

THE IMPACT OF INDOLE ON EARTHWORM REGENERATION

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

By

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CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

INTERNATIONAL RESEARCH CENTER

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **KESAVAMOORTHY V (41101006)** who carried out the project entitled "**The impact of indole on earthworm regeneration**" under my supervision from December 2022 to May 2023.

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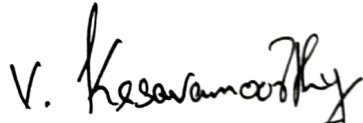
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DECLARATION

I **KESAVAMOORTHY V (41101006)** hereby declare that the project Report entitled **“THE IMPACT OF INDOLE ON EARTHWORM REGENERATION”** was successfully done by me under the guidance of **Dr. S. JOHNSON RETNARAJ SAMUEL**, Associate Professor (Research) and Head, Center for Molecular and Nanomedical Sciences is submitted in partial fulfillment of the requirements for the award of Master of Science Degree in Medical Biotechnology and Clinical Research.

DATE: 05.05.2023

PLACE: Chennai



SIGNATURE OF THE CANDIDATE

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ABSTRACT

Indole is a heterocyclic aromatic organic compound widely distributed in nature. Indole is studied to lay a role in gene expressions, cell growth, biofilm formation and virulence in bacteria and also known to be the precursor of the serotonin in mammals. Regeneration is the biological process where the damaged and lost tissues or organs are restored to their original form and function. This process is widespread throughout the animal kingdom and gradually decreases as the complexity of the organisms increases. In this study, indole's role in the regeneration of *Perionyx excavatus*, their morphological, gene and protein expression pattern of normal and regenerated worms were studied. Additionally, the embryotoxicity, cytotoxicity and wound healing properties of the indole in C2C12 cell lines were studied. Based on the morphological observation on the 11th day, a significant difference was observed in the 25µg indole injected regenerated bud of the earthworm. Indole exhibited a gradual increase in the cytotoxicity level ranging from 5-100 µg/ml and they exhibit wound healing properties. Expressions of actin, Sox-C, Wnt-11, TCTP, USP, H2A, and Hedgehog in normal and indole injected regenerating worm is also studied. Proteins namely sox2 and oct4 expression is reduced in control. However, the expression was increased in regeneration control and indole injected worm. Based on this study, the importance of indole in regeneration has been proven.

Keywords: Regeneration, SOXc, WNT11, TCTP, USP, H2A AND HEDGEHOG

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CHAPTER 1

1. INTRODUCTION

1.1 Indole

Indole is a heterocyclic aromatic organic compound with the chemical formula C_8H_7N . It is widely distributed in nature and is a key building block for the synthesis of many important natural products and pharmaceuticals.

1.1.1 Physical Properties of Indole

Indole is a colourless to pale yellow solid with a melting point of 52-56°C and a boiling point of 253-254°C. It has a characteristic odour that is often described as musty or earthy. Chemically, indole is a weak base, with a pKa of approximately 16.5, and is soluble in a variety of organic solvents.

1.1.2 Biological Properties of Indole

Indole has been found to play important roles in various organisms. In bacteria, indole has been shown to act as a signaling molecule, regulating processes such as biofilm formation and virulence (Lee & Lee et al., 2010). In mammals, indole is a precursor for the synthesis of the neurotransmitter serotonin, which is involved in regulating mood, appetite, and sleep (Walsh & Hourani et al., 1974).

Indole is a metabolic product that is produced by cells through the breakdown of the amino acid tryptophan, or by gut bacteria. Indole can act as a signaling molecule within cells, playing a role in regulating gene expression, cell growth, and other cellular processes. It also plays an important role in the synthesis of proteins and other biomolecules. It contains antibacterial activity, anti-oxidant activity, and anti-inflammation activity and it is used in drug development and cancer studies. It is believed that indole and its derivatives have regeneration activities.

Indole is used in various fields of applications such as

- **Pharmaceutical industry:** Indole serves as a key building block for the synthesis of a wide range of pharmaceuticals, including antihypertensive drugs, antipsychotics, and antimicrobial agents (Batra et al., 2017).

- **Agricultural industry:** Indole derivatives have been used as growth regulators for various crops, and have been shown to increase crop yields and improve resistance to environmental stresses (Gao et al., 2019).
- **Material science:** Indole-based compounds have been investigated for their potential as luminescent materials and as components of organic electronic devices (Huang et al., 2020).
- **Chemical synthesis:** Indole is used as a starting material for the synthesis of various chemicals, such as dyes, flavours, and fragrances (Sarma & Mohan et al., 2017).
- Indole was used in diverse fields of applications.

1.2 Earthworm

Earthworms are burrowing animals. They are classified into 23 families, more than 700 genera, and 7,000 species, among them 424 species have been identified in India and 98 species have been reported in the Western Ghats. (Narayanan et al., 2019). The recently mapped human genome revealed humans only have approximately 35,000 genes. A team of international researchers revealed the new finding that humans share about 70 percent of the genes with the worms that is approximately two-thirds of human genes have 4 counterparts in earthworms and it exists in all countries except Antarctica. Earthworms are farmers' friends and it is a segmented worm species that has a crucial role in maintaining soil health and fertility. Earthworms are invertebrates, it burrows through the soil, improving soil aeration and water retention. It supports breaking down organic matter and compounds to release nutrients in the soil as vermicompost. By breaking down organic compounds earthworms can increase soil carbon storage by 33% (González-Pérez et al., 2014). Earthworms can increase plant growth above 25% by the decomposition of organic compounds and improving nutrient availability in soil (Edwards and Lofty et al., 1972).

The number and the position of the earthworm structures are listed as follows (Reinecke and Pieters et al., 1997):

Segments present above the clitellum were the anterior region

Segment 7 – 9: A pair of spermathecae and a pair of spermathecae aperture

Segment 10: A pair of testes and a pair of sperm funnel

Segment 11: A pair of testes, A pair of seminal vesicles, and a pair of sperm funnels.

Segment 12: A pair of seminal vesicles.

Segment 13: A pair of ovaries, a pair of ovum funnels connected to one pair of short oviducts.

Segment 14: A common oviduct and one female reproductive opening

Segment 18: A pair of prostate glands, a pair of male reproductive openings, and associated penial chaetae.

Segment 10-18: one or two pair(s) vasa deferentia running through.

Segments present below the clitellum were posterior regions.

Earthworms have been used in various medicinal fields for the treatment of various diseases since 1340 AD (Cooper et al., 2009).

Earthworm has been used in traditional medicine for centuries in various countries and it has anti-inflammation properties, anti-oxidant properties, and also anticancer effects, a journal titled Food and Chemical Toxicology founds that earthworm extract has high antioxidant activity and it also has inhibition properties for cancer cell growth (Yang et al., 2013), and earthworm extract release some pain in osteoarthritis and improves knee function (Gao et al., 2012).

1.3 *Perionyx excavatus*

Perionyx excavatus is also known as African Nightcrawler, Composting worms, Indian blues, and blue worms, they are mostly founded in tropical and subtropical regions of Asia, Africa, and America. It is mostly used as a composting worm for commercial uses because it is commonly used in vermiculture, which is a practice of converting organic matter or wastes into nutrient-rich soil, vermicompost produced from blue worms helps to boost plant growth rate Eg- maize (Garcia-Gomez et al., 2017).

It has unique characteristics to tolerate extreme changes in environmental conditions and it can also survive in the acidic soil environment. it can sustain around 40-90 degrees Fahrenheit. *Perionyx excavatus* is very quick in reproduction once the earthworm hatched from the cocoon it can grow mature after two months and starts to reproduce and it produces 16-19 cocoons per week according to their environmental conditions and food source. *Perionyx excavatus* has excellent regeneration capacity.

