompasses a wide range of metabolic changes in cells. The cytotoxicity and cell viability can be measured using a variety of tests. The use of animals is eliminated and *in vitro* testing for safety assessment and cytotoxicity is more affordable. 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 (Riss *et al.*, 2016).

CHAPTER-3

AIM AND SCOPE OF INVESTIGATION

3.1 AIM

The purpose of this research was to investigate the dual regulatory role of enhancing anticancer and cell survival potential of earthworm tissue extract.

3.2 SCOPE OF INVESTIGATION

- ❖ To prepare earthworm body extract and evaluate its role in determining cell viability against MCF-7 and C212 cell lines.
- ❖ To analyze cleaved Caspase-3 expression against MCF-7 and C212 cell lines in control and earthworm body extract treated samples
- ❖ To determine the asymmetric expression of cell fate determinant protein TCTP, p53, CoxD3, in MCF-7 and C2C12 cell lines.
- ❖ To analyze the cellular localization of cleaved caspase-3 and Tubulin in MCF-7 and C2C12 using Immunofluorescence.

CHAPTER-4

MATERIALS AND METHODS

4.1 EARTHWORM REARING

The culture stocks of earthworm *Eudrilus eugeniae* was reared at the Centre for Molecular and Nanomedical Sciences, International Research Centre, Sathyabama Institute of Science and Technology, Chennai, Tamilnadu, India. Earthworms are maintained according to the established protocol developed by Karthikeyan chellathurai et al (Chelladurai *et al.*, 2020). The lower layer, which includes coir, effectively preserved the required moisture level. On a weekly basis, the earthworm compost was replaced with a mixture of soil, dried cow dung, and leaf litter (Johnson Retnaraj Samuel *et al.*, 2012).

4.2 COLLECTION, PREPARATION OF TISSUE SAMPLES FROM EARTHWORM BODY SEGMENTS

A group of adult earthworms were selected for amputation. Afterwards, they are placed in individual plastic containers and given time to adjust for a maximum of 2 days. The worms were amputated at the 30th to 37th segment (weight: 30-35mg) using a sterile scalpel blade (Lister Surgical Blade; Size 15, Kanpur, India). The dissected samples were rinsed with distilled water three times before being transferred into 1X Phosphate Buffer Saline (PBS) for surface sterilization. After that, the body extracts were stored in a solution consisting of 45ml of 0% DMEM media and 5ml of Fetal Bovine Serum (FBS). The DMEM used was from Sigma-Aldrich (Catalogue: D1152-10L) and the FBS was from Gibco. Catalogue: 10270106) and crushed using a tissue homogenizer (Bel-Art Disposable Plastic Pestle; Spectra Services, Wayne County, Michigan. Catalogue: SKU: 19923-0000) at 4°C. The crushed tissue samples underwent a brief spin in the centrifuge, and the resulting supernatant was collected and stored in a freezer at -20°C. The prepared mixture of body segments was utilized for cell culture studies and animal studies.

4.3 PROTEIN ISOLATION

Beta-Mercaptoethanol (β -ME-reducing agent) used for breaking of disulfide bonds of protein. 2X sample buffer were added with 5% of Beta-Mercaptoethanol (reducing agent) (stock 1). Samples were transferred into the fresh 1.5ml vial which has an equal amount of sample buffer at 4°C. Homogenizer was used for homogenization manually by hands after that samples were transferred into the dry bath at 100°C for 5 minutes, then centrifuge the samples at 10000 rpm for 5 minutes and supernatant is transferred to the fresh 1.5ml via and stored them into -20°C.

4.4 CELL CULTURE

The MCF7 human breast cancer cell line and C2C12 mouse myoblast cells were acquired from the National Center for Cell Science in Pune, India. The MCF7 and C2C12 cells were cultured in T-flasks with DMEM supplemented with 10% FBS and 1% antibiotics, including penicillin, streptomycin, and amphotericin B. The cells were maintained in a CO₂ incubator set at 37°C with a 5% CO₂ supply. Cells were sub-cultured once they reached 90% confluence. The culture maintenance was performed in a biosafety cabinet level II to ensure a sterile and aseptic environment. Haier, a company based in Qingdao, China, presents the HR40-IIA2 model.

4.5 MTT ASSAY

In order to assess the cell viability, C2C12 cells (stock 1×10^5 cells/ml) were placed in the 96-well plate (10,000 cells/well) and kept in an incubator at 37°C with CO₂. Following a 24-hour incubation period, the plates underwent three washes using 0% DMEM media, devoid of serum. In addition to using 10% DMEM medium, the body segments (30th-37th) of the tissue extracts were subjected to different percentages of treatment (5, 25, 50, 75, 100%) and incubated at 37°C in a CO₂ incubator. After incubation, MTT solution was added to each well of a 96-well plate and left to incubate for 2 hours in a dark environment. Following incubation, the media was gently removed and DMSO (Himedia; Mumbai, India. Catalogue: MB058-100ml; 100 μ l/well) was added to the plate to induce the formation of a purple color, which correlates with the quantity of viable cells. The 96 plate was analyzed for OD value at 570 nm using a microplate reader (BioTek Epoch Microplate Spectrophotometer;

United States). The percentage of cell viability was determined by calculating the ratio of the treatment average optical density to the control average optical density, multiplied by 100.

