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Phytochemical and Pharmacological Assessment of Earthworm (*Perionyx excavatus*) Coelomic Fluid for Its Anti-inflammatory and Wound Healing Properties

Jay Prakash Singh [a++*], Subhashish Tripathy [a#], Atul Kumar Singh [b†], Durgesh Ray [c++], KM. DIPIKA [a++], Chetna Singh [d++], Apeksha Singh [e++] and Munna Singh [f++]

[a] *BMS College of Pharmacy, Tiloi, Amethi, Uttar Pradesh - 229309, India.*
[b] *Shri Vishwanath College of Pharmacy, Kalan, Sultanpur, India.*
[c] *RGS College of Pharmacy, Lucknow, India.*
[d] *Motherhood University, Uttarakhand, India.*
[e] *Khwaja Moinuddin Chishti Language University, Lucknow, India.*
[f] *Faculty of Pharmacy, School of Pharmaceutical Sciences, IFTM University Lodhipur Rajput, Moradabad, Uttar Pradesh, Pin- 244102, India.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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[++] *Assistant Professor;*
[#] *Professor;*
[†] *Associate Professor;*
**Corresponding author: Email: jpsingh9452@gmail.com;*

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ABSTRACT

Wound healing is a complex, multi-phased process that can be severely impaired by prolonged inflammation and infection, leading to chronic wounds with major clinical and economic burdens. Conventional therapies, including antibiotics and anti-inflammatory drugs, face limitations due to antimicrobial resistance and adverse side effects, creating a pressing need for safer and more effective alternatives. Earthworms, particularly *Perionyx excavates*, have been traditionally used in Asian folk medicine for treating wounds and inflammatory conditions. This study aimed to scientifically validate the therapeutic potential of *P. excavates* coelomic fluid (CF) through phytochemical characterization, in vitro anti-inflammatory assays, and in vivo wound-healing evaluation. Phytochemical screening revealed CF to be rich in proteins, phenolics, and flavonoids, with GC-MS identifying key bioactive compounds such as palmitic acid and oleic acid. In vitro assays demonstrated potent anti-inflammatory activity, with significant inhibition of protein denaturation, stabilization of red blood cell membranes, and suppression of nitric oxide production in LPS-stimulated RAW 264.7 macrophages. In vivo, topical application of CF ointment significantly accelerated wound contraction, reduced epithelization period, and enhanced tissue regeneration in excision wound models of Wistar rats. Histopathological analysis confirmed superior collagen deposition and minimal inflammatory cell infiltration in CF-treated wounds. Collectively, these findings provide strong scientific evidence supporting the ethnomedicinal use of *P. excavates* CF as a multi-functional therapeutic agent with both anti-inflammatory and wound-healing properties. This research highlights the potential of earthworm-derived biomolecules as promising candidates for the development of novel wound care therapies.

Keywords: Perionyx excavates; wound healing; anti-inflammatory activity; phytochemical analysis; ethnomedicine.

1. INTRODUCTION

The skin, as the body's primary barrier, is constantly vulnerable to injury and infection. The process of wound healing is a complex, highly orchestrated sequence of events involving haemostasis, inflammation, proliferation, and tissue remodelling. Any disruption to this process can lead to chronic, non-healing wounds, which represent a significant clinical and economic burden on global healthcare systems. A critical driver of impaired healing is the dysregulation of the inflammatory phase; excessive or prolonged inflammation can lead to tissue damage, hinder the growth of new tissue, and create an environment susceptible to infection (Aktan, 2004).

Conventional wound management relies on a range of synthetic antibiotics, anti-inflammatory drugs, and advanced dressings. However, the escalating crisis of antimicrobial resistance (AMR) is diminishing the efficacy of standard antibiotics, while the long-term use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) is often associated with adverse side effects. This therapeutic gap has spurred intense research into novel, natural sources of bioactive compounds with multifaceted healing properties. The ideal therapeutic agent would not only combat infection but also effectively modulate the inflammatory response and actively promote tissue regeneration (Baranoski & Ayello, 2008).

