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Original Research Article

A comprehensive study on the biological activities of protein-rich earthworm (*Perionyx excavatus*) extracts collected in Vietnam

Nguyen T. Dung, Pham H. Son, Nguyen T. N. Trinh, Vo N. T. Thao, Bui B. Thinh*

Food Biotechnology Division, Biotechnology Center of Ho Chi Minh City, Ho Chi Minh City, 70000, Vietnam

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ABSTRACT

Earthworm extracts are increasingly recognized for their bioactive compounds and therapeutic potential. This study evaluated the bioactive properties of acetone extracts (AC1:2 and AC1:4) from *Perionyx excavatus*, emphasizing their potential applications. Both extracts demonstrated high protein content (70.247–71.150%), with AC1:4 showing significantly higher amino acid levels (31.697 g/100 g) compared to AC1:2 (12.727 g/100 g). Key amino acids identified included aspartic acid, glycine, alanine, leucine, tyrosine, lysine, and histidine, known for their physiological and therapeutic benefits. AC1:4 exhibited superior antioxidant activity, with half maximal inhibitory concentration (IC_{50}) values of 1.077 ± 0.088 mg/mL and 0.892 ± 0.037 mg/mL in DPPH and ABTS assays, respectively. It also showed potent anti-inflammatory effects by inhibiting nitric oxide production in lipopolysaccharide-stimulated RAW264.7 macrophages (IC_{50} = 0.352 ± 0.025 mg/mL). Additionally, AC1:4 demonstrated strong inhibitory activity against elastase (IC_{50} = 5.372 ± 0.333 mg/mL), tyrosinase (IC_{50} = 0.12 ± 0.013 mg/mL), and MMP-1 (IC_{50} = 4.885 ± 0.228 mg/mL), highlighting its potential for skincare and anti-aging applications. These findings underscore the promise of *P. excavatus* acetone extracts, particularly AC1:4, as a sustainable and natural source of bioactive compounds for pharmaceuticals, cosmetics, and nutraceuticals.

Keywords: Earthworm, *Perionyx excavatus*, biological activities, acetone extract, amino acid.

Introduction

Earthworms, belonging to the phylum Annelida and class Oligochaeta, play a crucial role in terrestrial ecosystems, particularly in enhancing soil fertility and facilitating the decomposition of organic matter.[1] Among the diverse species of earthworms, *Perionyx excavatus*—commonly known as the blue worm or compost worm—is of particular ecological and economic interest due to its remarkable efficiency in vermicomposting and its ability to convert organic waste into nutrient-rich vermicast rapidly.[2–4]

Native to tropical and subtropical regions, including Vietnam, *P. excavatus* thrives in surface soil layers abundant in organic material.[5] This small, epigeic species is characterized by its adaptability, rapid reproduction, and high metabolic rate, which enable it to process significant amounts of organic waste into a potent organic fertilizer.[6,7] Vietnam's favorable climatic conditions, with warm temperatures and high humidity, provide an ideal habitat for the proliferation of *P. excavatus*. Consequently, the species is widely distributed in agricultural areas and is increasingly cultivated in controlled environments to meet the growing demand for sustainable agricultural practices.[8]

Beyond its role in soil enrichment, earthworm-derived products have gained attention for their biomedical and industrial applications. Protein-rich extracts from earthworms contain bioactive compounds such as enzymes, peptides, and antimicrobial substances.[9–12] These compounds exhibit diverse therapeutic properties, including anti-inflammatory, antioxidant, anticoagulant, and antimicrobial activities.[13–19] For instance, the proteolytic enzyme lumbrokinase, derived from earthworms, has demonstrated potential in treating thrombotic disorders by promoting fibrinolysis.[20] Additionally, earthworm extracts have shown promise in wound healing, cancer therapy, and as functional biomolecules in the pharmaceutical and nutraceutical industries.[11–13]

Emerging research also suggests that these bioactive compounds may modulate the immune system, enhancing the body's ability to defend against infections and diseases.[13] Given their wide-ranging applications, there is increasing interest in systematically exploring the biological potential of earthworm-derived compounds. However, most studies to date have focused on species like *Lumbricus rubellus* and *Eisenia fetida* in regions outside Vietnam, with limited attention to *P. excavatus*.[20–23]