Taxonomy and Nomenclature

Kingdom - Animalia

Phylum - Annelida

Class - Clitellate

Order – Crassiclitellata

Family – Megascolecidae

Genus – *Perionyx* Perrier, 1872

The taxonomy and nomenclature of *Perionyx excavatus* were studied and published by Perrier. (*Perionyx excavatus* perrier, 1872).

1.4 Regeneration

Regeneration is the biological process where damaged or lost tissues or organs are restored to their original form and function. This process is widespread throughout the animal kingdom and can occur in response to various types of injuries, including cuts, burns, and even the loss of whole limbs. Regeneration occurs through a complex interplay of cellular mechanisms, including cell proliferation, differentiation, migration, and signalling pathways. Regeneration has been studied in many model organisms, such as planarians, salamanders, zebrafish, planarians, newts, axolotls, and earthworms, mostly these species were used in the field of regenerative medicine. The ability to regenerate tissue and organs has important implications for the treatment of various diseases and injuries. For example, researchers are investigating the use of stem cells and other regenerative therapies to treat conditions such as spinal cord injuries, heart disease, and diabetes (Tanaka, et al., 2011).

Regeneration has given rise to new research fields, including regenerative medicine, which aims to create new treatments for injuries and diseases. Progress in stem cell research, gene editing, and the discovery of signalling pathways and genetic factors involved in regeneration have opened up possibilities for new therapies that can replace damaged tissues and organs. These advances offer hope to patients with debilitating conditions.

While researchers have made significant progress in understanding the mechanisms of regeneration, many questions remain unanswered, such as the factors that govern the regeneration process and the contribution of the immune system to this process. To tackle these challenges, scientists are now adopting innovative technologies like single-cell sequencing and genome editing to gain deeper insights into the intricate biology of regeneration (Tanaka, et al., 2016).

CHAPTER 2

2. Review of literature

2.1 Biological activities of indole and its derivatives

Indoles are majorly present in various bioactive compounds of alkaloids as well as agrochemicals and pharmaceuticals. The indole C₈H₇N is a valuable structural moiety having a wide range of pharmacological activities such as antimicrobial, antifungal, antihistaminic, antioxidant, analgesic, anticonvulsant, anti-inflammatory, and plant growth regulator, etc. Among the heterocycles in the discovery of drugs, indoles occupy the place of the most important component. Some examples of Indole derivatives as a biological – are tryptophan used as a nutritive compound, mitomycin and its derivatives used in cancer therapy, serotonin as a neurotransmitter, indole-3-acetic acid as a plant growth hormone, reserpine used as a tranquilizer and blood pressure controlling compound. Indole derivatives and the compounds belonging to the indole moiety are well-established as potential pharmaceutical agents (Singh, et al., 2018).

2.2 Anti-Inflammatory and Analgesic agents

Anti-inflammatory drugs work by inhibiting the cyclooxygenase enzymes and prostaglandins. A series of indole-oxazole hybrids were tested for their anti-inflammatory activity against carrageenan-induced oedema in albino rats at a dose of 50 mg/kg by mouth. the tested compounds, showed strong anti-inflammatory activity around 53.3%, and analgesic around 51.4% (Singh, et al., 2008). (2-chlorophenyl) (1-phenyl-2,3-dihydropyrazolo[3,4-*b*] indol-8(1*H*)-yl methanone was also reported to exhibit both anti-inflammatory and analgesic agent (Mandour, et al., 2010). It is reported that an indole compound containing pyrazole possesses anti-inflammatory activity with an IC₅₀ value of 48MM as compared to indomethacin belonging to the indole moiety (Sharath, et al., 2013)

It is reported the synthesis of spirooxindoles and their anti-microbial activity against *Bacillus subtilis*, *Aspergillus niger*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* by determining the minimum inhibition concentration (MIC) by disc diffusion method (Nandakumar, et al., 2010). Anti-microbial activity and antifungal activity of *Escherichia coli* and *Candida albicans* respectively were evaluated with the synthesized N-1, C-3, and C-5 substitutes of indole. (Singh, et al., 2011) Indole derivatives indol-pyrandicarbonitrile was revealed to possess strong antibacterial

activity against *Klebsiella pneumonia* (16.1MIC), *Escherichia coli* (16.5MIC), *Bacillus cereus* (16.4MIC), *S. Aureus* (12.4 MIC). (Lakshmi, et al., 2010).

2.3 Regeneration

The role of indole in regeneration has been studied and demonstrated and reported to be a promoter and inhibitor in some cases. The role of microbiome-derived metabolite indole -3-propionic acid was analyzed for its regenerative ability. It showed to facilitate the regeneration and functional recovery of sensory axons through an immune-mediated mechanism (Sergey Elizabeth, et al., 2022). The study showed that intermittent fasting (IF) promotes axonal regeneration after sciatic nerve crush in mice through an unexpected mechanism that relies on the gram-positive gut microbiome and an increase in the gut bacteria-derived metabolite indole-3-propionic acid (IPA) in the serum. The study stated that the production of indole-3-propionic acid produced by *Clostridium sporogenes* is required for efficient axonal regeneration and delivery after an injury enhances and accelerates the recovery of sensory function (Borowski, and Vuong, et al., 2022). It is reported that the bacterial metabolite indole inhibits the regeneration of the planarian flatworm *Dugesia japonica*. The study found that the microbial metabolite indole is produced in significant quantities by the bacteria present in the microbiome of *D.japonica* which delays regeneration. (de Melo, Mariza Pires et al., 2004).

Folkes. et.al evaluated the cytotoxicity of indole-3-acetic acid in fibroblasts and neutrophils and several cancer lineages such as breast and leukemia cell lines. The cytotoxic effect of Indole-3-acetic acid was studied in rat neutrophils and lymphocytes by examining the loss of membrane integrity (necrosis), DNA fragmentation, chromatin condensation, and mitochondrial transmembrane potential (apoptosis). The cytotoxic effects of indole alkaloids were determined against AGS, L1210, BC, and HepG2 cancer cell lines using the MTT method. The compound exhibited moderate cytotoxicity against cancer cell lines at concentrations between 1.1 and 20 µg/ml (Oh, et al., 2006). Cytotoxic effects of newly isolated bisindole alkaloids were evaluated against cancer cell lines -breast cancer MCF-7, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, colon cancer SW480, and lung cancer A-549 cells. The structures were elucidated using spectroscopic methods. The alkaloid compounds exhibited inhibitory effects with IC50 values more than the known standard indole alkaloids cisplatin and vinorelbine. (Feng Teo, et al., 2010)

CHAPTER - 3

Aims and Objectives

3. Aim

To investigate the effect of indole on regeneration in earthworms and determine its potential as a regenerative agent.

3.1 Objectives:

- Indole's role in the regenerative study of earthworms.
- To study the invitro wound healing activity of indole on myoblast cells.
- Analysis of regeneration-specific genes upon indole treatment.
- Studying the protein expression pattern on indole-treated regenerated samples.

CHAPTER – 4

4. MATERIALS AND METHODS

4.1 Earthworm culture and maintenance:

Earthworm species *Perionyx excavatus* were brought from SK Farms and Organics, Kongananchery village, Chengalpattu. Then the earthworms were cultured in the Earthworm rearing unit which is maintained by Regeneration and Stem cell biology, International Research Centre of Sathyabama Institute of Science and Technology. The worms were maintained in the plastic tub. which contains cow dung, organic wastes, soil, and leaf litter. *Perionyx excavatus* is simple to feed, earthworms are fed with mixed cow dung, leaf litter, jaggery water, and soil. The incubation room was kept at 22-26°C. The incubation room was kept at 22-26°C.