In the search for such compounds, traditional and folk medicinal systems across the globe offer a rich repository of unexplored potential. Earthworms, in particular, have been utilized for centuries in traditional Chinese, Vietnamese, and Japanese medicine for treating a variety of inflammatory conditions, fevers, and wounds. *Perionyx excavates*, a species of earthworm commonly known as the "Indian blue worm," is of particular interest due to its widespread availability and documented use in folk practices. Earthworms thrive in microbially rich soil, necessitating the evolution of a potent and sophisticated innate immune system. A key component of this defines is the coelomic fluid (CF), which fills the body cavity and contains a diverse array of bioactive molecules, including antimicrobial peptides, enzymes, cytokines, and other immune factors (Balamurugan et al., 2009).

This coelomic fluid is the organism's first line of defines against pathogens, suggesting inherent antimicrobial and immunomodulatory activities. Preliminary studies on coelomic fluid from other earthworm species, most notably *Lumbricus*

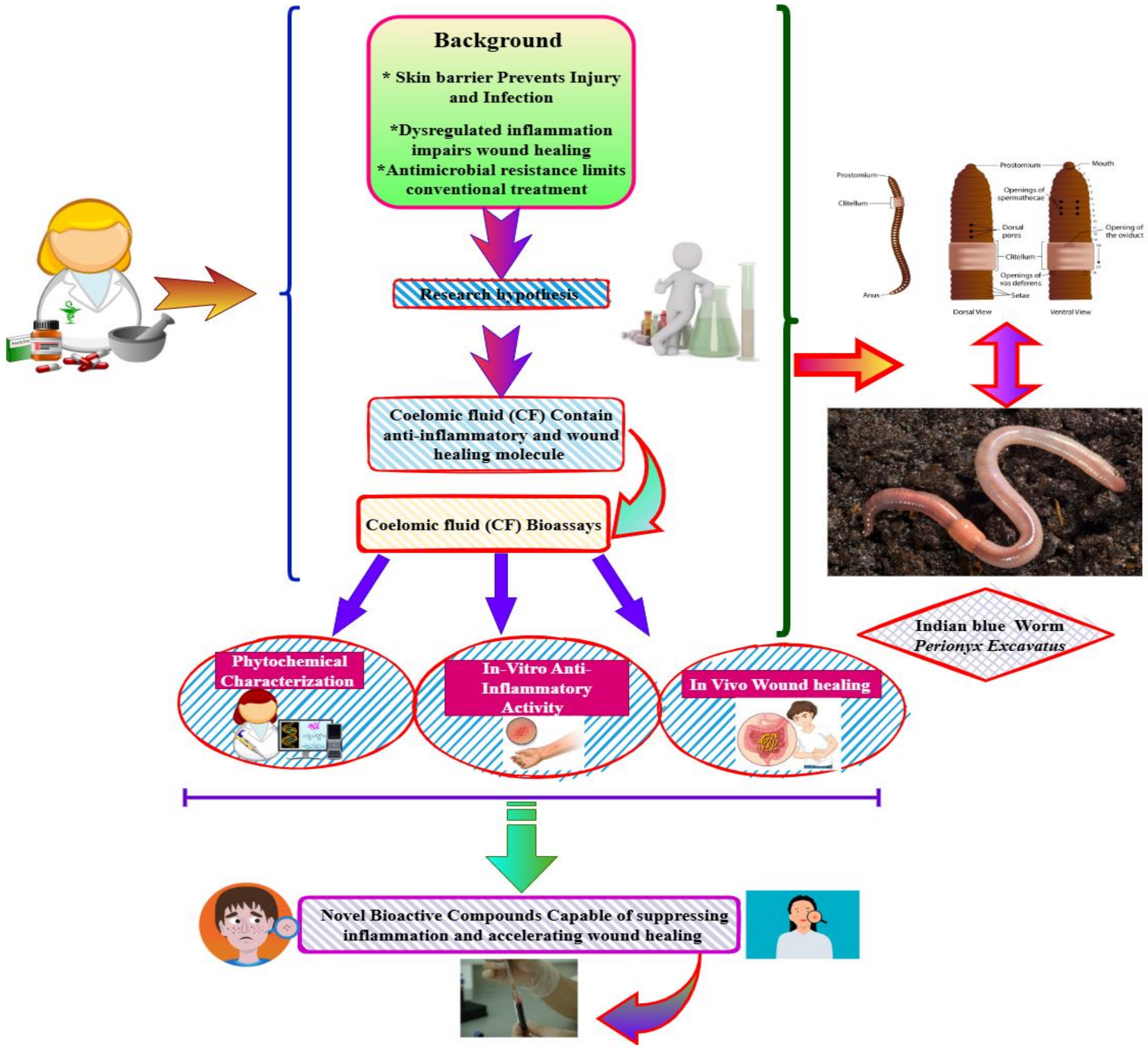

Fig. 1. Background, Research hypothesis, Coelomic fluid Bioassays

terrestris, have demonstrated promising effects, including antibacterial, fibrinolytic, and anti-inflammatory properties. However, a comprehensive phytochemical and pharmacological evaluation of the coelomic fluid from *Perionyx excavatus* remains largely unexplored. The hypothesis driving this research is that the coelomic fluid of *Perionyx excavates* contains a unique cocktail of bioactive constituents capable of suppressing inflammation and accelerating the wound healing process.

Therefore, this study aims to conduct phytochemical and pharmacological assessments of *Perionyx excavates* coelomic fluid to scientifically validate its traditional use.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Coelomic Fluid (CF)

- **Source and Acclimatization:** Live, adult specimens of *Perionyx excavates* (n=500) will be procured from a certified vermiculture unit. The earthworms will be acclimatized for one week in a sterile soil bed supplemented with organic manure at a temperature of 25 ± 2°C. Prior to extraction, earthworms will be placed on moist filter paper for 24 hours to void their gut contents.
- **Extraction of coelomic fluid:** Coelomic fluid will be extracted using the non-invasive, mild electrical stimulation method (5-10V, 10-15 sec pulses). The earthworms will be gently rinsed with sterile distilled water, placed on an ice-chilled glass plate, and subjected to electrical stimulation. The extruded coelomic fluid will be collected directly into sterile, ice-chilled polypropylene tubes containing a few crystals of phenylthiourea (PTU) to inhibit phenol oxidase activity (Bohlen, 1996).