In Vietnam, the farming of *P. excavatus* has expanded significantly in recent years, driven by the growing demand for organic fertilizers and eco-friendly waste management solutions.[24] Vermiculture, or the practice of earthworm farming, has become an integral component of organic farming initiatives, supported by governmental and non-governmental organizations.[4] Farmers and entrepreneurs are adopting vermicomposting as a cost-effective method to manage agricultural waste while enhancing soil fertility.[25]

Despite these advancements, the potential of *P. excavatus* as a source of bioactive compounds remains underexplored. Investigating the protein-rich extracts of this species could unlock valuable opportunities for developing novel health-related products. Comprehensive studies are needed to elucidate the biological activities of these extracts to maximize their utility. This study is among the first to systematically examine the biological activities of acetone extracts from *P. excavatus* collected in Vietnam. The study employs acetone-based extraction methods, which are widely recognized for their effectiveness in isolating bioactive molecules, including polyphenols, peptides, and

*Corresponding author. E-mail: buibaothinh9595@gmail.com
Tel: +84379536135

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enzymatic compounds. By analyzing these extracts, the research aims to uncover novel bioactive compounds with potential applications in pharmaceuticals, cosmetics, and nutraceuticals.

Materials and Methods

Earthworm samples

Living earthworms (*P. excavatus*), aged 7–8 weeks, were collected from a farm in Cu Chi District (11°07'15.0"N 106°30'04.0"E), Ho Chi Minh City, Vietnam. Immediately after collection, the earthworms were transported to the Biotechnology Center of Ho Chi Minh City for further processing. The earthworms were prepared according to the method described by Azmi *et al.*,[26] with minor modifications. First, the earthworms were thoroughly washed under running water to remove dirt and debris from their body surface. They were then immersed in 1.0% NaCl solution for 15 min to eliminate impurities, followed by immersion in 0.3% citric acid solution for 20 min to enhance cleanliness and reduce microbial load. After these treatments, the earthworms were freeze-dried to produce earthworm powder, which was subsequently stored at 40°C until further use.

Preparation of earthworm extract

Processed earthworm powder (50 g) was weighed and placed into a stomacher bag containing 250 mL of distilled water. The mixture was homogenized using a stomacher machine for 25 min to obtain a crude extract, which was then centrifuged to separate the supernatant. Protein precipitation was carried out using cold acetone at two different ratios of crude extract to acetone: 1:2 (AC1:2) and 1:4 (AC1:4). The mixtures were incubated at 4°C using a cooled incubator (MIR-254 PHCbi, Japan) for 20 min to allow protein precipitation. Subsequently, the mixtures were centrifuged at 7500 rpm for 40 min to collect the precipitate. The resulting precipitate was dissolved in phosphate buffer (pH 6.5) and then freeze-dried to obtain the earthworm extracts AC1:2 and AC1:4.

Determination of protein content

The total protein content of the earthworm extracts was determined using the Kjeldahl method.[27] Each extract (1 g) was placed in a Kjeldahl digestion flask containing 25 mL of concentrated H_2SO_4 and a catalyst mixture comprising 9 g K_2SO_4 and 1 g $CuSO_4 \cdot 5H_2O$. The sample was digested for 2.5 h using an electric heater equipped with a fume removal system. After digestion, the mixture was cooled to room temperature, and 80 mL of NaOH solution was added to neutralize the acid and release ammonia. The resulting mixture was distilled, and the liberated ammonia was collected in a receiving solution. The ammonia concentration was determined by titration with standardized HCl solution. The total protein content was calculated using the formula (1):

$$\text{Protein content (\%)} = \frac{(V \times C \times 14 \times 6.25)}{W} \quad (1)$$

Where: V: Volume of HCl used in the titration (mL); C: Concentration of HCl (mol/L); W: Weight of the sample (g); 14: Atomic weight of nitrogen (g/mol); 6.25: Conversion factor to estimate protein content.

