Wound Healing Effects of Blastema Extract from Amputated Earthworm Adults *Eisenia fetida* on Mouse Fibroblast Cell Line L929

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Abstract. Natural compounds of biological origin have gained immense interest in wound healing applications due to their natural tissue regenerative properties without inducing any systemic toxicity. It has been reported previously that earthworm extracts of whole animals are exhibiting high wound healing index. It has also been reported that the newly regenerated tissues of animals are highly enriched with growth factors, cytokines and regenerative proteins and peptides. Combining both these aspects, the present study explores the utility of blastema tissue- the regenerated tissue from the amputated region of adult earthworms, *Eisenia fetida* in its proliferative and healing properties in vitro on mouse fibroblast cell line L929. *Eisenia fetida* exhibits remarkable regenerative capabilities, making it an excellent model for studying tissue regeneration and developing novel wound healing agents. Five segments from the anterior region of the earthworms were amputated, and regeneration was observed over a 12-day period. The tissue extract from the regenerated blastema was prepared and tested for its efficacy in wound healing on mouse fibroblast (L929) cells using a scratch assay. The experimental observations were remarkable, that the earthworm blastema extract significantly accelerated wound closure, by enhancing fibroblast migration, proliferation, and with enhanced collagen synthesis. The high index in cell migration on treatments projects regenerated blastema extract as a promising candidate for wound healing applications especially for degenerated resistant wounds like Diabetic foot ulcer.

Keywords: *Eisenia fetida,* Natural compounds, Wound healing, Tissue regeneration, Fibroblast

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1 Introduction

Wound healing is a complex biological process that requires the coordination of numerous cellular activities, including inflammation, tissue proliferation, and remodelling. Even then, several factors reduce wound recovery such as malnutrition, chronic diseases like Diabetics, and infections as observed in diabetic wounds (1). To minimize thse complications there are several strategies are under intense research (2). Compounds that are of biological origin like natural compounds from plants and animals, have been extensively studied for their wound healing and regenerative properties (3). Annelids are one among those animals with highest regeneration capacity n the animal world recent research on annelid regeneration has revealed that these organisms share fundamental molecular and cellular pathways of repair (4).

Earthworms, one of the widely used annelids in clinical research have exhibited a wound healing property in its whole tissue extract . It was shown to be promoting wound healing process by enhancing collagen synthesis, fibroblast proliferation, and tissue repair Moreover, newly regenerating tissues are observed to have high regeneration index ([5];[6]). Earthworms, particularly *Eisenia fetida*, are known for their regenerative and tissue repair abilities The elevated and unafforbale expense for advanced therapies of wound healng has spurred interest in alternative treatments that are both effective and affordable. (7). So, this research aims to investigate the wound healing potential of a natural tissue extract from the adult earthworms *Eisenia fetida* prepared from the regenerated blastema from the anterior region of amputated animals.

2 Materials and Methods

2.1 Materials Used

Eisenia fetida adult worms, Leica EZ 4 Stereo zoom microscope. Vermicompost, Cow dung, culture chambers lined with wet clothes, ice and sterile dissection tools

2.2 Methodology

The methodology of the study was carefully designed to investigate the therapeutic potential of the regenerated tissue extracts from Eisenia fetida. The experimental procedure was divided into several stages, including the collection, acclimatization and amputation of earthworms, preparation of blastema tissue extracts and the assessment of its potential in wound healing properties.

2.2.1 Collection and Acclimatization of Earthworms

Adult earthworms (*Eisenia fetida*) were brought to the laboratory from the Kerala Agricultural University, Thrissur, Kerala in vermicompost and acclimatized in the laboratory for one week in optimal conditions by maintaining adequate moisture, food (cow dung), and temperature levels to ensure the health and sustainability of the earthworms and are fed cow dung and kept at 25-degree Celsius moist environment to promote healthy growth.