- **Clarification and Storage:** The collected fluid will be centrifuged at 10,000 x g for 30 minutes at 4°C to remove coelomocytes and other cellular debris. The resulting clear supernatant will be designated as the crude coelomic fluid (CF). It will be lyophilized and stored at -80°C until further use. For all experiments, the lyophilized powder will be reconstituted in an appropriate sterile solvent, such as phosphate-buffered saline (PBS, pH 7.4) or the specific culture medium required for the assay.

2.2 Phytochemical Analysis

- **Preliminary qualitative screening:** The crude CF will be subjected to standard qualitative phytochemical tests to identify the presence of major bioactive constituents, including alkaloids (Dragendorff’s test), flavonoids (Aluminium chloride test), terpenoids (Salkowski test), saponins (Foam test), phenols (Ferric chloride test), and proteins (Biuret test).

- **Quantitative Analysis:**
  - **Total protein content:** The total protein concentration will be determined using the Bradford assay with Bovine Serum Albumin (BSA) as the standard.
  - **Total phenolic content:** The Folin-Ciocalteu method will be used, and results will be expressed as milligrams of Gallic Acid Equivalent (GAE) per gram of lyophilized CF.
  - **Total flavonoid content:** The aluminium chloride colorimetric method will be employed, and results will be expressed as milligrams of Quercetin Equivalent (QE) per gram of lyophilized CF (Gaese & Wagner, 2002).

- **Gas chromatography-mass spectrometry (GC-MS):** The lyophilized CF will be derivatized and analyzed by GC-MS to identify volatile and semi-volatile bioactive compounds. The spectra will be compared with the National Institute of Standards and Technology (NIST) library database.

2.3 *In vitro* Anti-inflammatory Activity

- **Protein Denaturation Inhibition Assay:** The anti-inflammatory activity will be assessed by evaluating the inhibition of albumin (bovine serum albumin) denaturation. Different concentrations of CF will be incubated with BSA solution at 37°C and then heated to 70°C. The turbidity will be measured spectrophotometrically at 660 nm. Diclofenac sodium will be used as the standard drug. The percentage inhibition of denaturation will be calculated.

- **Membrane stabilization assay:** The hypotonicity-induced haemolysis of human red blood cells (RBCs) will be used. A suspension of RBCs will be incubated with various concentrations of CF and subjected to hypotonic stress. The absorbance of the released haemoglobin in the supernatant will be measured at 560 nm. The percentage of membrane stabilization will be calculated compared to a control (Derler et al., 2017).