Determination of amino acid composition

The amino acid composition of the earthworm extracts was analyzed using high-performance liquid chromatography (HPLC Agilent series 1200, Agilent Technologies, USA) based on the method described by Istiqomah *et al*.[28]. A 2 g sample of earthworm extract was mixed with 10 mL of 6 N HCl in a test tube and hydrolyzed at 110°C for 20 h. After hydrolysis, the sample was cooled to room temperature and evaporated at 50°C to remove excess HCl. The residue was neutralized with 6 N NaOH and filtered through a 0.2 µm membrane. Prior to injection into the HPLC system, a derivatization reaction was performed. The reaction mixture consisted of 275 µL of OPA solution (prepared by dissolving 0.01 g phthalaldehyde in 9 mL methanol, 40 mL borax buffer at pH 9.1, and 100 µL 2-mercaptoethanol) and 25 µL of the sample. The mixture was vortexed and reacted for 5 min. The HPLC analysis was performed using a C18 column and a binary solvent system. Solvent A was sodium acetate buffer (pH 5), and solvent B was a mixture of methanol, acetate buffer, and tetrahydrofuran (80:15:5, v/v/v). The column was eluted at a flow rate of 1.5 mL/min, and detection was performed using a fluorescence detector (excitation: 340 nm; emission: 450 nm).

Antioxidant activity assay

The antioxidant activity of earthworm extracts was determined by DPPH and ABTS assays as described by Dung *et al*.[29].

For the DPPH assay, various concentrations of earthworm extracts were prepared in methanol. Each sample (0.9 mL) was mixed with 4 mL of a 0.1 mM DPPH solution in methanol. The mixture was incubated at 25°C for 30 min in the dark to allow the reaction to occur. Following incubation, the absorbance was measured at 517 nm using a microplate reader (VersaMax, Molecular Devices, USA). Vitamin C (Sigma-Aldrich, Germany) was used as the positive control. The percentage of DPPH radical scavenging activity was calculated using the formula (2):

$$\% \text{ Inhibition} = \frac{A_C - A_S}{A_C} \times 100 \quad (2)$$

Where: A_C: Absorbance of the control sample; A_S: Absorbance of the test sample.

For the ABTS assay, the ABTS radical cation ($ABTS^+$) was generated by reacting a 7 mM ABTS solution in methanol with 2.45 mM potassium persulfate in a 1:1 ratio. The reaction mixture was kept in the dark at 24°C for 16 h to allow the formation of the ABTS stock solution. Before use, the stock solution was diluted with methanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. Earthworm extracts, dissolved in methanol at varying concentrations, were mixed with 5 mL of the ABTS solution and incubated in the dark for 15 min. Absorbance was measured at 734 nm using a microplate reader (VersaMax, Molecular Devices, USA). Vitamin C (Sigma-Aldrich, Germany) was used as the positive control. The percentage of ABTS radical scavenging activity was calculated using the formula (3):

$$\% \text{ Inhibition} = \frac{A_C - A_S}{A_C} \times 100 \quad (3)$$

Where: A_C: Absorbance of the control sample; A_S: Absorbance of the test sample.

Anti-inflammatory activity assay

The ability of earthworm extracts to inhibit nitric oxide (NO) production was evaluated as an indicator of anti-inflammatory activity.[30] RAW 264.7 cells, obtained from the American Type Culture Collection (ATCC), were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h under standard culture conditions (37°C, 5% CO_2). Following incubation, the medium was replaced with fresh medium containing various concentrations of earthworm extract or 20 µM dexamethasone, which served as a positive control. Cells were incubated for an additional hour before adding 1 µg/mL lipopolysaccharide (LPS) to induce inflammation. After 24 h of further incubation, 50 µL of supernatant from each well was transferred to a new 96-well plate, and 50 µL of Griess reagent was added. The mixture was incubated for 10 min at room temperature, and the optical density (OD) was measured at 540 nm using a microplate reader (VersaMax, Molecular Devices, USA). The percentage of NO inhibition was calculated using the formula (4):

$$\% \text{ NO inhibition} = \frac{\text{OD of LPS group} - \text{OD of treated group}}{\text{OD of LPS group}} \times 100\% \quad (4)$$

The cytotoxicity of earthworm extracts on RAW 264.7 macrophage cells was assessed both before and after inflammatory stimulation with LPS using the MTT assay.[30] RAW 264.7 cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h under standard culture conditions (37°C, 5% CO_2). After incubation, the medium was replaced with fresh medium containing different concentrations of earthworm extract. At the end of the treatment, MTT reagent was added to each well, followed by a 4 h incubation to allow for the formation of formazan crystals. The crystals were subsequently dissolved in DMSO, and the OD was measured at 570 nm using a microplate reader (VersaMax, Molecular Devices, USA). Cell viability was calculated using the formula (5):

$$\% \text{ Cell Viability} = \frac{\text{OD of treated group}}{\text{OD of untreated group}} \times 100\% \quad (5)$$