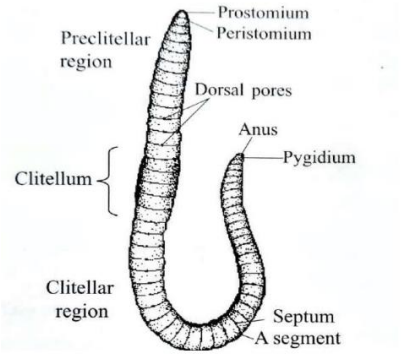


Fig. 1. Earth worm Eisenia fetida

2.2.2 Amputation Procedure

To study the regenerative properties of Eisenia fetida, the animals were grouped in to anterior region amputated -Adult Anterior (AA) as well as posterior region amputated ones- Adult Posterior (AP). The anterior amputated groups were selected later for wound healing studies. Five segments were removed from the anterior region of the animals using sterile surgical instruments, kept the separately in individual containers and the regeneration process was monitored over a period of 12 days.

2.2.3 Regeneration Monitoring

The animals were observed daily under a compound microscope for the blastema development, and the regeneration process was documented on days 3, 6, 9, and 12. The growth of the new segments were photographed using Leica EZ 4 Stereo zoom microscope and the blastema lengths were measured to determine the extent of regeneration using the software Leica Application Suite. Tissue samples were collected from the fully regenerated segments on day 12 for further analysis.

2.2.4 Preparation of Regenerated Tissue Extract

After regeneration of the blastema cells, on 12 day of amputation, the newly formed anterior segments were excised using sterile scalpels, followed by thorough washing in phosphate-buffered saline (PBS, pH 7.4) to remove any residual vermicompost. The excised blastema tissue was then homogenized using a mechanical homogenizer in ice-cold PBS buffer to prevent degradation of bioactive compounds. The homogenate was centrifuged at 5000 rpm for 10 minutes to separate the supernatant containing the bioactive compounds from the cellular debris and the tissue extract was quantified using UV-Visible spectroscopy to estimate the total protein concentration in the preparation which was used to standardize the extract for subsequent wound healing experimentations.

2.2.5 Scratch Wound Healing Assay

The wound healing efficacy of the regenerated tissue (blastema) extract from the amputated earthworms were assessed using a scratch wound assay on mouse fibroblast

(L929) cells. This assay is commonly used to evaluate the ability of compounds to promote cell migration and wound closure, which are key processes in skin regeneration. Briefly, L929 mouse fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were then maintained in optimal conditions in a humidified incubator at 37°C with 5% CO_2.

Scratch wound assay has been performed once the L929 cells reached 90% confluency, a uniform scratch was created across the monolayer using a sterile pipette tip. The cells were then washed with PBS to remove any cell debris, and fresh DMEM containing different concentrations of the Eisenia fetida tissue extract was added to the wells at varying concentrations (50, 100, 250, 500 µg/mL). Sericin protein extracted from Silk cocoons were used as positive control for fibroblast migration and wound healing.
Images of the wound area were captured at 0 hours, 12 hours, 24 hours, and 36 hours using an inverted microscope. By comparing the wound area at each time point with the initial wound area the percentage of cell migration and wound closure was calculated using ImageJ software.

2.2.6 Statistical Analysis

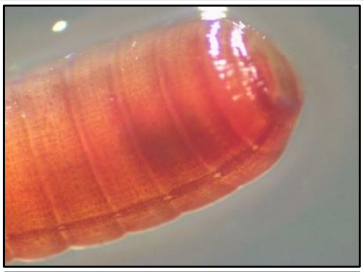
All experiments were conducted in triplicates to ensure the reliability of the results. The data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc pairwise test to determine the significance of differences between groups. A *p*-value of less than 0.05 was considered statistically significant.

3 Results and Discussion

The results of this study demonstrated the regenerative potential of *Eisenia fetida* tissue after anterior 5 segment amputation and the wound healing potential of blastema tissue extract.

3.1 Regeneration of Eisenia fetida Anterior Segments after amputing 5 segments

The anterior segments of *Eisenia fetida* exhibited robust regenerative ability efficiently over a 12-day period. Observations revealed that new segments began forming within the first few days' post-amputation, with noticeable growth by day 6. By day 12, the regenerated segments closely resembled the original anterior structure.