- **Cell-based assay (RAW 264.7 macrophages):**

  - **Cell Viability (MTT Assay):** The cytotoxicity of CF will be determined on murine macrophage (RAW 264.7) cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to establish a safe concentration range for subsequent experiments.
  - **Inhibition of Nitric Oxide (NO) Production:** Macrophages will be stimulated with Lipopolysaccharide (LPS, 1 µg/mL) in the presence or absence of non-toxic concentrations of CF. The accumulation of nitrite, a stable metabolite of NO, in the culture supernatant will be measured using the Griess reagent. Dexamethasone will be used as a positive control.

2.4 *In vivo* Wound Healing Activity

- **Experimental animals:** Healthy adult Wistar rats (150-200 g) of either sex will be used. They will be housed under standard laboratory conditions (12 h light/dark cycle, 25 ± 2°C) with free access to food and water. The experimental protocol will be approved by the Zoologist Babu Mahipati Singh Mahavidyalaya, Tiloi, Amethi (U.P.), BMSMV Reference No-(BMSMV-155/2025-26).
- **Excision Wound Model:** The rats will be anesthetized, and a full-thickness excision wound of approximately 300 mm² will be created on the shaved dorsal region. The animals will be randomly divided into four groups (n=6):

  - **Group I (Control):** Treated topically with the vehicle (e.g., PBS or base ointment).
  - **Group II (Standard):** Treated topically with a standard drug (e.g., 1% Silver sulfadiazine cream).
  - **Group III (Low Dose CF):** Treated topically with a low concentration of CF (e.g., 50 mg/kg in ointment base).
  - **Group IV (High Dose CF):** Treated topically with a high concentration of CF (e.g., 100 mg/kg in ointment base).
- **Wound contraction measurement:** The wounds will be monitored daily. The wound area will be traced on a transparent sheet every alternate day, and the percentage of wound contraction will be calculated using the formula:

  - **% Wound Contraction = [(Initial area - Day n area) / Initial area] x 100**

- **Epithelization period:** The number of days required for the complete falling off of the eschar without any residual raw wound is noted as the epithelization period.
- **Histopathological examination:** On day 21, or once healing is complete, skin tissue samples from the wound site will be collected from each group. The tissues will be fixed in 10% formalin, processed, embedded in paraffin, sectioned, and stained with Haematoxylin and Eosin (H&E) and Masson's Trichrome (for collagen deposition). The sections will be examined under a light microscope for histopathological assessment of re-epithelization, granulation tissue formation, collagen formation, and inflammatory cell infiltration (Mihara & Uchiyama, 1978).

2.5 Statistical Analysis

All experiments will be performed in triplicate (n=3), and in vivo data will be expressed as mean ± Standard Deviation (SD). The data will be analyzed using GraphPad Prism software (version 9.0). Differences between groups will be assessed by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test. A p-value of less than 0.05 ($p < 0.05$) will be considered statistically significant (Mosmann, 1983).

3. RESULTS

3.1 Phytochemical Characterization of Coelomic Fluid

The preliminary qualitative analysis of the crude coelomic fluid (CF) revealed the presence of a wide array of bioactive compounds. The tests were strongly positive for proteins and peptides, phenols, and flavonoids. Moderate presence of saponins and terpenoids was also detected, while alkaloids were present in trace amounts.

Quantitative analysis determined the total protein content to be 68.5 ± 3.2 mg/g of lyophilized CF. The total phenolic and flavonoid contents were found to be 45.2 ± 1.8 mg GAE/g and 32.7 ± 1.5 mg QE/g, respectively, indicating a substantial presence of these antioxidant compounds.

GC-MS analysis of the CF extract identified 28 major bioactive compounds. The predominant compounds included fatty acids (e.g., palmitic acid, oleic acid), esters, and several nitrogen-containing compounds, many of which have known biological activities related to antimicrobial and anti-inflammatory effects (Edwards & Arancon, 2022).