Anti-elastase activity assay

The elastase inhibition activity of earthworm extracts was determined using the method described by Azmi *et al*.[26]. The reaction mixture

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consisted of 100 µL of 0.2 M Tris-HCl buffer (pH 8.0), 25 µL of 10 mM N-(methoxysuccinyl)-ala-ala-pro-val-4-nitroanilide (MAAPVN) substrate solution, and 100 µL of the test sample. The mixture was thoroughly mixed and incubated at 25°C for 15 min. Subsequently, 50 µL of elastase enzyme solution (0.3 U/mL) was added, and the mixture was further incubated at 25°C for 15 min. Absorbance was measured at 410 nm using a microplate reader (VersaMax, Molecular Devices, USA). Epigallocatechin gallate (EGCG) served as the positive control. The percentage of elastase inhibition was calculated using the formula (6):

$$\%\ \text{Elastase inhibition} = \frac{A_C - A_S}{A_C} \times 100 \quad (6)$$

Where: A_C: Absorbance of the control sample; A_S: Absorbance of the test sample.

Anti-tyrosinase activity assay

The tyrosinase inhibition activity of earthworm extracts was determined using the method described by Azmi *et al*.[26]. A 2 mM solution of L-tyrosine was prepared in 50 mM phosphate buffer (pH 6.8). Phosphate buffer served as the negative control, and deionized water was used as the blank. The sample was incubated with tyrosinase (250 U/mL) for 10 min. Then, 50 µL of L-tyrosine solution was added to each well of a 96-well plate, and the mixture was incubated at 25°C for 30 min. Absorbance was measured at 492 nm using a microplate reader (VersaMax, Molecular Devices, USA). The percentage of tyrosinase inhibition was calculated using the formula (7):

$$\%\ \text{Tyrosinase inhibition} = \frac{A_C - A_S}{A_C} \times 100 \quad (7)$$

Where: A_C: Absorbance of the control sample; A_S: Absorbance of the test sample.

Anti-MMP-1 activity assay

The matrix metalloproteinase-1 (MMP-1) inhibition activity of earthworm extracts was determined using the method described by Tien *et al*.[31]. The sample was incubated with 20 µL of MMP-1 enzyme solution (0.5 µg/mL) at 37°C for 1 h. Following incubation, 40 µL of collagen solution (5 mg/mL) and 75 µL of collagenase buffer (50 mM Tris-HCl, 10 mM CaCl2, 0.15 M NaCl, pH 7.4) were added to the reaction mixture, which was then incubated at 37°C for 4 h. After incubation, 100 µL of Coomassie Brilliant Blue (CBB) solution (0.1%) was added to stain the collagen. The reaction mixture was centrifuged, and the collagen precipitate was dissolved in 200 µL of DMSO. EGCG served as the positive control. The OD was measured at 600 nm using a microplate reader (VersaMax, Molecular Devices, USA). The percentage of MMP-1 inhibition was calculated using the formula (8):

$$\%\ \text{MMP} - 1\ \text{inhibition} = \frac{(A_S - A_B) - (A_C - A_B)}{(A_{CO} - A_B)} \times 100 \quad (8)$$

Where: A_S: Absorbance of the sample containing collagenase and the test compound; A_B: Absorbance of the sample containing only buffer, CBB, and DMSO; A_C: Absorbance of the sample containing collagenase without the test compound; A_{CO}: Absorbance of the sample containing collagen and buffer.

Statistical analysis

All experiments were performed in triplicate to ensure reliability and reproducibility. Data were expressed as the mean ± standard deviation (SD) and calculated using Microsoft Office Excel 2016. The half maximal inhibitory concentration (IC_{50}) value, which indicates the sample concentration required to achieve 50% inhibition, was determined by plotting the percentage of inhibition against the logarithmic scale of sample concentrations. A dose-response curve was generated using non-linear regression analysis in Microsoft Office Excel 2016. The IC_{50} value was derived from the fitted curve.

Results and Discussion

Total protein and amino acid contents of earthworm extracts

The analysis of total protein content and amino acid composition in earthworm extracts was conducted to explore their potential applications in various fields, including pharmaceuticals, nutrition, and agriculture.[32] The total protein content of the acetone extracts AC1:2 and AC1:4 was 70.247% and 71.150%, respectively, with no statistically significant difference between the two (Figure 1). This high protein content aligns with findings from previous studies on different species of earthworms.[10,28] Such protein-rich extracts are highly valued for their nutritional and therapeutic potential.[10]

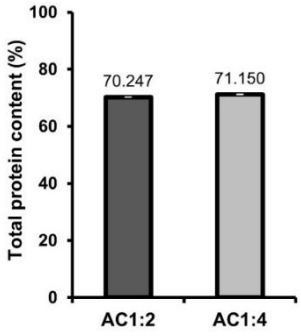

Figure 1: Total protein content of earthworm extracts.