4.2 Collection of earthworms

Sexually matured *Perionyx excavatus* were collected from Regeneration and Stem cell biology Lab, International Research Centre of Sathyabama Institute of Science and Technology. Totally 10 worms were collected for each concentration and maintained separately in a plastic box that contains cow dung, and organic soil. For the adaptation of environmental changes earthworms were maintained separately for 3 days before starting the experiment.

4.3 Solubility of Indole

Indole is a colourless to pale yellow solid with a melting point of 52-56°C and a boiling point of 253-254°C. 100mg of indole was measured and dissolved in 100µl of absolute ethanol (stock), and it is diluted in different concentrations and injected in earthworm.

4.3 Indole dosage standardization:

To study the regeneration growth kinetics different dosages of indole is given to the worms. Different dosage of indole was given to earthworm by injection method. Indole has injected 5-6 segments below the clitellum, different concentrations were used 5µg, 25µg, 50µg for injecting earthworms. Insulin injections were used for injecting earthworms.

4.4 Phenotypic observation of earthworm on Regenerative studies

Following a three-day incubation period for acclimatization to the environment. For each set of concentrations, five sexually matured worms were selected for the experiment, and for control, non-injected earthworms were used. During the first three days of the experiment, each worm was injected with 5µl of indole at varying concentrations. On the second day, precisely two hours before injection, each worm was amputated at the 10-11 segment. And continuously observed its morphological changes, mortality rate, and behaviour pattern continuously for 11 days. Images were photographed each day.

4.5 Embryotoxicity assay

4.5.1 Preparation of E3 media (60X)

60x stock for 2litre

- 34.8gm sodium chloride
- 1.6gm potassium chloride
- 5.8gm calcium chloride
- 9.7gm magnesium chloride

pH - 7.2 and autoclaved and stored at 4°C.

4.5.2 Preparation of 1X E3 media

- From the 60X stock media, 16.5ml were taken and made it up into 1000ml.
- 3 to 4µl of 1% methylene blue were added to prevent fungal contamination and stored at 4°C.

Note – Before adding 16.5ml of 60X stock media, the distilled water was autoclaved and cooled down.

4.5.3 Procedure

Embryotoxicity studies were carried out as soon after the fertilization of embryos occurs. The test was carried out based on the OECD guidelines.

For analysis of embryotoxicity, indole was treated with embryos in various concentrations - 5µg, 25µg, 50µg, and 100µg.

4.6 Cytotoxicity assay (MTT assay protocol)

4.6.1 Reagent preparation

4.6.2 Prepare MTT solution

MTT is soluble in water (10 mg/mL), ethanol (20 mg/mL), buffered salt solutions, and culture media (5 mg/mL).

MTT was taken at 5 mg/mL solution in PBS and mixed by vortexing.

4.6.3 Prepare MTT solvent

DMSO Solvent

4.6.4 Procedure

1. Cells were grown in DMEM media supplements with 10% FBS, collected using trypsin
2. Cells were counted using a haemocytometer.
3. Cells were seeded in the 96 wells plate at the concentration of 5000 to 10000 cells per well.
4. The cells were incubated at 37°C in CO₂ Incubator.
5. After the cells were attached, the media was removed from the plate and washed with 1X PBS twice.
6. The sample was then added in triplicate along with 2% FBS in various concentrations.
7. Cells were incubated at 37°C in the CO₂ Incubator for 24 – 48 hours.
8. Discard media from cell cultures. For adherent cells, carefully aspirate the media. Images should be photographed at each concentration.
9. Add 50 µL of serum-free media and 50 µL of MTT solution into each well.
10. Incubated the plate at 37°C for 3 hours.
11. After incubation, added 100 µL of DMSO to each well.

12. Wrapped the plate in foil and kept the plate on the racker for 15 minutes. Occasionally, pipetting of the liquid may be required to fully dissolve the MTT formazan.

13. The absorbance at OD 590 nm was read. The plate was read within 1 hour

4.6.5 Calculation

1. Average the duplicate reading for each sample.

2. Subtract the culture medium background from your assay readings. This is the corrected

absorbance.

3. Calculate percentage cytotoxicity with the following equation, using corrected absorbance:

Percentage of cytotoxicity = $(100 \times (\text{control} - \text{sample}))$.

4.6.6 DMEM Media Preparation

DMEM (Dulbecco's Modified Eagle Medium) was used in most of the cells. Sodium bicarbonate at a concentration of 3.75 g/L was added to maintain the pH of the media. Three antibiotics, namely 1% Streptomycin, 1% Penicillin G sodium salt, and 1% Amphotericin B, were used.

- One liter of Millipore water was taken and sterilized.
- 17.4g of DMEM medium was added and mixed well until completely dissolved.
- Then, 1g of Streptomycin, 1g of Penicillin, and 1 ml of Amphotericin B were added and mixed thoroughly.
- 3.75g of sodium bicarbonate was added, resulting in a reddish-pink color change in the media.
- The pH was adjusted from 7.2 to 7.4.
- The media was then filtered through a 0.22µm Millipore filter using a Filtration unit.
- The media were stored at 4°C for future use.

4.7 Passaging or Subculture/ Seeding:

A T-25 flask with 70 to 80% confluence was taken.

- The used media was removed, and the flask was washed twice with 1X PBS.
- 2 ml of trypsin was added for the enzymatic technique for cell dissociation.
- The flask was incubated at 37°C for 1 to 2 minutes in a CO2 incubator.
- The diamond-shaped cell structure changed to an oval shape, after which the trypsin was quickly removed and 2 ml of 10% serum media was added.
- The media was pipetted out to detach the cells from the bottom of the flask.
- The cells with media were transferred into a 15 ml tube and centrifuged at 800 rpm for 2 minutes.
- The supernatant was removed, and 1 ml of fresh media was added to the cell suspension, gently vortexing until the cells were well mixed.
- The cells were counted using Haemocytometer.
- The cells were seeded into a new T-25 flask or well plates containing 10% serum media

4.7.1 Calculation

X Conversion = Required volume * Required Concentration/ Available Concentration.

4.8 Wound healing assay

C2C12 cells (1×10^5 cells/well) were seeded with a complete medium at 37°C and maintained at 5% CO2 for 24 hours. After 24 hours, the monolayer confluent cells were scraped horizontally with sterile 200uL pipette tips. The debris was removed by washing with 1x PBS.

The cells were treated with indole of various concentrations (5µg, 25µg, 50µg, and 100µg) by diluting with a complete medium. The cells along with distilled water were used as vehicle control for this experiment. Images were photographed at 0h, 12h, 24h, and 48h post-wounding under Phase contrast microscopy.

4.9 Total RNA Isolation Using BIO-RAD Kit

The regenerated earthworm blastema was amputated and washed with cold 1XPBS.

4.9.1 Animal Tissue

1. The amputated earthworm tissue was cut into small pieces (<5 mm long) and ground into a fine powder with a mortar and pestle containing liquid nitrogen. Liquid nitrogen was periodically added to the mortar to prevent the thawing of the tissue.
2. Up to 20 mg of hard animal tissue, and up to 40 mg of soft animal tissue, were transferred into an RNase-free 2.0 ml capped microcentrifuge tube. The tissue was allowed to thaw before adding the lysis solution to avoid precipitation.
3. 700 µl of lysis solution was added to the tube, and the sample was disrupted by pipetting up and down. A rotor-stator homogenizer or other equivalent methods were used to disrupt the sample for 30-60 seconds.
4. The lysate was centrifuged for 3 minutes, and the supernatant was transferred into a new 2.0 ml capped microcentrifuge tube.
5. 700 µl of 70% ethanol for plant tissue or 60% ethanol for animal tissue was added to the supernatant. Thorough mixing was performed by pipetting up and down or using a rotor-stator homogenizer. Care was taken to ensure that no bilayer was visible.