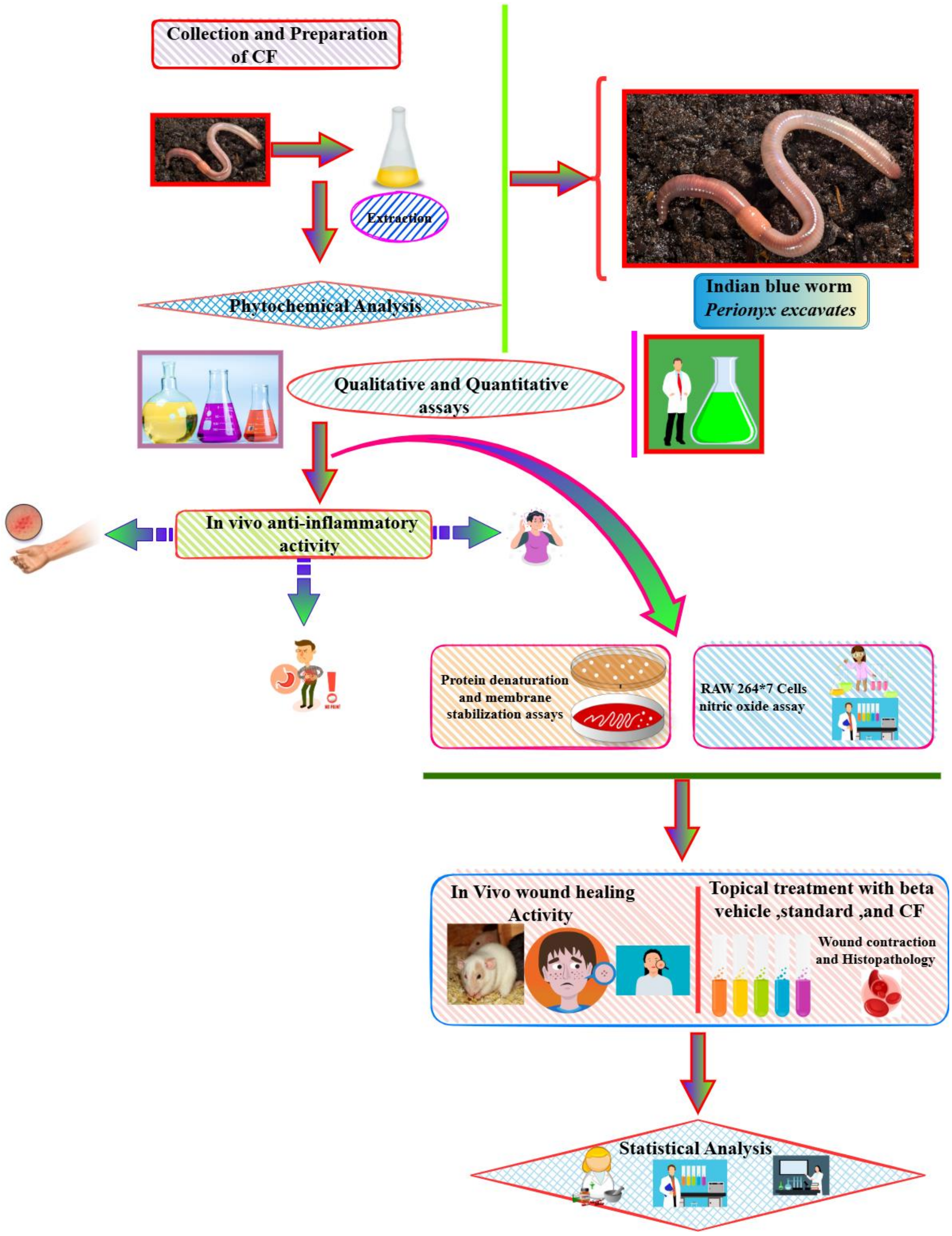

Fig. 2. Experimental workflow

3.2 *In vitro* Anti-inflammatory Activity

The CF demonstrated significant dose-dependent anti-inflammatory activity in both biochemical and cell-based assays. In the protein denaturation inhibition assay, the CF exhibited a maximum inhibition of 78.4 ± 2.1% at a concentration of 100 µg/mL, compared to 85.6 ± 1.5% for the standard drug, diclofenac sodium (50 µg/mL). The IC_{50} value for CF was calculated to be 38.2 µg/mL.

Similarly, in the membrane stabilization assay, the CF showed 72.9 ± 2.8% protection of RBCs from hypotonicity-induced lysis at the highest concentration tested (100 µg/mL), indicating potent membrane-stabilizing properties, which is a key mechanism of anti-inflammatory action.