The amino acid composition of AC1:2 and AC1:4 revealed notable differences. AC1:4 demonstrated higher concentrations of all analyzed amino acids, with total amino acid content reaching 31.697 g/100 g compared to 12.727 g/100 g in AC1:2 (Table 1). Among the identified amino acids, aspartic acid, glycine, alanine, leucine, lysine, and histidine were predominant. These results are consistent with the findings of Istiqomah *et al.*[28] and Hidayat *et al.*[33] for *Lumbricus rubellus*, Garczyńska *et al.*[34] for *Dendrobaena veneta*, Kostecka *et al.*[35] and Rufchaei *et al.*[36] for *Eisenia fetida*, and Kavle *et al.*[37] for *Eisenia andrei*. Interestingly, aspartic acid, glycine, alanine, and tyrosine were absent in the extract of *Eisenia fetida* as reported by Vital *et al.*[38]. This variability among species and extraction methods underscores the complexity of earthworm-derived bioactives and their unique benefits.[39]

The amino acids identified in these extracts contribute to various physiological functions, including protein synthesis, immune response enhancement, and cellular repair mechanisms.[28] The absence of certain amino acids, such as those noted in the study by Vital *et al.*,[38] may suggest selective bioavailability or differences in extraction methodologies, warranting further exploration. Overall, these findings highlight the significant potential of *P. excavatus* extracts as a valuable source of bioactive compounds, reinforcing their utility in advancing sustainable and effective bioresource applications.

Table 1: Amino acid composition of earthworm extracts.

Amino acid composition (g/100g)	Earthworm extract	
	AC1:2	AC1:4
Aspartic acid	3.279 ± 0.045	8.250 ± 0.026
Glycine	1.817 ± 0.015	3.277 ± 0.006
Alanine	2.330 ± 0.026	3.897 ± 0.025
Leucine	1.363 ± 0.015	6.047 ± 0.015
Tyrosine	0.213 ± 0.015	2.627 ± 0.015
Lysine	2.760 ± 0.026	5.630 ± 0.036
Histidine	1.137 ± 0.015	2.057 ± 0.035
Total	12.727 ± 0.186	31.697 ± 0.049

Antioxidant activity of earthworm extracts

The evaluation of antioxidant activity in earthworm extracts was conducted to explore their potential as natural antioxidant sources and to contribute to the understanding of earthworm-derived bioactive compounds.[13] The antioxidant activity was assessed through two mechanisms: scavenging of DPPH free radicals and ABTS free radicals. For DPPH radical scavenging, the extract AC1:2 exhibited an IC_{50} value of 2.073 ± 0.163 mg/mL, whereas AC1:4 demonstrated significantly higher activity with an IC_{50} value of 1.077 ± 0.088 mg/mL (Figure 2). Vitamin C, a well-known antioxidant, had an IC_{50} value of 0.006 ± 0.0002 mg/mL. Similarly, in the ABTS assay, the extract AC1:2 showed an IC_{50} value of 12.405 ± 0.459 mg/mL, while AC1:4 exhibited a remarkable IC_{50} value of 0.892 ± 0.037 mg/mL, compared to vitamin C's IC_{50} of 0.012 ± 0.002 mg/mL (Figure 3).

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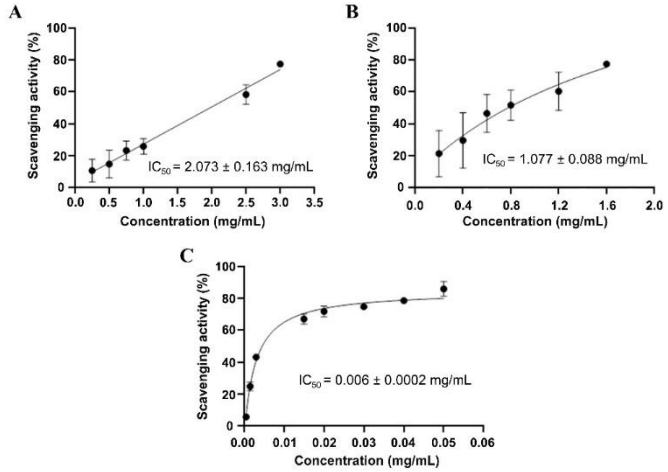

Figure 2: Antioxidant activity of earthworm extracts AC1:2 (A) and AC1:4 (B), compared to positive control Vitamin C (C), in the DPPH assay.