4.9.2 All Starting Sample Types

1. An RNA binding column was inserted into a 2 ml capless wash tube.
2. The homogenized lysate was decanted or pipetted into the RNA binding column. It was then centrifuged for 30 seconds (for cultured, bacterial, or yeast cells) or 60 seconds (for animal or plant tissue). The RNA binding column was removed from the wash tube, the filtrate from the wash tube was discarded, and the column was replaced with the same wash tube.
3. The low stringency wash solution, provided as a 5x concentrate, was prepared by adding 4 volumes (80 ml) of 95-100% ethanol to the concentrate.
4. 700 µl of low stringency wash solution was added to the RNA binding column. It was centrifuged for 30 seconds. The low-stringency wash solution was discarded from the wash tube, and the column was replaced with the same wash tube.
5. The RNase-free DNase I, provided as a lyophilized powder, was reconstituted by adding 250 µl of 10 mM Tris, pH 7.5 (not provided), to the vial and briefly pipetting up and down to mix.

6. For each processed column, 5 µl of reconstituted DNase I was mixed with 75 µl of DNase dilution solution in a 1.5 ml microcentrifuge tube (not provided). If processing more than one column, the proportions were scaled up accordingly. 80 µl of diluted DNase I was added to the membrane stack at the bottom of each column. The digest was allowed to incubate at room temperature for 15 minutes (25 minutes for animal tissue).
7. 700 µl of high stringency wash solution was added to the RNA binding column. It was centrifuged for 30 seconds. The high-stringency wash solution was discarded from the wash tube, and the column was replaced in the same wash tube.
8. 700 µl of low stringency wash solution was added to the RNA binding column. It was centrifuged for 1 minute (for cultured, bacterial, or yeast cells) or 30 seconds (for animal or plant tissue). The low-stringency wash solution was discarded from the wash tube, and the column was replaced in the same wash tube.
9. An additional centrifugation step was performed for 2 minutes to remove the residual wash solution.
10. The RNA binding column was transferred to a 1.5 ml capped microcentrifuge tube. 80 µl (or 40 µl) of the elution solution was pipetted onto the membrane stack at the bottom of the RNA binding column, and 1 minute was allowed for the solution to saturate the membranes. The column was centrifuged for 2 minutes to elute the total RNA.
11. Note: The eluted total RNA samples could be used immediately in downstream applications. Alternatively, the total RNA could be stored at -20°C or -80°C for later use.

4.10 RNA Quantification using Qubit Fluorometer

4.10.1 Preparing samples and standards

This protocol assumed that you were preparing standards for calibrating the Qubit Fluorometer. If you planned to use the last calibration performed on the instrument, fewer tubes (step 1) and less working solutions (step 4) were needed.

1. The required number of Qubit tubes for standards and samples was set up. Thin-wall, clear, 0.5-mL PCR tubes were used for the Qubit 4 Fluorometer.

Note: Only thin-wall, clear, 0.5-mL PCR tubes should be used for the Qubit 4 Fluorometer.

2. The Qubit working solution was prepared by diluting the Qubit RNA BR Reagent 1:200 in Qubit RNA BR Buffer. A clean plastic tube was used each time the Qubit working solution was prepared.
3. Qubit working solution was added to individual assay tubes so that the final volume in each tube after adding the sample was 200 μL .

	Standard assay tubes	User sample assay tubes
Volume of working solution	190 μL	180–199 μL
Volume of standard	10 μL	—
Volume of user sample	—	1–20 μL
Total volume in each assay tube	200 μL	200 μL

4. 10 μL of each Qubit™ standard was added to the appropriate tube.
5. 1–20 μL of each user sample was added to the appropriate tube. The tubes were vigorously vortexed for 3–5 seconds.

All tubes were allowed to incubate at room temperature for 2 minutes, and then the standards and samples were read to quantify the RNA using the Qubit Fluorometer.

4.11 cDNA Synthesis using BIO-RAD Kit

Component

5x iScript Reaction Mix	- 4 μl
iScript Reverse Transcriptase	- 1 μl
Nuclease-free water	- Variable
RNA template (100fg – 1 μg total RNA)	- Variable
Total Volume	- 20μl

4.11.1 Reaction Protocol

Incubate the complete reaction mix in a thermal cycler using the following protocol:

- | | |
|--------------------------|-------------------------------|
| 1. Priming | - 5 min at 25 ⁰ C |
| 2. Reverse transcription | - 20 min at 46 ⁰ C |
| 3. RT inactivation | - 1 min at 95 ⁰ C |
| 4. Optional step | - Hold at 4 ⁰ C |

Note- When using larger amounts of input RNA (>1µg), the reaction should be scaled up (for example, 40µl reaction for 2µg, or 100µl reaction for 5µg) to ensure optimum synthesis efficiency.

4.12 Quantification of cDNA using Qubit Fluorometer

4.12.1 Prepare standards and samples

1. The required number of assay tubes (or tube strips) for standards and samples was set up.
2. 10 µL of each Qubit standard was added to the appropriate tube.
3. 1–20 µL of each user sample was added to the appropriate tube.
4. The Qubit 1X dsDNA working solution was added to each tube, ensuring a final volume of 200 µL.
5. Each sample was vigorously mixed by vortexing for 3–5 seconds.
6. All tubes were allowed to incubate at room temperature for 2 minutes. The cDNA was quantified using the Qubit Fluorometer.

4.13 Gene Expression using qPCR

Conventional PCR involves running DNA on an agarose gel once the reaction is complete in order to detect the amplified product or amplicon. In contrast, real-time PCR (RT-PCR) allows the accumulation of the amplified product to be detected and measured as the reaction progresses that is, in real-time.

4.13.1 Principle

Real-time detection of PCR products is made possible by incorporating a fluorescent molecule in the reaction that reflects an increase in DNA amount with a corresponding increase in fluorescent signal. The fluorescent chemistries used for this purpose include DNA-binding dyes and fluorescently labelled sequence-specific primers or probes. Fluorescence is monitored when amplicon occurs using specialized thermal

cyclers that have fluorescence-detecting modules. The amount of amplified product in each cycle is represented by the measured fluorescence.

Real-time PCR has several advantages over traditional PCR, but its ability to estimate the beginning template copy number with precision and excellent sensitivity over a broad dynamic range is its key benefit. Results from real-time PCR might be quantitative or qualitative. Quantitative RT-PCR is also referred to as qPCR.

Contrarily, traditional PCR is only semi-quantitative. Additionally, gel electrophoresis is not necessary for the evaluation of real-time PCR results, reducing experiment time and increasing throughput. Also, chances for contamination are very less because reactions are carried out and data are analysed in a closed tube system.

4.13.2 SYBR Green Dye

SYBR Green is the most commonly used double-stranded binding dye for RT-PCR. The dye exhibits minimal fluorescence when free in solution, but when bound to dsDNA, its fluorescence increases up to 1000-fold (Fig 1). In RT-PCR, its fluorescent signal increases in proportion to the amount of dsDNA generated during amplification. The excitation and emission maxima of SYBR Green dye are 494nm and 521 nm respectively. This SYBR green Supermix is a ready-to-use 2x reaction cocktail that contains an optimized concentration of SYBR Green I dye, Taq DNA polymerase, dNTPs, and buffers for detecting a wide range of target DNA.

DNA-binding SYBR Green dye in RT-PCR. Fluorescence dramatically increases when SYBR Green binds to dsDNA.