Adult Anterior (AA) at Day 0 after amputation

Fig. 2. Showing amputated adult *Eisenia fetida* immediately after amputation of first 5 segments Adult anterior (AA)

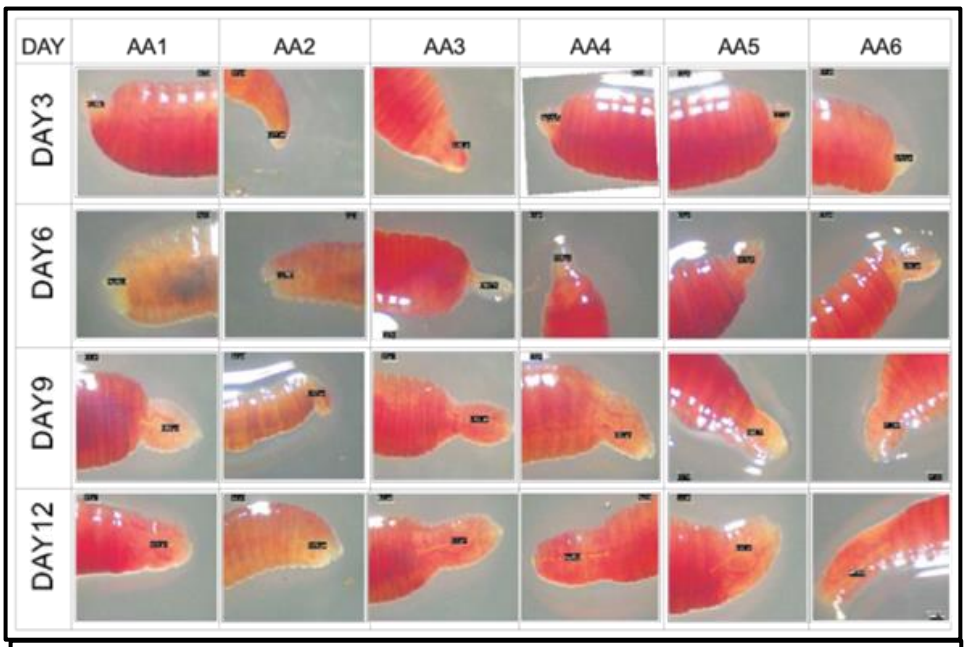


Image showing the regenerated anterior region after amputation in adut *Eisenia fetida* on day3, 6, 9 and 12

Fig. 3. Showing images of newly regenerated blastema on individual animals (AA-Adult anterior) after amputation of first 5 segments on day 3,6, 9 and 12. Images taken on Leica EZ 4 Stereo zoom microscope and measured the length of regenerated portion by using the software Leica Application Suite.

3.2 Adult Anterior (AA)

Adult worms after amputation post 5 segments from the anterior exhibited comparatively better regeneration potential than posterior region amputated adults. On day 3, the average regenerated length was measured as 0.373 cm which found to be 1.01 cm by day12. One-way ANOVA showed a strong effect of day on average length, $F(3, 143) = 133.87$, $p < 1\times10^{-15}$, $\eta^2 = 0.74$. Post-hoc pairwise tests with Holm adjustment indicated that each consecutive day differed significantly (Day 3 < Day 6 < Day 9 < Day 12; all adjusted $p < .001$). A weighted least-squares trend confirmed a robust linear increase of ≈0.073 cm per day ($t = 10.95$, $p = .008$). Error bars show SE.

Table 1 showing Mean growth of Blastema tissue over time

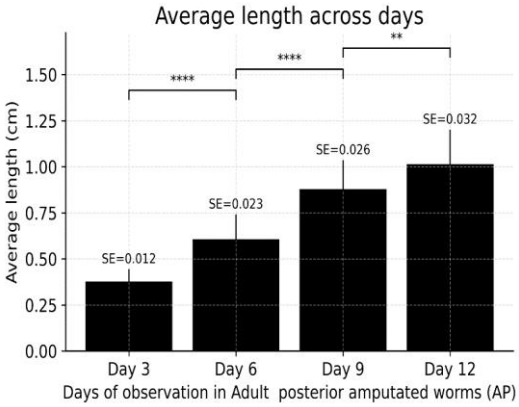


Fig. 4. Graph showing average length of newly regenerated portion in adult *Eisenia fetida* after anterior portion amputated on fifth segment (Day 3, 6, 9and 12).