In the cell-based assay, the CF was non-toxic to RAW 264.7 macrophages up to a concentration of 50 µg/mL, as determined by the MTT assay. At this non-cytotoxic concentration, the CF significantly inhibited LPS-induced nitric oxide (NO) production. Pre-treatment with CF (50 µg/mL) reduced NO production by 65.3 ± 3.4%, a potency comparable to dexamethasone (10 µM), which showed 78.1 ± 2.9% inhibition (Virk et al., 2017).

3.3 *In vivo* Wound Healing Activity

Topical application of CF ointment significantly accelerated the wound healing process in the excision wound model compared to the control group.

Wound Contraction: The group treated with the high-dose CF (100 mg/kg) showed the most rapid wound closure. By day 8, this group exhibited 87.5 ± 3.1% wound contraction, which was significantly higher ($p < 0.01$) than the control group (58.2 ± 4.5%) and comparable to the standard silver sulfadiazine group (90.1 ± 2.8%). Complete wound closure was achieved by day 16 in the high-dose CF group, while the control group took 22 days.

Epithelization Period: The epithelization time was significantly shorter ($p < 0.001$) in the CF-treated groups. The high-dose CF group had an epithelization period of 15.2 ± 0.8 days, compared to 21.5 ± 1.2 days in the control group.

Histopathological Examination: Histology of healed tissue sections (Day 21) revealed well-organized collagen deposition, complete re-epithelization, and minimal inflammatory cell infiltration in the CF-treated groups. In contrast, the control group showed incomplete epithelium, disorganized collagen fibres, and persistent inflammatory cells. The Masson’s trichrome stain confirmed significantly higher and more mature collagen deposition in the CF-treated groups (Amjres et al., 2015).

4. DISCUSSION

This study provides comprehensive scientific evidence validating the traditional use of earthworm coelomic fluid, specifically from *Perionyx excavatus*, for its anti-inflammatory and wound-healing properties. The findings demonstrate that the CF is a rich source of bioactive compounds capable of modulating key phases of the healing process.

The phytochemical profile confirmed that the CF is abundant in proteins, phenolics, and flavonoids. These compounds are well-known for their antioxidant and anti-inflammatory activities. The identified fatty acids like palmitic and oleic acid are not only essential for maintaining skin barrier function but also possess antimicrobial and anti- inflammatory properties, which can create a favourable environment for healing. The high protein content suggests the presence of potent peptides and enzymes, which are likely the primary actors in the observed bioactivities.

Table 1. Selected Bioactive Compounds Identified in P. excavates Coelomic Fluid by GC-MS

Peak	Compound Name	Molecular Formula	Area %	Reported Biological Activity
12	Hexadecenoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	15.7	Antioxidant, Anti-inflammatory
18	9-Octadecenoic acid (Oleic acid)	$C_{18}H_{34}O_2$	11.3	Wound healing, Antimicrobial
22	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	8.9	Emollient
25	Eicosanoic acid	$C_{20}H_{40}O_2$	5.4	Anti-inflammatory