4.13.2 Materials required for setting qPCR

- SYBR Green SuperMix
- Template DNA (cDNA)
- Forward and Reverse Primers
- Pipettes, Pipettes tips
- Sterile Microcentrifuge tubes
- Vortex, Microcentrifuge
- qPCR microplates or tubes
- Nuclease free water

4.13.3 Protocol for gene expression analysis using qPCR

The important application of RT-PCR was the study of gene expression. Studying gene expression was of fundamental importance in many areas of modern biomedical research, ranging from basic science to practical applications in industry and medicine. Most of the gene expression studies were determined by comparing the expression level of test samples vs control samples. For studying the gene expression studies, the first step was to isolate RNA from the particular samples, which was followed by the reverse transcription of isolated RNA to cDNA. The expression of the target genes and the reference genes (Actin) was determined in each cDNA sample using a qPCR assay. Further data was analyzed, and the relative expression of the target genes in the samples was calculated.

4.13.4 Important points while designing primers

1. The primers were designed in such a way that they had a GC content of 50-60%.
2. The melting temperature (T_m) was best kept between 50 and 65°C.
3. Repeats of G's or Cs longer than 3 bases were avoided, and G's and C's were placed on the ends of the primers.
4. The sequence of the forward and reverse primers was checked to ensure no 3' complementarity to avoid primer-dimer formation.

4.13.5 Primer Dilution

1. The primer tubes were centrifuged at 8000 rpm for 30 sec.
2. The primers were dissolved with the appropriate volume of nuclease-free water as mentioned in the primer datasheet.
3. The tubes were vortexed and allowed to settle for 2 minutes at room temperature.
4. The tubes were kept in a boiling water bath for 10 minutes at 60°C.
5. Afterward, the tubes were centrifuged at 8000 rpm for 30 sec.
6. To aliquot the primers, 10 μ L of the primer was taken and mixed with 90 μ L of nuclease-free water in a sterile centrifuge tube. The tubes were properly labeled and stored at -20°C.

4.13.6 Preparation of reaction mixture

1. The frozen 2X SYBR Green Supermix, template DNA, and primers were thawed on ice. The tubes were briefly vortexed and spun to collect the contents at the bottom.
2. The components were added to each reaction tube as given in Table 1. Each reaction mix was briefly vortexed and the tubes were spun at low speed in a microcentrifuge (2000 rpm for 2 min).

Table 1: Reaction Mix - for 10 μ l

Step	Volume	Final Concentration
Nuclease free water	1 μ l	-
Forward Primer	1 μ l	300 nM (100-500nM)
Reverse Primer	1 μ l	300 nM (100-500nM)
2x SYBR Green s mix	5 μ l	1x
Template DNA	2 μ l	cDNA equivalent to 1pg to 100 ng of total RNA
Total reaction volum	10 μ l	

Important Notes:

1. 10% more reaction mix was prepared than required to compensate for potential pipetting loss. Triplicate samples were prepared as a master mix with a single addition of the sample template.
2. A no template control (NTC) was included to detect possible contamination or primer-dimer formation.
3. The reaction triplicates were loaded into PCR tubes or microplates and properly sealed. To remove any bubbles, the microplates or tubes were briefly spun in a microcentrifuge at low speed. If necessary, the sealed reactions were stored on ice or at 4°C until the start of the RT-PCR.
4. The thermal cycler was programmed with the recommended RT-PCR protocol, with or without the melt curve step (Table 2).
5. The sealed reaction vessels were placed in the thermal cycler block, and the RT-PCR protocol was started.

Table 2. RT-PCR protocol

Cycling Step	Temperature	Hold Time (min: sec)	No. of cycles
Initial denaturation and enzyme activation.	95 ° C	02:00	1
Denaturation	95° C	00:15	40
Annealing/ Extension	55-60° C	00:20	
Melt curve (Optional)	65-95° C (in 0.5 increments)	00:05	1

4.13.7 qPCR Analysis Methods

The choice of reaction conditions for qPCR was important to ensure reliable and effective amplification for both relative and absolute measurements. The CT or Cq values obtained during qPCR were used for absolute and relative quantification.

In absolute quantification, the CT or Cq values of test samples were compared to those of standards of known quantity using a standard curve. This analysis provided the copy number or concentration of the sample. This method was widely used for the detection of pathogens or copy number analysis.

In relative quantification, the CT or Cq values of the test samples were compared to those of the control samples. This analysis yielded the ratio of the relative amount, i.e., the fold difference of the target gene in the test vs control samples. It was commonly used for gene expression studies. This analysis could be done using software, and manual calculations could also be performed in an Excel sheet using formulas.

4.14 Protein sample isolation:

Beta-Mercaptoethanol (β -ME-reducing agent) is used for breaking disulfide bonds of protein. 2X sample buffers were brought to room temperature before use. After that, 2X sample buffer was 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 which was done manually. 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 the supernatant was transferred to the fresh 1.5ml tube and stored at -20°C.

4.15 SDS-PAGE

SDS PAGE is an analytical technique that is used to separate the proteins based on their molecular weight. 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 the 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 proteins. During PAGE electrophoresis linear or primary structures of the proteins were stacked or arranged properly, once reached the separating gel, proteins started to separate based on their size.

4.15.1 Materials:

Eppendorf's; Micro-pipettes; Micro-tips; Centrifuge; Water bath; Vortex and Distilled water.

4.15.2 Chemical preparation of SDS-PAGE:

30% Acrylamide and 0.3% Bis acrylamide:

Acrylamide - 30g

N, N' Methylene Bisacrylamide - 0.3g

Makeup into 100ml with distilled water.

4X Separating Buffer:

Tris HCL - 18.2g

Distilled water - 60ml

pH was adjusted to 8.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4g

Makeup into 100ml with distilled water.

4X Stacking Buffer:

Tris Hcl - 6.05g

Distilled water - 40ml

pH was adjusted to 6.8 with 10N NaOH

Sodium Dodecyl Sulphate (SDS) - 0.4g

Makeup into 100ml with distilled water.

2X Sample Buffer:

Tris stacking buffer (pH 6.8) - 25ml

Sodium Dodecyl Sulphate (SDS) - 4g

20% Glycerol - 20ml

Makeup 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.

10% Ammonium Per Sulphate (APS):

APS - 100mg

Makeup into 1ml with distilled water.

1X Running Buffer:

Tris Hcl - 3.94g

Glycine - 15.014g

SDS -1g

Makeup in 1L with distilled water.

Staining Solution:

Commassie Brilliant Blue R-250 - 200mg

Methanol - 50ml

Glacial Acetic acid - 7ml

Makeup into 100ml with distilled water.

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

Destaining Solution:

Methanol - 30ml

Glacial Acetic acid - 7ml

Makeup into 100 ml with distilled water.

Table.1: Separating gel concentration

Stock Solution	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
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
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
Distilled water (ml)	17.5	16.5	15.5	15.0	14.5	13.5	12.5	10.5	9.5	7.5
10% APS (μl)	200	200	200	200	200	200	200	200	200	200
TEMED (μl)	20	20	20	20	20	20	20	20	20	20

Table.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 (μ l)	100	100	100	100
5.	TEMED (μ l)	10	10	10	

4.15.3 Preparation of gel:

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

4.15.4 Sample loading and electrophoresis:

A protein sample of 80 μ g and the appropriate molecular markers were loaded into the wells. The apparatus was connected to the power pack with proper polarity and 60v 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 pulled away from the gel.

4.15.5 Staining and Destaining the Gel:

The gel was carefully taken out from the glass plate and the stacking gel was removed by using a cleaver. The separating gel was removed by gently grasping the two corners of the gel and placed in the container containing the Coomassie brilliant blue R250 (stainer solution). The gel should be fully submerged in the staining solution for 60 minutes by gentle agitation on a Rocker. Then it was washed with distilled water and transferred to a destaining solution until protein bands are visualized clearly. Commonly, the first destainer wash is about 30 minutes, then the second wash is about overnight.