4.6. SDS-PAGE:

4.6.1. Principle:

SDS PAGE is an analytical technique which is used to separate the proteins based on their molecular weight. Predominantly Electrophoresis is the study of the movement of charge molecules in an electric field. Their rate of movement depends on the strength of the field, net charge, size and shape of the molecules and also the ionic strength, viscosity and temperature of the medium in which the molecules are moving. Polyacrylamide gels are widely used for separating larger molecules like proteins. Based on the resistance of the gel matrix proteins are separated viz., smaller proteins generally have less resistance as well as larger proteins have more resistance. The molecular weight of protein was estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS) and a reducing agent β -Mercaptoethanol, it can bind with the protein backbone and denaturing effect of SDS is very strong and that disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by reducing all disulfide bonds (both inter-disulfide and intra-disulfide bonds). Polyacrylamide creates a mesh like matrix that helps in the separation of protein. During PAGE electrophoresis linear or primary structure of the proteins were stacked or arranged in the proper way, once reached the separating gel, proteins start to separate based on their size.

4.6.2. Materials

Eppendorfs; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.6.3. Chemical preparation of SDS-PAGE

4.6.3.1 30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide	- 30 g
------------	--------

N,N' Methylene Bisacrylamide - 0.3 g

Make up into 100 ml with distilled water.

4.6.3.2 4X Separating Buffer

Tris HCL - 18.2 g

Distilled water - 60 ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

4.6.3.3 4X Stacking Buffer:

Tris HCl - 6.05 g

Distilled water - 40 ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4 g

Make up into 100 ml with distilled water.

4.6.3.4 2X Sample Buffer:

Tris stacking buffer (pH 6.8) - 25 ml

Sodium Dodecyl Sulphate (SDS) - 4 g

20% Glycerol - 20 ml

Make up into 100 ml with distilled water. During protein sample preparation, 5% β -mercaptoethanol and during loading 0.001% (w/v) of Bromo-phenol blue was added.

4.6.3.5 10% Ammonium Per Sulphate (APS):

APS - 100 mg

Make up into 1 ml with distilled water.

4.6.3.6 1X Running Buffer:

Tris Hcl - 3.94 g

Glycine - 15.014 g

SDS - 1 g

Make up into 1L with distilled water.

4.6.3.7 Staining Solution:

Coomassie Brilliant Blue R-250 - 200 mg

Methanol - 50 ml

Glacial Acetic acid - 7 ml

Make up into 100 ml with distilled water.

The above mixture was filtered using Whatman No 1 filter paper.

4.6.3.8 Destaining Solution:

Methanol - 30 ml

Glacial Acetic acid - 7 ml

Make up into 100 ml with distilled water.

Table.4.1: Separating gel concentration

Sl. No	Stock Solution	5%	6%	7%	7.5 %	8%	9%	10%	12%	13%	15%
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	5.0	6.0	7.0	7.5	8.0	9.0	10.0	12.0	13.0	15.0
2.	4X Tris Separating Buffer (ml) pH 8.8	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
3.	Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
4.	10% APS (μl)	200	200	200	200	200	200	200	200	200	200
5.	TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table.4.2: Stacking gel concentration

Sl.No	Stock Solution	5 ml	10 ml	15 ml	20 ml
1.	30% Acrylamide + 0.3% Bis-acrylamide (ml)	0.65	2.5	3.75	5.0
2.	4X Tris Stacking Buffer (ml) pH 6.8	1.25	2.5	3.75	5.0
3.	Distilled water (ml)	3.05	5.0	7.5	10.0
4.	10% APS (μl)	100	100	100	100
5.	TEMED (μl)	10	10	10	10

4.6.4 Preparation of gel:

The glass plates were cleaned by using ethanol and manually assembled. Glass plates fixed in the proper equalize manner with each corners covered by using spacers so that used as a primary baseline on gel which helps in a process of agarose sealing or second level baseline system. 1% agarose was prepared and boiled until liquidy then corners sealed in both surface and bottom area of spacer. Separating gel was manually prepared and loaded on the gel with careful manner. The gel was overlaid with isopropyl alcohol so that it excludes the air. Then IPA was removed and washed with distilled water, after drain stacking gel was prepared and added, instantly comb was fixed on the stacking gel. The gel was allowed few minutes to solidifying then comb were carefully removed and wells manifestly washed with water to eliminate the bubbles and the bottom of the spacer were carefully removed and setup was fixed in the bottom chamber which carried 1X running buffer as well as upper chamber.