As the well documented regeneration in annelids, the anterior segments from *Eisenia fetida* exhibited its remarkable regenerative capacity, The successful regenerative potential form the basis for its use in wound healing applications.

3.3 Wound Healing Efficacy – Scratch wound healing assay

The scratch assay creating a "scratch" in a monolayer of fibroblast cells L929 demonstrated the effectiveness of *Eisenia fetida* tissue extract in promoting wound healing. In the control group (without extract), the wound closure was slower, with only about 40% wound closure observed at 36 hours of treatment whereas the treatment groups at varying concentrations in a dose-dependent manner.

Table 1. Average wound area in treated L929 cells after 36 hours of treatment

Sample Conc.in µg /ml	0 hours	12 hours	24 hours	36 hours
Control	91.471	69.092	53.088	45.969
Positive Control	91.641	47.951	24.245	1.977
5	84.844	72.635	60.976	43.833
50	86.719	68.571	49.606	32.772
500	89.599	62.662	33.737	14.828

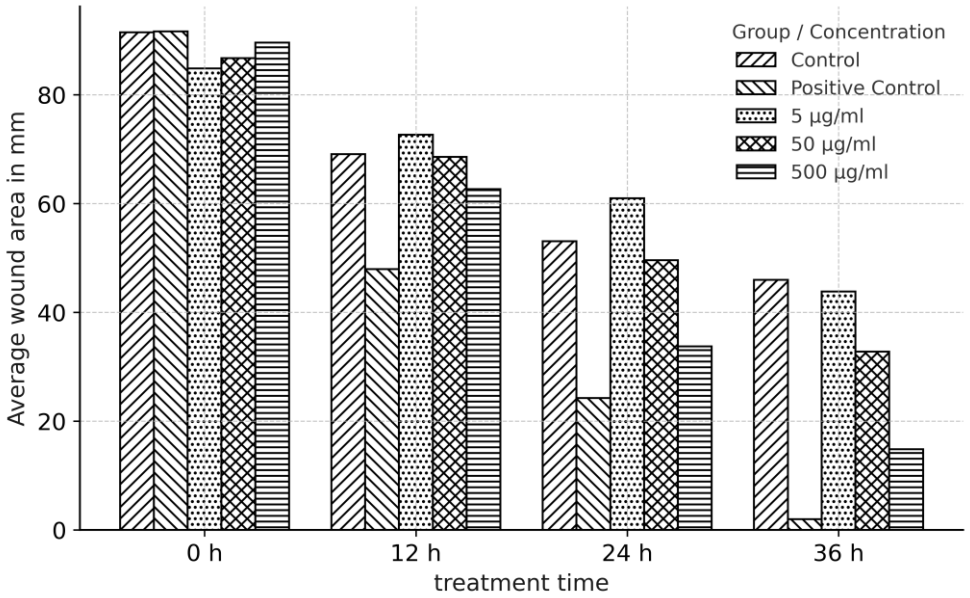


Fig. 5. Showing average wound area in experimental groups (L929 mouse fibroblast cell line) over the period of 36 hours; Control- without treatment; Positive control- The silk protein Sericin at 100 µg /ml Concentration; Blastema extract concentrations 5,50 and 500 µg/ml. Bars represent average wound area (mm) at each treatment time. Values provided are means only, so inferential significance was not computed. Across time, average wound area decreased in all groups. By 36 h, the ordering (smallest→largest) was: Positive Control (1.98), 500 µg/ml (14.83), 50 µg/ml (32.77), 5 µg/ml (43.83), Control (45.97). Among the concentrations, **500 µg/ml** showed the greatest reduction by 36 h