4.16 Immunoblotting

4.16.1 Buffer preparation

10X Transfer Buffer (Stock)

Glycine - 72.1g

Tris-base - 15.2g

Distiller water- 500ml

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

For a Total Volume of 1 litre

100ml - 10X Transfer Buffer

200ml – Methanol

700ml of Distilled water

4.16.2 Running the gel:

Prepare 12% SDS gel and place it in SDS – PAGE setup. Pour 1X running buffer (100ml of 10X running buffer + 900ml of distilled water) into the setup. A protein ladder of 5 μ l is loaded at the first lane of the gel for molecular weight determination. In other

wells, the prepared protein samples were added (60µg/well) concentration. After loading all the samples, the setup started to run at a 60V unit. The samples were left to run till it reaches the bottom line.

4.16.3 Membrane transfer:

After the protein runs fully till the end, take off the glass slides from the setup and cut off the stacking gel. Then gently take the gel and keep it in the transfer buffer for 5 minutes and simultaneously, cut the PVDF membrane by the same gel size. Soak the PVDF membrane in the methanol for 1 – 2 minutes for activation of the membrane. Then, keep the membrane in 1X transfer buffer until preparing the sandwich for blotting.

Prepare blotting apparatus, sponge, and filter paper to arrange the sandwich setup and soak it in 1X transfer buffer before using.

4.16.4 Blocking

A blocking buffer was used to block all proteins which would bind with targetable and non-targetable proteins. 5% of Bovine serum albumin (BSA) with 20ml of 1X TBST buffer was prepared for Blocking and let in for 2 hours.

4.16.5 Primary Antibody

For studying protein expression of treated indole earthworm's various Primary antibodies were used for the experiment and these primary antibodies were added to the 5% of bovine serum albumin (BSA) at (:5000 dilutions) and incubated overnight in cold conditions.

- Actin (produced from mouse polyclonal Abcam, ab8226) - (1:5000 5%BSA).
- Sox2 (produced from Rabbit polyclonal Abcam, ab97959) - (2:5000 5%BSA).
- Oct4 (produced from Rabbit polyclonal Abcam, ab181557) - (1:5000 5%BSA).
- Wnt3a (produced from Rabbit polyclonal Abcam, ab19925) - (1:5000 5%BSA).
- Caspase3 (produced from mouse polyclonal Abcam, 208161) - (2.5:5000 5%BSA).

After undergoing an incubation period overnight keep the membrane at room temperature for 30mins and start the washing process.

4.16.6 Secondary Antibody

Secondary antibody (Anti Rabbit IgG (Produced in goat) Abcam, AF568 – ab175471 was added in the 5% Bovine serum albumin (BSA) (1:1000 dilutions) and incubated for 1 hour at room temperature. (NOTE: starting from adding a secondary antibody, every step should be done in the dark condition as these antibodies are light sensitive and if subjected to light causes denaturation). After undergoing a dark incubation period overnight, start the washing process.

4.16.7 Gel imaging

Gel imaging has been carried out using the sophisticated BioRad ChemiDoc™ XRS⁺ imaging system for faster and more accurate western blot quantification. only stain-free enabled instrument capable of chemiluminescent, multiplex fluorescent, and routine gel imaging and it is used to quantify total protein for normalization purposes after transfer to the membrane.

After the incubation with a secondary antibody for 2 hours has been completed, the PVDF membrane is washed with 1X PBST every 5 minutes for 5 times. Then, the ECL substrate solution was prepared by adding equal amounts of ECL solution A and ECL solution B mixed in (a 1:1 Ratio), after the secondary antibody process the blot was washed more than 5 times in the same box and transferred into the new one with few washes to avoid patchy spots on the blot. Then, turn on the BioRad ChemiDoc™, and connect to the PC. The membrane is kept inside in the correct position after wiping the place provided for placing the membrane with tissue paper. Add the prepared ECL substrate over the membrane in drops/spraying covering all the areas of the membrane. Open BioRad image Lab 6.1 software and select New protocol → Application → select → blots → chemi, In the imaging area à select gel type → BioRad criterion gel. In the Image exposure area, manually set the exposure time to 150 seconds – 300 seconds and select Run protocol. The results will be displayed on the monitor with clear bands based on the protein luminance at different exposure times. The results were documented by File → export for publication → set the resolution → save. The entire procedure was carried out in a dark environment.

CHAPTER - 5

5. RESULTS AND DISCUSSION

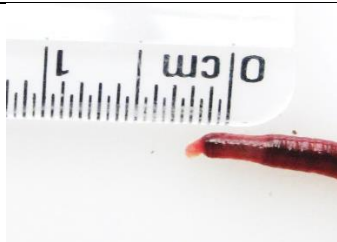
5.1 Earthworms treated with different concentrations of indole

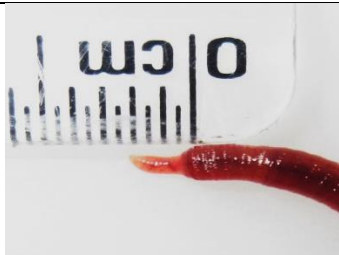
The matured worms were taken for the experiment (Fig: 5.11). The drug indole was injected into the earthworm, *Perionyx excavatus* at 5 μ g, 25 μ g, and 50 μ g concentration, and distilled water as a control for continuously three days. On the second day, the earthworms were amputated at the 10th segment in the anterior region. The experimental worms were maintained for up to 11 days for observation. The amputated worm started regenerating buds and it was measured with a scale every day and documented.



Fig: 5.1 Sexually matured *perionyx excavatus* (Day 01) Fig 5.2: Amputated worm (Day 02)

Table 5.1: Phenotypic observation of indole on Day 11

Indole at different concentration	Indole-treated earthworm regeneration at 11 th Day
Control Regeneration	
5 μ g	

25µg	
50µg	

Control and indole-treated injected worms were measured on different days (Table 5.1). Indole-injected worms were compared with control worms. On the 11th day, a significant difference was observed in the regenerated bud of the earthworm. When comparing 5µg, 25µg, and 50µg concentration 25µg promote the regeneration process based on the bud observation. For further molecular evidence, wound healing, qPCR and immunoblotting studies were carried out with the regenerated bud protein samples.

5.2 Wound healing assay

C2C12 cells (1×10^5 cells/well) were seeded with a complete medium at 37°C and maintained at 5% CO₂ for 24 hours. After 24 hours, the monolayer confluent cells were scraped horizontally with sterile 200µL pipette tips. The debris was removed by washing with 1x PBS.

The cells were treated with indole of various concentrations (5µg, 25µg, 50µg, and 100µg) by diluting with a complete medium. The cells along with distilled water were used as vehicle control for this experiment. Images were photographed at 0h, 12h, 24h, and 48h post-wounding under Phase contrast microscopy.

Wound-healing migration assay was applied to assess the migration. C2C12 mouse myoblast cell line was used in this study. Indole at the concentration of 5µg, 25 µg, 50 µg, and 100µg were compared with the control group. The migration of C2C12 cells was observed at different times (Fig 5.3). The results indicate that indole enhances the migration of C2C12 cells at 36thh whereas the 100µg cells were not healed, which

proves that it promotes wound healing in mouse myoblast cells and concentration which exceeds 100 μ g are not fully healed at 48th hour.