4.6.5 Sample loading and electrophoresis:

Protein sample of 10 µg and the appropriate molecular markers were loaded in the wells. The apparatus was connected to the power pack with proper polarity and 5 to 6 v/cm was applied. After electrophoresis, the glass plate assembly was taken from the buffer chamber. The spacer was gently twisted so that the upper glass plate pulls away from the gel.

4.6.6 Staining and destaining the gel:

The gel was carefully taken out from the glass plate and the stacking gel was removed by using cleaver. Separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the commassie brilliant blue R-250 (stainer solution). The gel should be fully submerged in the staining solution for 30 minutes by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to destaining solution until protein bands are visualized clearly. Commonly first destainer wash is about 30 minutes then second wash is about overnight.

4.7 WESTERN BLOTTING

Protein samples were prepared from the control and body extract treated (50%) MCF7 and C2C12 cells using RIPA buffer. After that, samples were measured using Lowry's method. Afterwards, 80µg of each protein sample were separated using 12% SDS-PAGE. The proteins that have been resolved in the SDS-PAGE gel are then transferred to the PVDF membrane. Next, the protein membrane underwent treatment with a 5% BSA in TBST buffer to block any unwanted interactions. After that, the membrane underwent treatment with primary antibodies, including Anti-p53 antibody (Abcam, ab26), Anti-TCTP antibody (Abcam, ab37506), Anti-Caspase-3 (Abcam, ab208161), and Anti-H3 (Abcam, ab1791), at a dilution of 1:5000. This was done at a temperature of 4°C overnight. After incubation, the membrane is rinsed three times with 1×PBST buffer and then exposed to the secondary antibodies. The secondary antibodies used are anti-rabbit IgG, HRP (Sigma, A0545), and Anti-Mouse IgG, HRP (Abcam, ab6789), diluted at a ratio of 1:10,000. This incubation step is carried out for 2 hours at room temperature. The washing step was repeated three times, followed by the addition of ECL substrate onto the membrane. The luminescence signals from the membrane were then captured using the ChemDoc system (Bio-Rad ChemiDoc XRS+; United States. Catalogue: 170-8265).

4.7.1 CHEMICAL PREPARATION

4.7.1.1 Buffer preparation

10X Transfer Buffer (Stock)

Glycine - 72.1g

Tris-base - 15.2g

Distiller water- 500ml

The 10X transfer buffer was stored in the room temperature, and during blotting the 1X transfer buffer were prepared from the 10X in the ratio of preparation viz.,

For Total volume 1 liter

100ml - 10X Transfer Buffer

200ml - Methanol

700ml - Distilled water

4.7.1.2 Blocking

Blocking buffer was used to block all proteins which would bind with targetable and non-targetable proteins. 5% BSA with 20ml of 1X TBST buffer was prepared for Blocking.

4.7.1.3 Primary Antibody

Primary antibodies was added in the 5% BSA solution (1:5000 dilutions). Only could be binds with specific antibody.

4.7.1.4 Secondary Antibody

Secondary antibodies was added in the 5% BSA solution (1:10000 dilutions). Only could be binds with primary antibody.

4.7.1.5 ECL Preparation

Mix equal volume of Peroxide solution and Luminol /enhancer solution (200 µl + 200 µl)

4.8 IMMUNOFLUORESCENCE ASSAY

Cells were cultured in a six well plate with the supplement of body extract (50%) and control (10% FBS). After 24 hours of treatment, they were washed three times with ice-cold 1XPBS buffer. The cells were treated with a fixative solution and a permeabilizing agent to prepare them for further analysis. The cells were treated with a 1% solution of Bovine Serum Albumin (BSA) for 30 minutes to block them. Afterwards, they were incubated overnight at 4°C with primary antibodies, including Anti-Caspase-3 and Anti-α-tubulin. The cells were rinsed with 1X TBST and then exposed to appropriate secondary antibodies for 2 hours at room temperature in a dark environment. Following that, the cells were rinsed three times with 1X TBST buffer and then stained with DAPI (40,6-diamidino-2-phenylindole) (Sigma Aldrich, United States Catalogue: 10236276001). They were then mounted using DPX

mount (SRL; Maharashtra, India. Catalogue: 88147) and examined using an EVOS fluorescence microscope.

4.8.1 Chemical Preparation

4.8.1.1 1XPBS

Take 30ml of 10X PBS +
270ml Distilled water

4.8.1.2 TBST Buffer (volume 100ml)

1XPBS buffer = 100ml
0.1% Tween 20 = 100µl

4.8.1.3 4 % Paraformaldehyde

1XPBS = 50ml
Paraformaldehyde = 2grams

4.8.1.4 Permeabilization (For 50ml)

Triton X100 = 10µl
1X PBS = 10ml

4.8.1.5 Blocking Buffer

BSA = 50mg
Glycine = 114.25mg
1X PBS buffer = 5ml

4.8.1.6 Primary antibody

1:100 Concentration
1 µl antibody with 100 µl TBST buffer.