Table 2. Percentage of fibroblast migration rate in treated L929 cells after 36 hours of treatment

Sample Conc. µg/ml	0 hours	12 hours	24 hours	36 hours
Control	0	24.5	42	49.7
Positive Control	0	47.7	73.5	97.8
5	0	14.4	28.1	48.3
50	0	20.9	42.8	62.2
500	0	30.1	62.3	83.5

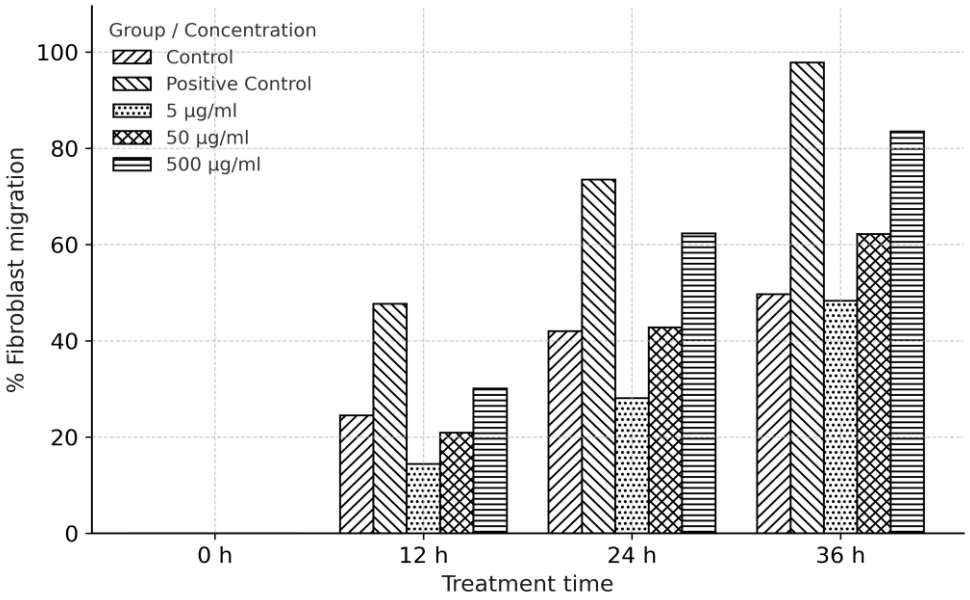


Fig. 6. Showing the % of fibroblast cell (L929 mouse fibroblast cell line) migration in experimental groups over the period of 36 hours; Control- without treatment; Positive control- The silk protein Sericin at 100 µg /ml Concentration; Blastema extract concentrations 5,50 and 500 µg /ml. % Fibroblast migration increased over time in all groups. By **36 h**, the ordering (lowest→highest) was: Control (49.7%), 5 µg/ml (48.3%), 50 µg/ml (62.2%), 500 µg/ml (83.5%), and **Positive Control** (97.8%). Among the test concentrations, **500 µg/ml** produced the largest migration at 36 h, with a clear dose-responsive pattern evident at 24–36 h (5 < 50 < 500 µg/ml).