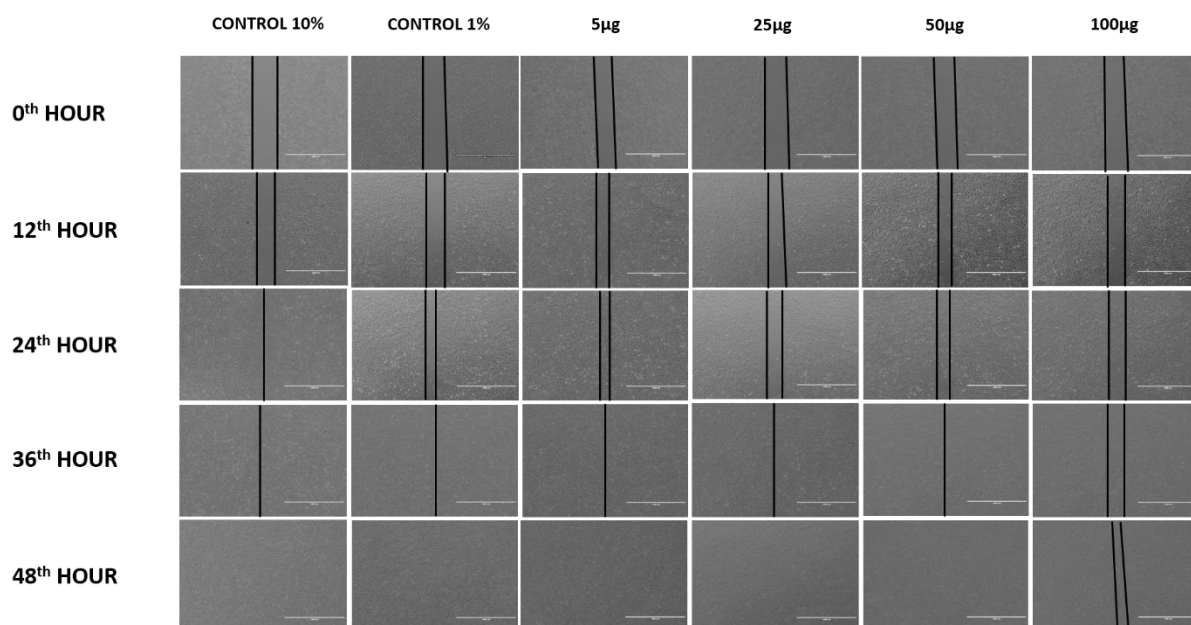


Fig 5.3: Wound healing assay treated with Indole

5.3 Cytotoxicity test (MTT Assay)

Cytotoxicity of indole was evaluated against myoblast cell lines (C2C12). The following concentrations were used to examine the toxicity – 5, 25, 50, and 100 μ g/ml and for control, 2% DMEM media were used. The compound exhibited a gradual increase in the cytotoxicity level ranging from 5-100 μ g/ml (Fig 5.4).



Fig 5.4: Cytotoxicity assay treated with Indole

5.4 Embryo Toxicity Assay

Embryotoxicity studies were carried out in zebrafish embryos as soon after the fertilization of embryos occurs. For analysis of embryotoxicity, indole was treated with embryos in various concentrations - 5 μ g, 25 μ g, 50 μ g, and 100 μ g. The compound exhibited the toxicity of indole in embryo increases gradually (Fig 5.5)

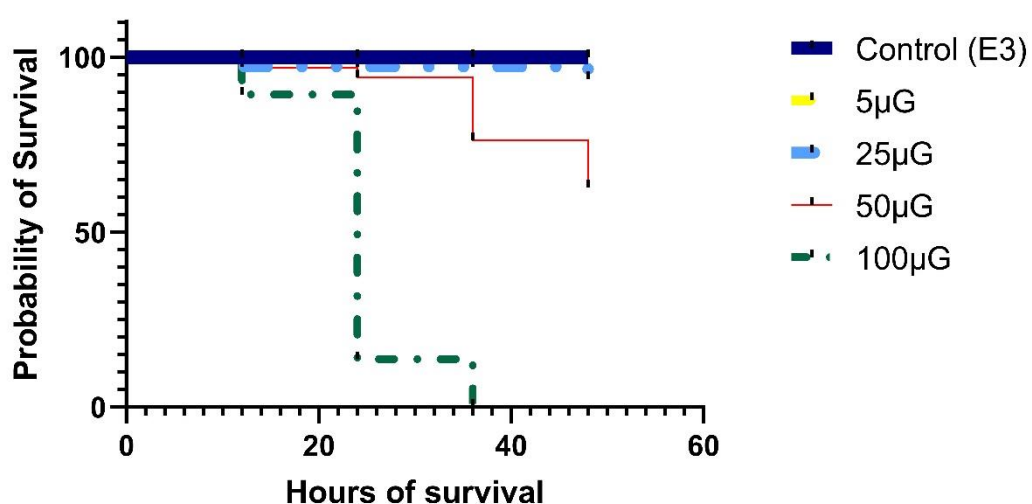


Fig 5.5: Embryotoxicity Assay treated with Indole

Group	Dose	Dose difference (a)	Dead	Mean (b)	Product a*b
1	100 µg		30		
2	50 µg	50	16	26	1300
3	25 µg	25	4	10	250
4	5 µg	20	0	2	40
				Total	1590

Table 5.2: LD50 value of indole-treated Embryotoxicity Assay

LD50 =Maximum Dose – (Total/maximum dead)

$$= 100 - (1590/30)$$

$$= 100 - (53)$$

$$\text{LD50} = 47$$

The embryotoxicity results show that the higher the concentration the higher the toxicity.

5.5 Gene expression studies

Gene expression studies were performed from the following samples viz control, 3rd day regenerated control, 5µg, 25µg, and 50µg, indole treated groups. The gene expression studies were resolved with the following gene, ACTIN, SOX-C, WNT-11, TCTP, USP, H2A, and HEDGEHOG. Results show that (Fig 5.6) compared to the control the gene expressions in the 3rd-day regenerated control were increased in ACTIN, SOX-C, WNT-11, TCTP, USP, H2A, and HEDGEHOG. Wnt11 gene is expressed during early embryonic development and has a critical role in the regulation of gastrulation movements and wnt11 expression is highly increased at 5µg compared to 3rd-day regenerated blastema control and indole-treated other samples 25 and 50µg, by analysing the gene expression wnt11 plays an important role in an early stage of embryo development.

Hedgehog functions as a chemical signal that is essential for embryonic development, Sonic Hedgehog plays a role in cell growth, cell specialization, and the normal shaping

(patterning) of the body. This protein is important for the development of the brain and spinal cord (central nervous system), eyes, limbs, and many other parts of the body. Hedgehog expression is highly increased at 5 μ g compared to 3rd-day regenerated blastema control and indole-treated other samples 25 and 50 μ g. TCTP plays a role in the development, immune system, tumour reversion, and maintenance of stem cell expression and is increased at 5 μ g compared to control, 3rd-day regenerated blastema control, and indole-treated other samples 25 and 50 μ g and it shows that indole-treated samples play an important role in stem cell maintenance. H2A gene which plays a unique role in determining the structure of chromatin and DNA modification, indole treated sample has a high expression of 5 μ g compared to the control and 3rd-day blastema regenerated control, interestingly similar to other genes SOX and USP has a high expression on 5 μ g of indole treated groups compared to a control sample. By evaluating the gene expression pattern of these genes' indole treated sample 5 μ g has more expression on regeneration and embryogenesis mechanism.