4.8.1.7 Secondary Antibody

1:200 Concentration
0.5 µl antibody with 100 µl TBST buffer.

CHAPTER-5

RESULTS

5.1 HISTOLOGY

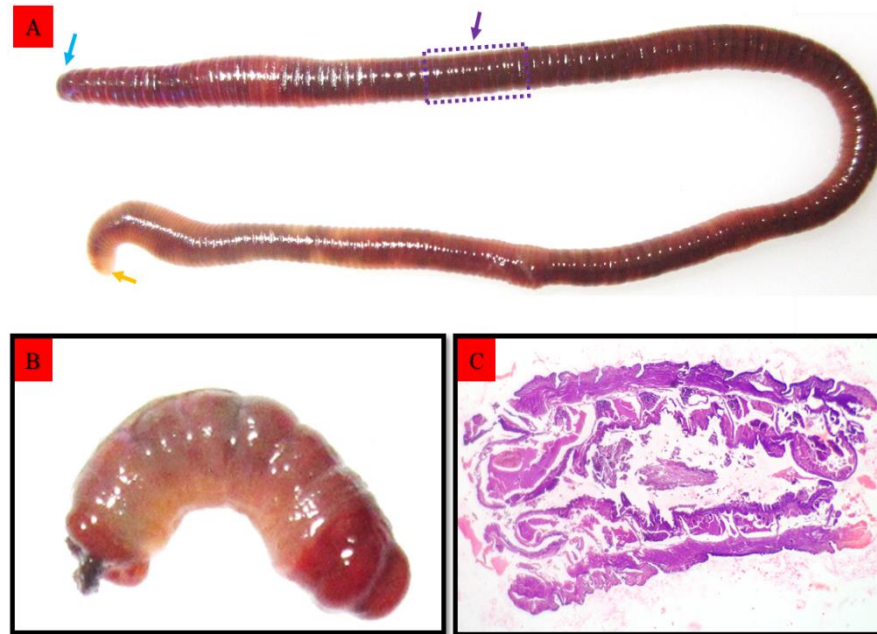


Fig:5.1: Morphological and Histological characteristics of 30th - 37th body segments

In this study, we have chosen the regeneration animal model *Eudrilus eugeniae*. It's a clitellum dependent earthworm. The epigeous *E. eugeniae* has a cylindrical shape, length is 80mm-210mm, width is 3.5mm-5.0mm. Due to the presence of clitellum, the body of an earthworm is divided into pre-clitellar region (1st-13th segments), clitellar region (14th to 17th segments) and post-clitellar region (after 17th segment) that have shown in Figure 1A. For this study, we have selected the intestine region (30th-37th segment) of an earthworm. This particular region is of interest due to its composition, which includes the intestine tract and various tissue layers such as the chloragogen layer (responsible for carrying coelomic fluid), epithelial layer, circular muscle layer, and longitudinal layer. It not contains any specific organs in this region that have shown in Figure 1B & 1C.