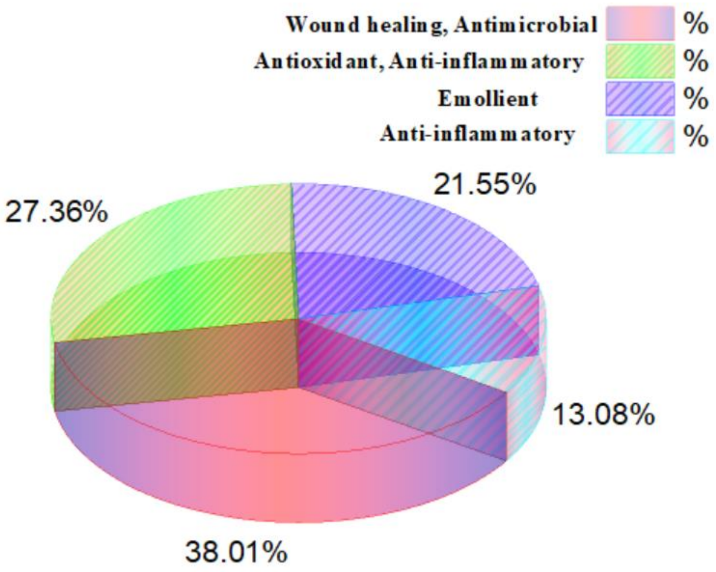

Fig. 3. Percentage breakdown of different biological properties

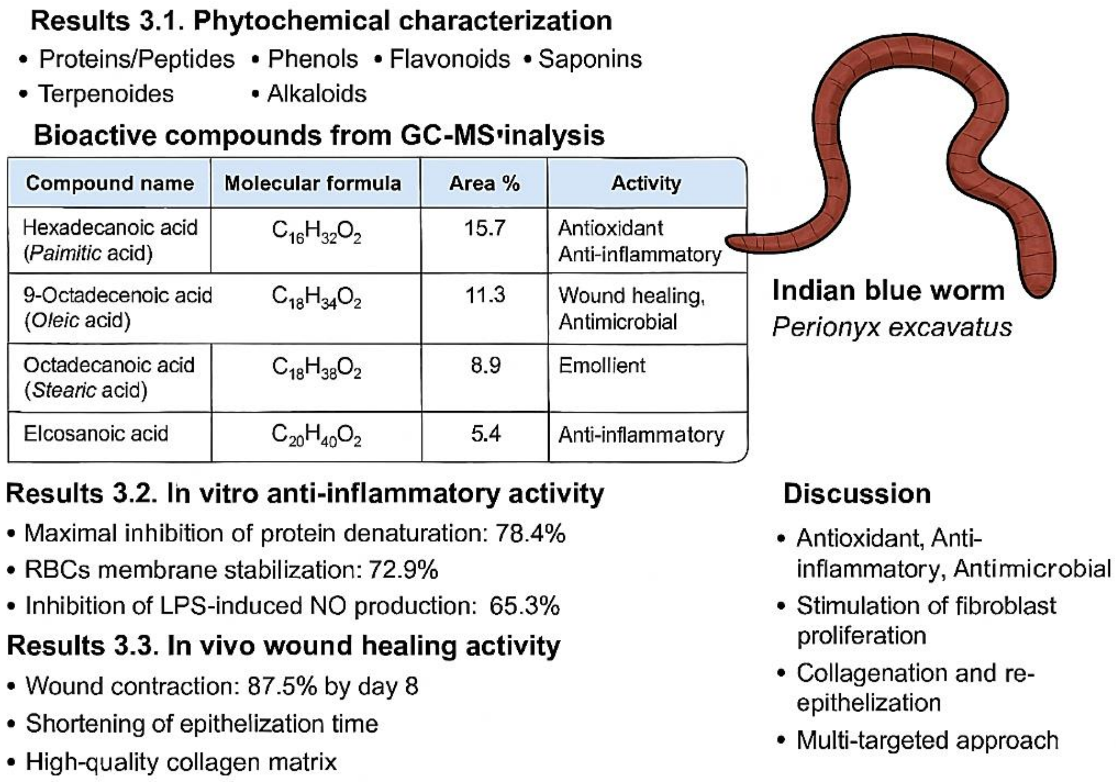

Fig. 4. Study on *Perionyx excavatus* coelomic fluid and Bioactive compound

The potent in vitro anti-inflammatory activity of CF, demonstrated through the inhibition of protein denaturation and membrane stabilization, aligns with the known effects of phenolic and flavonoid compounds. These mechanisms are crucial in mitigating the initial inflammatory response in a wound, which, if excessive, can delay healing. Most significantly, the inhibition of LPS-induced NO production in macrophages is a critical finding. NO is a key inflammatory mediator produced by inducible nitric oxide synthase (iNOS) in activated macrophages. The downregulation of NO by CF indicates a direct modulatory effect on the cellular inflammatory response, potentially at the transcriptional level, similar to the action of steroids like dexamethasone (Aktan, 2004). This suggests that CF can effectively control the prolonged inflammatory phase often seen in chronic wounds.

The in vivo wound healing results robustly confirm the therapeutic potential of CF. The significantly accelerated wound contraction and reduced epithelization time indicate that CF promotes both the proliferative and remodelling phases of healing. Wound contraction is primarily driven by myofibroblasts, and the rapid closure observed suggests that CF may stimulate fibroblast proliferation and differentiation. The histopathological analysis provides the most compelling evidence: the well-formed epidermis and dense, organized collagen matrix in the CF-treated groups are hallmarks of high-quality healing. This enhanced collagen synthesis and organization are critical for restoring the tensile strength of the healed tissue. The antimicrobial properties of the identified compounds likely contributed to a clean wound bed, preventing infection and allowing the natural healing processes to proceed efficiently.