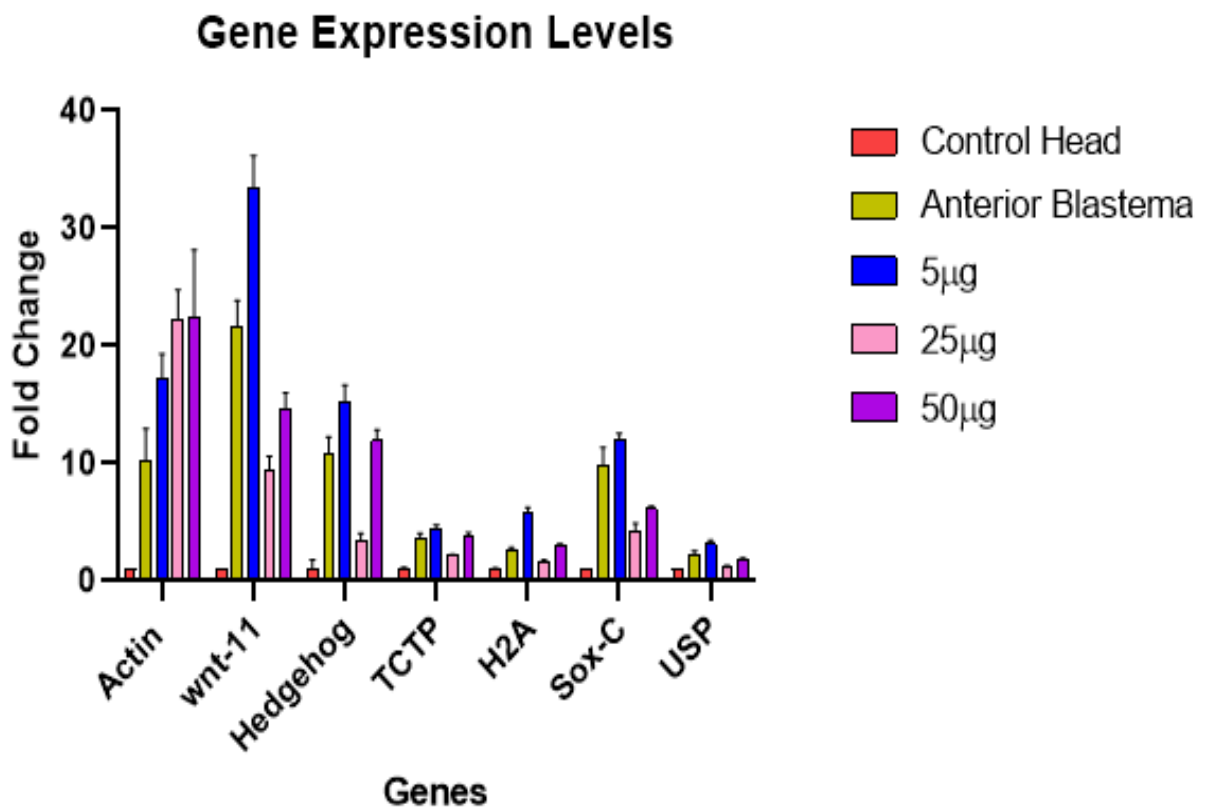


Fig 5.6: Expression pattern of genes in the indole-treated sample

5.6 Western blotting

The protein lysate was prepared from the following samples viz, control, 3rd day regenerated control, 5µg, 25µg, and 50µg, indole treated groups. The protein lysate was resolved in SDS PAGE and western blotting was performed with the following antibodies were used wnt, sox2, oct4, and caspase. Results show that (Fig 5.7) sox2 and oct4 expression is reduced in control however the expression was increased in regeneration control. The expression of sox2 and oct4 was increased serially from 5 µg, 25 µg, and 50 µg of indole injected samples. The data implies that sox 2 and oct data imply that sox 2 and oct4 are stem cell markers whose expression are high in control regeneration and indole-treated samples.

Western blotting with caspase antibody shows that the expression was increased in control compared to regeneration control, however, the decreased expression is noted in 5 and 50µg indole injected samples compared to control, caspase is an apoptotic marker whose expression is reduced upon indole-treated samples compared to control. Western blotting with wnt antibodies shows that (fig 5.7) the expression was increased in control compared to regeneration control, interestingly upon indole treatment the wnt expression was decreased upon increasing the concentration of indole.

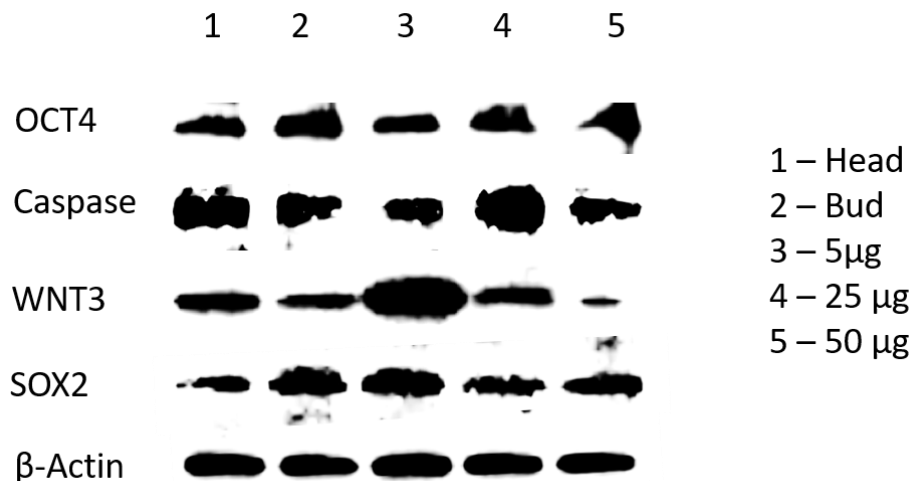


Figure 5.7: Expression of Protein

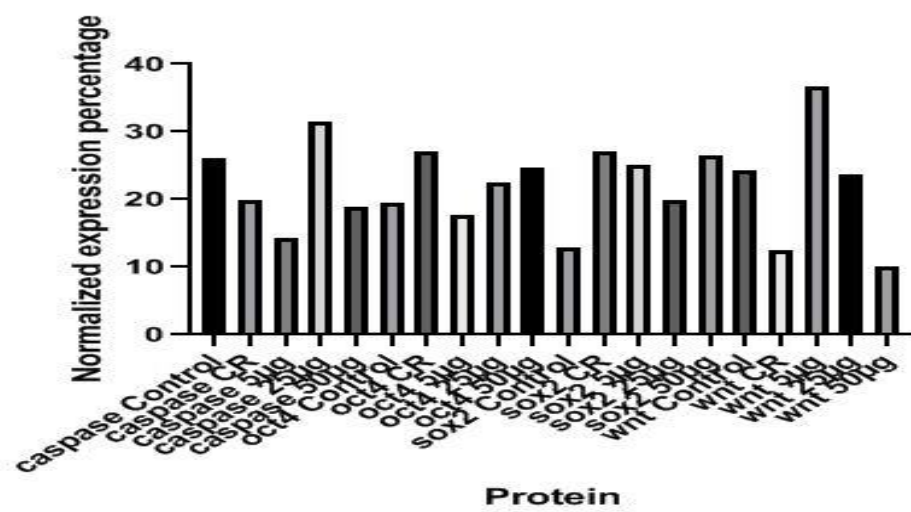


Fig 5.8: Expression pattern of proteins in the indole-treated sample

CHAPTER – 6

Summary and Conclusion

Indole is a metabolic product that contains wonderful wound-healing activity, the cytotoxicity and embryotoxicity of the compound indole were evaluated, and was found when the concentration of indole increases the toxicity also increases. In addition, the gene expression studies were performed on the following genes Sox-c, WNT, HEDGEHOG, TCTP, and USP were overexpressed in indole-treated worms compared to the control and blotting result of caspase concluding that indole helps to inhibit apoptosis and stem cell markers like Sox-c and OCT4 are overexpressed compared to control, interestingly WNT3 expression is reduced upon increasing the concentration of indole treated worms on earthworm. Hence, we conclude that indole not only plays a major role in the regeneration process but also the embryonic developmental process. It can be used for therapeutic purposes for healing wounds and burn wounds.

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