5.2 MTT ASSAY

5.2.1 Earthworm body segments effects on MCF7 and C2C12

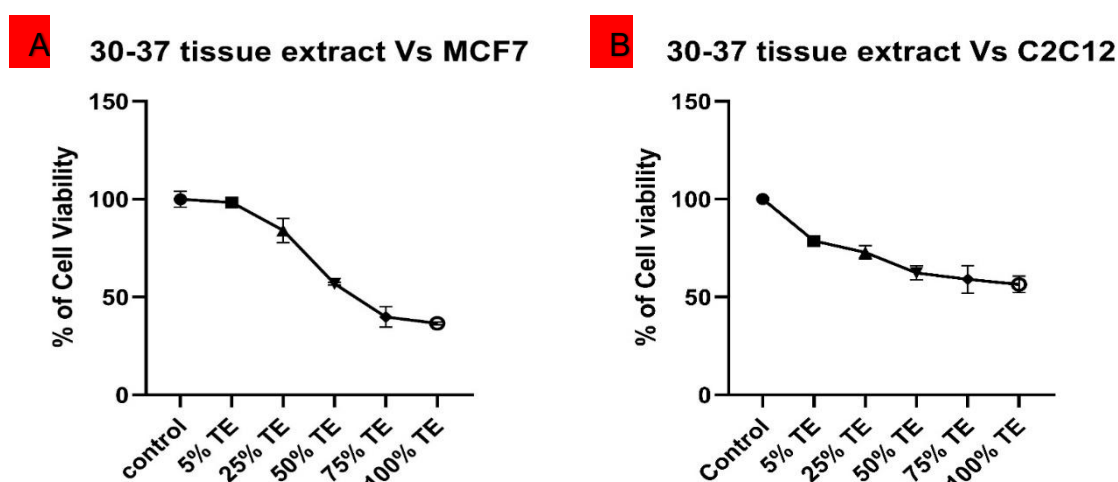


Fig: 5.2.1: Earthworm body segments effect on MCF7 and C2C12 cell lines

The tissue mixture from the 30th to 37th segments of the prepared earthworm body, which includes the gastric region, exhibited a greater negative impact on the cell viability of MCF7 cells compared to C2C12 cells. The observed phenomenon entailed a notable reduction in cellular proliferation of MCF 7, specifically when subjected to treatment concentrations of 50%, 75%, and 100% (as depicted in Figure 2A). Remarkably, a significant proportion of cellular entities, specifically 36.5%, exhibited viability solely at the concentration of 100% treatment. The observed trend exhibited a gradual decline, with a decrease in cellular survival observed in approximately 56.8% of the cells subjected to the 50% treatment. Similarly, at a concentration of 75% treatment, approximately 39.8% of the cells demonstrated survival. Based on the findings, it can be observed that the treatment exerted a moderate influence on the growth of C2C12 cells (Fig. 2B). The lowest observed viability of cellular organisms was recorded at a rate of 57.8%, specifically when subjected to a treatment concentration of 100%. At the specified concentrations of 5% and 25%, the treatment did not exert any discernible impact on cellular proliferation, as evidenced by the survival of approximately 80% to 70% of cells. Conversely, at concentrations of 50%, 75%, and 100%, the treatment facilitated cell viability, resulting in survival

rates of 66.3%, 59.7%, and 57.8%, respectively. In a comparative analysis, it was observed that the cell viability of C2C12 myoblast cells exhibited a relatively higher percentage (21.3%) of unaffected viability compared to MCF7 human breast cells.

5.2.2 Cell survivability of tissue parts

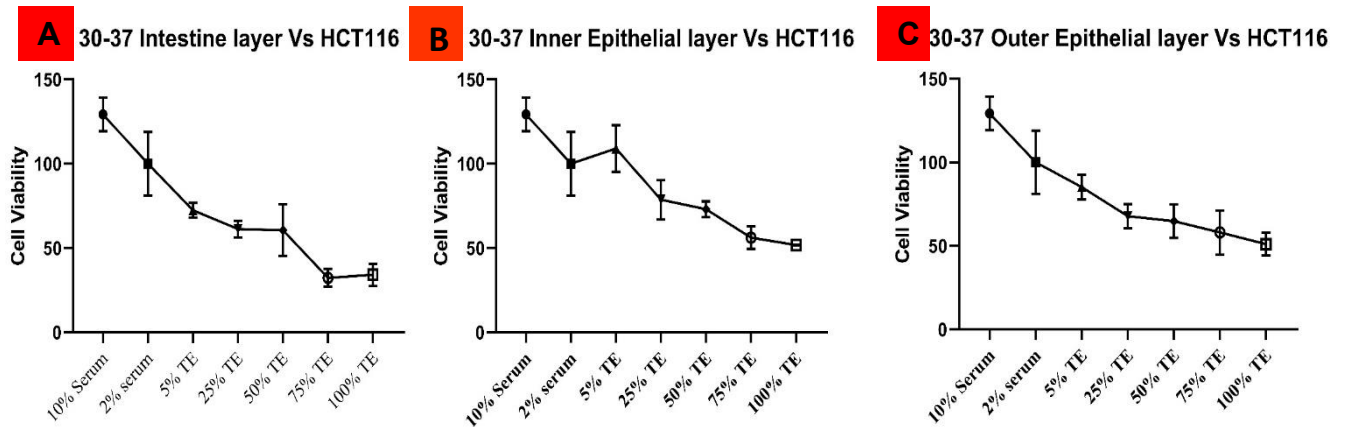


Fig:5.2.2 Cell survivability of tissue parts

A. Cell viability assay was connected between inner intestine layer with HCT116 cancer cell line in various dilution (5%,25%,50%,75%,100%) and as a control 10% and 2% DMEM has employed. It shows that growth of HCT116 cells was negative response in the all the above concentrations, there was a least cell growth of 32% was observed in 75%. **B and C.** The moderate negative response was seen in the inner and outer epithelial layer of the 30-37 segment.