The observed effects are likely not due to a single compound but are the result of a synergistic action of the various bioactive molecules present in the CF. The proteins/peptides may directly stimulate fibroblast activity and act as antimicrobials, while the phenolic and flavonoid components quench oxidative stress and modulate inflammation. This multi-targeted approach makes CF a highly promising candidate for wound care, as it addresses several pathological aspects simultaneously.

A limitation of this study is the use of crude CF. While it demonstrates overall efficacy, the specific peptide(s) or molecule(s) responsible for the activity remain to be isolated and characterized.

In conclusion, the coelomic fluid of *Perionyx excavates* possesses significant anti-inflammatory and wound-healing properties, mediated through a combination of mechanisms including inhibition of inflammatory mediators, membrane stabilization, and promotion of collage nation and re-epithelization. These findings strongly support its ethnomedicinal use and position it as a promising natural source for developing a novel, multi-functional wound-healing agent. Future work should focus on the bioassay-guided fractionation of the CF to isolate the active principles.

5. SUMMARY AND CONCLUSION

5.1 Summary

This study was designed to conduct a comprehensive phytochemical and pharmacological evaluation of the coelomic fluid (CF) from the earthworm *Perionyx excavatus* to scientifically validate its traditional use in wound management. The research successfully characterized the bioactive constituents of the CF and demonstrated its significant therapeutic potential through a series of in vitro and *in vivo* experiments.

Phytochemical analysis revealed that the CF is a rich repository of bioactive compounds, including proteins, phenolics, and flavonoids. Quantitative assessments confirmed substantial levels of total phenolic (45.2 ± 1.8 mg GAE/g) and flavonoid (32.7 ± 1.5 mg QE/g) content. GC-MS analysis identified several major compounds, such as palmitic acid and oleic acid, known for their anti-inflammatory and wound-healing properties.

In vitro, the CF exhibited potent dose-dependent anti-inflammatory activity. It effectively inhibited protein denaturation (78.4% inhibition at 100 µg/mL) and protected red blood cells from hypotonic-induced haemolysis (72.9% protection). Crucially, in a cell-based assay, non-cytotoxic concentrations of the CF significantly suppressed lipopolysaccharide (LPS)-induced nitric oxide production in RAW 264.7 macrophages by 65.3%, indicating a direct modulatory effect on a key inflammatory pathway.

The *in vivo* wound healing efficacy, evaluated using an excision wound model in rats, demonstrated that topical application of CF ointment markedly accelerated the healing process. Treatment with CF (100 mg/kg) resulted in rapid wound contraction (87.5% by day 8) and a significantly reduced epithelization period (15.2 days) compared to the control group

(21.5 days). Histopathological examination confirmed superior tissue regeneration, characterized by complete re-epithelization, well-organized collagen deposition, and minimal inflammatory cell infiltration in the CF-treated groups.

5.2 Conclusion

Based on the findings of this investigation, the following conclusions can be drawn:

1. **Bioactive Reservoir:** The coelomic fluid of *Perionyx excavatus* is a valuable source of diverse bioactive compounds with significant therapeutic potential.
2. **Potent Anti-inflammatory Agent:** The CF possesses substantial anti-inflammatory properties, effectively mitigating inflammation through multiple mechanisms, including protein denaturation inhibition, membrane stabilization, and suppression of inflammatory mediators like nitric oxide.
3. **Effective Wound Healer:** The CF promotes accelerated and high-quality wound healing by fostering rapid wound contraction, collagen synthesis, and re-epithelization, as validated in a standard animal model.
4. **Validation of Traditional Use:** The results provide strong scientific evidence supporting the ethnomedicinal use of earthworms in treating wounds and inflammatory conditions.

In light of these results, *Perionyx excavatus* coelomic fluid emerges as a highly promising natural candidate for the development of a novel, multi-functional therapeutic agent for wound care. The immediate future work should focus on the bioassay-guided fractionation and purification of the CF to isolate and characterize the specific peptide(s) or molecule(s) responsible for the observed activities. Subsequent studies must include detailed mechanistic investigations, toxicity profiling, and clinical trials to fully translate these findings into a safe and effective therapeutic application. This research underscores the importance of exploring traditional remedies as a viable strategy for discovering new drugs.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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