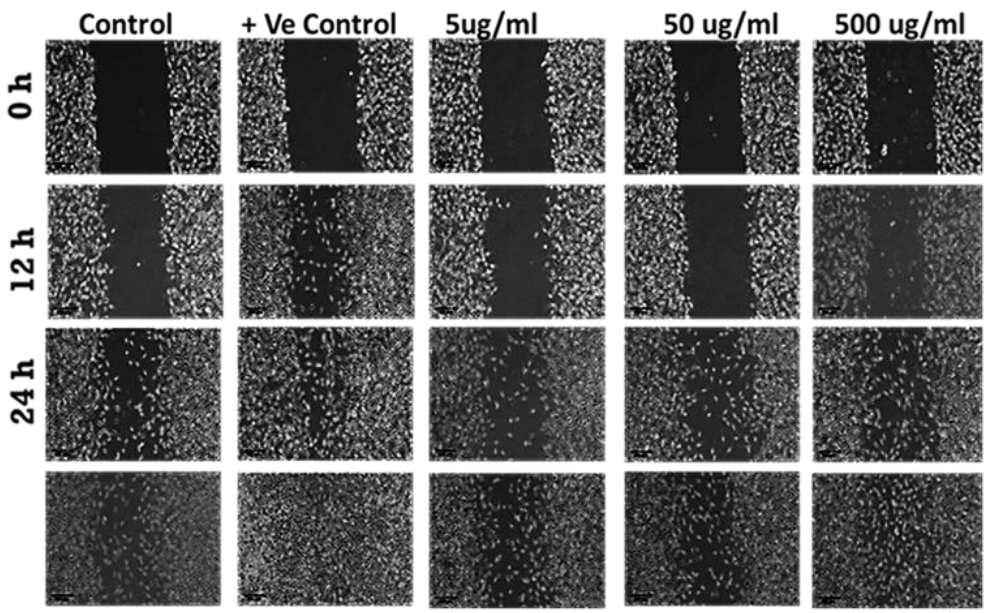


Fig. 7. Scratch wound assay images of cells under culture showing the fibroblast cell (L929 mouse fibroblast cell line) migration in experimental groups and wound closure over the period of 36 hours; Control- without treatment; Positive control- The silk protein Sericin at 100 µg /ml Concentration; Blastema extract concentrations 5,50 and 500 µg /ml.

The highest concentration of the extract (500 µg/mL) showed a significant improvement in wound closure, with nearly complete wound closure (~90%) observed within 36 hours. Lower concentrations (100 and 250 µg/mL) also promoted wound closure but at a slower rate compared to the 500 µg/mL group (Figure 2). The higher rates of migration of fibroblast cells on the wound area upon higher concentration suggests that the extract may contain factors that promote faster cell migration and proliferation, the key processes in tissue repair.

The wound-healing effects of earthworm blastema extract observed in this study also similar outcomes have been reported for a new collagen-like peptide, col4a1, Col4a1 enhanced the, proliferation, and migration of fibroblasts and the deposition of collage .Researchers have evaluated the bioactivities of the earthworm extract PvE-3 on the diabetic wound model and the diabetic related cell damage model and the results revealed that the PvE-3 promoted diabetic wound healing and protected fibroblast function in cell-damaged conditions ([8] :[9]). Wound healing potential of spidroin nanoparticles produced from the spider silk ([10] :[11]) and Rutin encapsulated decellularized earthworm granulation hydrogel and it promotes angiogenesis in wound healing of diabetic rabbit model by inhibiting TRAF1/NF-κB pathway. Sericin stabilized emulgel has been shown toact as a potent wound healing agent and this work emphasizes sericins natural wound healing properties ,which enhances the therapeutic potential of quercetin (12).

The present study limited the would healng experiments with mouse fibroblast cell line instead of experimenting with animals. There is a lack of research support from *in vivo*

experiments and molecular identification in wound repaire pathways ; additionally biochemical characterization to profile the specific bioactive peptides, proteins, and metabolites responsible for the observed regenerative effects can be done as an extention of the work.

3.4 Summary of the Findings

The wound healing experimentations conducted using the newly regenerated blastema tissue extract from the amputated adult earthworms *Eisenia fetida* tissue exhibited fibroblast proliferation and accelerated wound closure on mouse fibroblast cell line L929. Fibroblast migration is the key factor in wound healing process and the findings are consistent with the previously reported experiments on earthworm extracts which exhibited enhanced skin regeneration, collagen synthesis and angiogenesis (Deng et al., 2018).,

4 Conclusion

In conclusion the regenerated blastema tissue extract *from Eisenia fetida* anterior amputated animals have immense potential as a natural bioactive agent for promoting fibroblast migration and wound healing activity. Further experimentation both *in vivo* studies and molecular studies may unlock the full therapeutic potential of this extract. As the demand for natural, cost effective, sustainable and powerful wound healing regenerative therapies continues to grow, the observations of this study provide a strong foundation for developing earthworm-based wound healing and tissue regenerative products that harness natural regenerative properties in modern clinical practices.

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