5.3 WESTERN BLOTTING

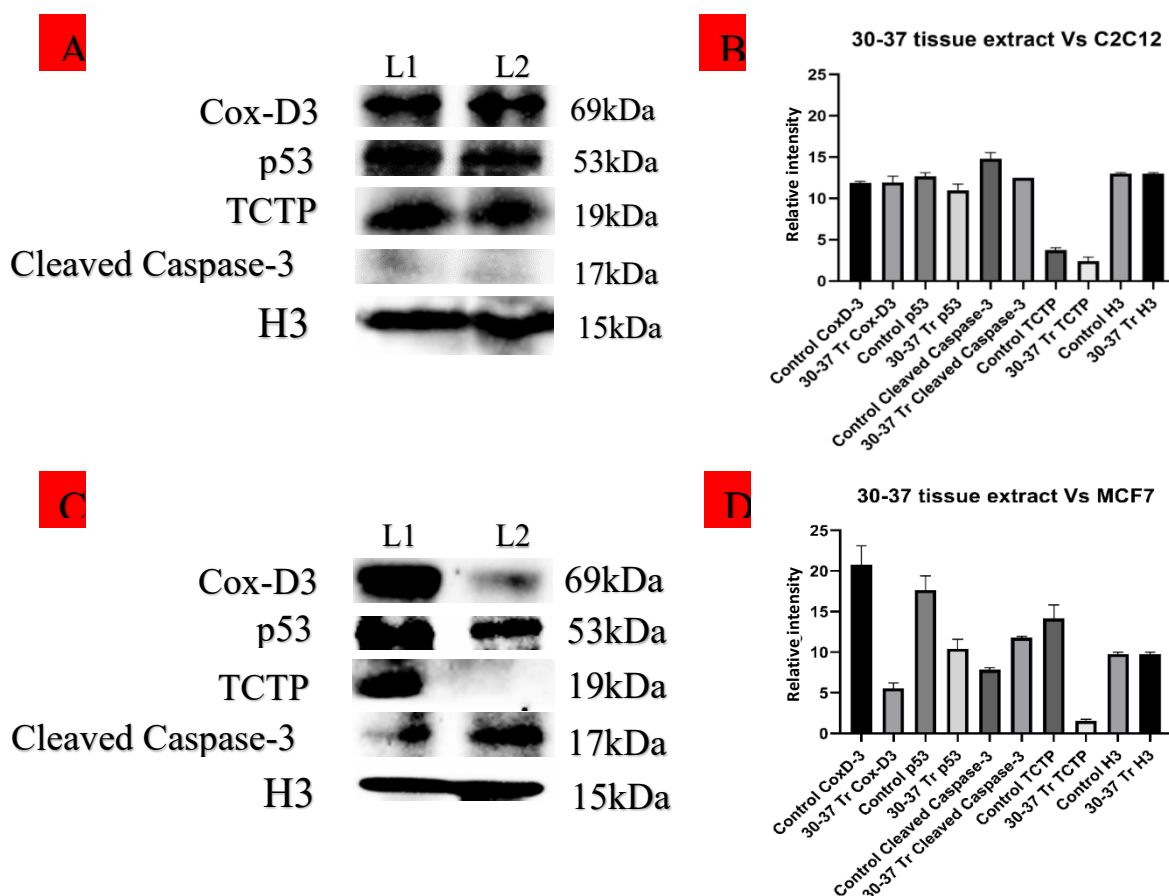


Fig: 5.3: Exploring the molecular changes caused by earthworm body segments on cancer cells and somatic cells.

In order to better understand the molecular processes underlying the anticancer activity of earthworm body segments, protein samples obtained from MCF7 and C2C12 cells are subjected to treatment with a 25% tissue extract. These samples are analyzed using western blotting, following the procedures outlined in the materials and methods section. According to the results of MCF7, there was a significant increase (3.93 fold) in cleaved caspase-3 levels in the samples treated with body extract compared to the control samples (Fig. 3B). The expression of TCTP was significantly decreased in the samples treated with body extract, while there was a slight reduction in p53 expression. Due to the expression of p53 and cleaved caspase-3, it has led to the hypothesis of apoptosis execution that is

dependent on p53. In a different scenario involving in C2C12 cells, data represents cleaved caspase-3 (apoptosis executor), p53 and TCTP (cell fate determinants) protein expressions were not changed while compare to the C2C12 control samples (Fig. 3B). It appears that the body segments of earthworms do not have any significant effects on the C2C12 cells (somatic cell line). The findings from the western blotting experiments suggest that the earthworm body extract has a more detrimental effect on MC7 Cells compared to C2C12 cells.

5.4 IMMUNOFLUORESCENCE ASSAY

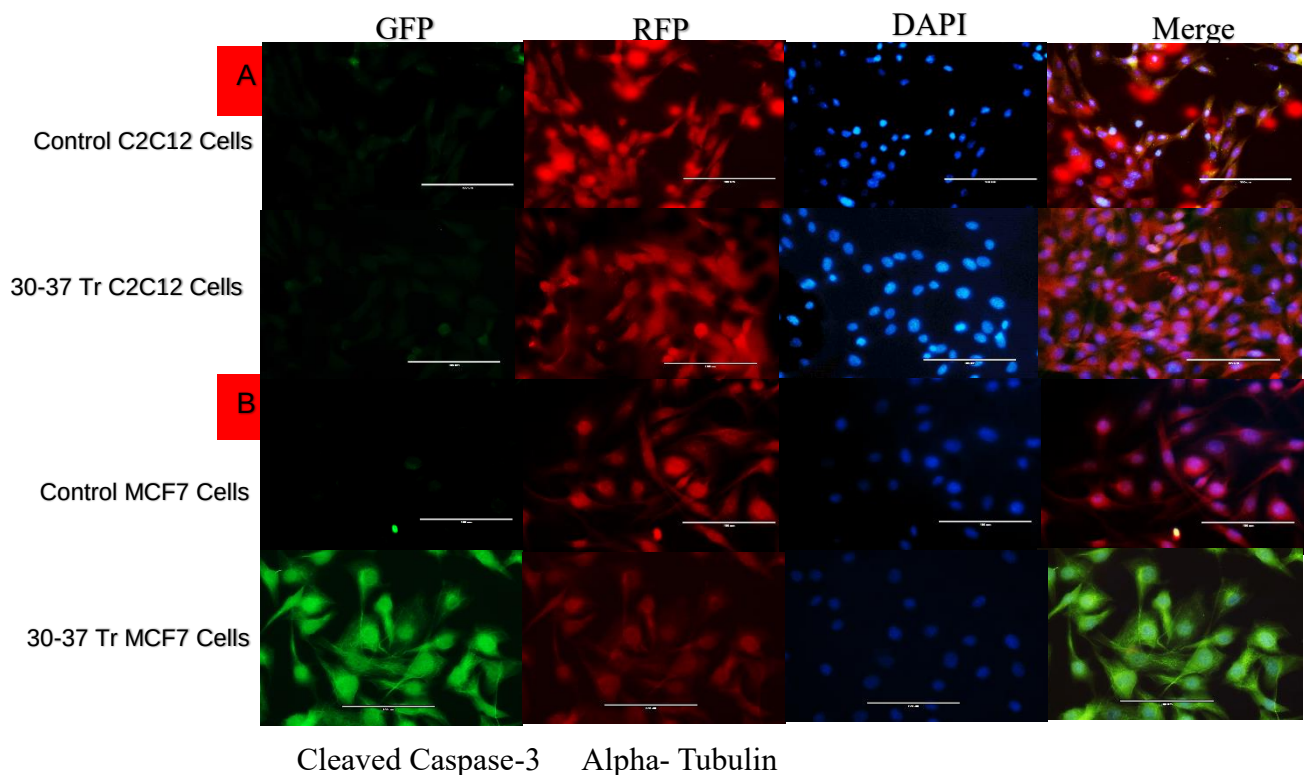


Fig: 5.4: Apoptosis effect of earthworm body segments on breast cancer cell line (MCF7) and somatic cell line (C2C12).

To gain insight into the signalling responses of earthworm tissue extracts on MCF7 and C212 cells, we examine the expression of cleaved caspase-3 in cellular level within the cells using immunofluorescence. The image capturing light intensity is adjusted equally for every protein sample to facilitate protein

expression comparison. The cleaved caspase-3 expression was notably increased in the earthworm body extract treated MCF7 cells than control MCF7 cells (Fig. 4A). The protein expression was recorded in the both cytoplasm and nucleus as well, it is indicating that cleaved caspase-3 was activated in the MCF7 cells by earthworm body extracts. Meanwhile, the cleaved caspase-3 was not activated notably in the C2C12 (somatic cells) but tiny expression was present (Fig. 4B). It's indicating that earthworm body extract which not intensively activating the cleaved caspase-3 in C2C12 cells.

DISCUSSION

In 2020, there were 2.3 million women who received a diagnosis of breast cancer and unfortunately, 685,000 lives were lost worldwide. By the end of 2020, there were 7.8 million women who had been diagnosed with breast cancer in the past 5 years, making it the most common type of cancer worldwide (WHO, 2020). Natural extracts from animal and plants are being an effective source for chemotherapeutic agents. In all form of earthworm extracts are well-known for anti-cancer activity against different types of cancers but how the earthworm extract carrying the capacity of anti-cancer activity and deep molecular level of evidence behind the anticancer activity is still not clear.

Interstitial stem cells are distributed throughout the gastric region in *Hydra*. We drew inspiration from the process of differentiation of stem cells in *Hydra*, which is controlled by the epithelial cells in the gastric region (Bosch *et al.*, 2010). We speculated that these epithelial cells can control cancer cells, suggesting that they may also have an impact on cancer cells, because growing experimental evidence indicates that regeneration and tumorigenesis are related processes, whereby dysregulated regeneration process may lead to tumour development (Schäfer and Werner, 2008). Additionally, it is crucial to note that they do not directly affect somatic cells. This is the rationale for focusing on the gastric region extract of earthworms.

According to the current study's findings, earthworm extracts are more harmful to cancerous cells than they are to healthy ones. Furthermore, the extract from the earthworm's body exhibited a more potent inhibitory impact on MCF7 cells, resulting in a 36% reduction in cell viability and a 58% death rate in somatic cells. Our reports and Shafi and Faleh's 2019 report bear a lot of similarities (Shafi and Faleh, 2019) whereas in our study we have use . Due to the *lumbricus* earthworm powder's IC₅₀ of 965.9µg/ml on MCF7, up to 56% of the cells' viability was controlled (Shafi and Faleh, 2019). Interestingly, Shafi and Faleh have shown that earthworm powder containing whole earthworm extract has anticancer action. However, in our investigation, we only included 7 segments, which includes the gastrointestinal region, so these 7 segments can equalize the activity of whole *lumbricus* earthworm extract. Many reports from earthworm *E. eugeniae* coelomic

fluid and bioactive compounds derived from coelomic fluid with nanomaterials also claimed for anticancer activity against various types of cancer cells (Haque *et al.*, 2023) such as A549 and MCF7 (Haque *et al.*, 2023), Hela, Colon cancer cells, WBC malignant tumor cells and brain tumor cells (Dinesh *et al.*, 2013).

Nevertheless, there is still a gap in our understanding of the molecular mechanism underlying the anticancer activity of earthworm extract and the rationale behind selecting earthworms as a target. Our findings focused on the analysis of apoptosis execution, specifically the expression of cleaved caspase-3. We examined how TCTP and p53, which are cell fate determinants, influenced this process. The TCTP protein is widely recognized for its role in preventing cell death (Rho *et al.*, 2011; Bommer, 2017). During the treatment of body extract on MCF7 cells, there was a significant suppression of TCTP, while at the same time, p53 and cleaved caspase-3 were activated. It has prompted us to consider p53-dependent apoptosis (Schuler and Green, 2001). Rho *et al.*, have discovered a fascinating finding regarding the regulation of the tumor suppressor p53 by TCTP (Rho *et al.*, 2011). In our reports, we found that the body extract molecules have a suppressive effect on TCTP. As a result, the stability of p53 is also reduced, as the expression of p53 was lower in the samples treated with body extract compared to the control samples. Interestingly, the protein expression pattern in C2C12 was not affected by the earthworm body extract. It is worth noting that the TCTP expression was observed in the control sample, which aligns with previous findings by Xiang Cheng *et al.* who documented the TCTP expression pattern in C2C12 at the RNA level. The TCTP expression was 2.5 fold increased in the C2C12 than stem cells (Cheng *et al.*, 2012). Importantly, cleaved caspase-3 was not activated in both control and treated samples. Meanwhile, TCTP and p53 protein expression was reduced in a bit but both are expressed in both samples. For further confirmation, cellular level protein expression studies were conducted to track the apoptotic signals. According to the results of immunofluorescence results reveals the caspase-3 expression in both nucleus and cytoplasm of MCF7 cells. Meanwhile, caspase-3 was not activated in the C2C12 (somatic cells) and the architect of the cells also not damaged while treating with earthworm body extract.

CHAPTER-6

SUMMARY AND CONCLUSION

In conclusion, our study shows that the potential anti-cancer properties of earthworm body segment extracts, focusing on a specific region within the gastric area. Through a series of experiments utilizing MCF7 (breast cancer) and C2C12 (somatic) cell lines, we unveiled intriguing insights into the cytotoxic effects of these extracts, as well as their underlying molecular mechanisms.

Our findings demonstrated a remarkable selectivity of the earthworm body extracts towards MCF7 breast cancer cells compared to C2C12 somatic cells. This selective cytotoxicity suggests a promising avenue for the development of targeted anti-cancer therapies that spare healthy tissues.

Moreover, our molecular analyses shed light on the mechanisms driving the observed anti-cancer effects. Treatment with earthworm body extracts resulted in significant alterations in protein expression profiles within MCF7 cells, notably marked by the activation of cleaved caspase-3, a key executor of apoptosis. This activation was accompanied by a suppression of TCTP expression, a known anti-apoptotic protein, and a decrease in p53 expression, implying potential involvement of p53-dependent apoptotic pathways.

The specificity of these effects to cancer cells, without significant impact on healthy somatic cells, underscores the potential therapeutic value of earthworm extracts in cancer treatment. Furthermore, our immunofluorescence assays provided visual confirmation of cleaved caspase-3 activation in MCF7 cells treated with earthworm extracts, corroborating the induction of apoptosis in cancer cells.

Overall, our study contributes valuable insights into the anti-cancer properties of earthworm body extracts, highlighting their potential as novel sources of anti-cancer agents. Further exploration of the underlying molecular mechanisms and rigorous preclinical studies are warranted to validate their therapeutic efficacy and safety for clinical applications in cancer treatment.

CHAPTER-7

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