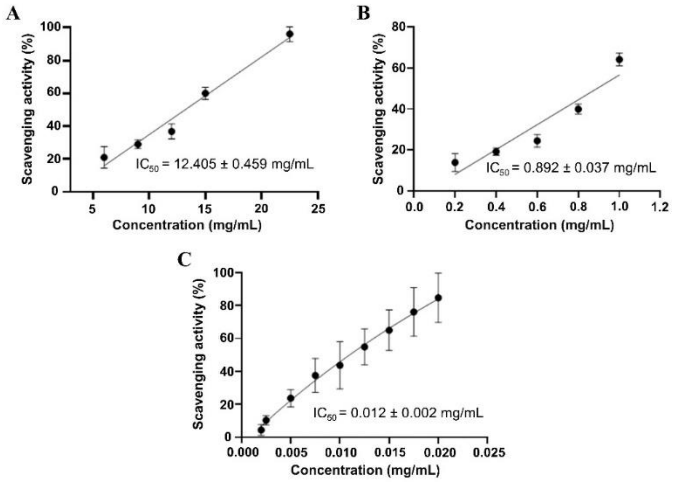

Figure 3: Antioxidant activity of earthworm extracts AC1:2 (A) and AC1:4 (B), compared to positive control Vitamin C (C), in the ABTS assay.

The results indicate that the AC1:4 extract had stronger antioxidant activity compared to AC1:2 in both assays. Furthermore, these findings suggest that *P. excavatus* exhibits better antioxidant potential compared to some earthworms studied previously. For example, Mustafa *et al.*[40] reported lower antioxidant activity in extracts from other earthworm species, and Dewi *et al.*[41] documented an IC_{50} value of 12.3 mg/mL for DPPH radical scavenging in *Lumbricus rubellus*. This highlights the distinctive bioactivity of *P. excavatus*, potentially attributable to differences in amino acid content and other bioactive constituents.

Amino acids such as aspartic acid, glycine, alanine, leucine, tyrosine, lysine, and histidine likely contribute to the observed antioxidant activity.[42] These compounds are known to function as free radical scavengers or metal chelators, stabilizing oxidative species through hydrogen donation or electron transfer.[19,42] The higher antioxidant activity in the AC1:4 extract could be due to a greater concentration of these amino acids, which enhance its capacity to neutralize reactive oxygen species (ROS) through multiple mechanisms. Additionally, the solvent ratio plays a critical role in isolating compounds with higher antioxidant potential.[29] These findings underscore the potential application of *P. excavatus* extracts in natural antioxidant development, offering an environmentally friendly alternative to synthetic antioxidants.

Anti-inflammatory activity of earthworm extracts

Inflammation is the body's defensive response to stimuli and can be classified as infectious or non-infectious.[43] In some cases, inflammation may be alleviated by anti-infective agents. This study investigates the anti-inflammatory activity of acetone extracts derived from *P. excavatus*, driven by the growing demand for natural therapeutic products and the need to explore sustainable bioresources. The anti-inflammatory potential was evaluated by examining the ability of these extracts to inhibit NO production in RAW264.7 macrophage cells stimulated with LPS.[30] Both acetone extracts (AC1:2 and AC1:4) effectively inhibited NO production at concentrations ranging from 0.2 to 0.8 mg/mL, with cell viability remaining consistently above 85% under these conditions (Figure 4 and Figure 5). This demonstrates the non-cytotoxic nature of the extracts, both before and after LPS stimulation. The IC_{50} value for AC1:2 was 0.465 ± 0.013 mg/mL, while AC1:4 exhibited a stronger inhibitory effect with an IC_{50} of 0.352 ± 0.025 mg/mL (Figure 6). In comparison, the positive control, dexamethasone (20 μM), achieved a 48% inhibition of NO production. The difference in IC_{50} values suggests that the composition of the extracts influences their anti-inflammatory efficacy. The superior activity of AC1:4 may be attributed to its higher concentration of amino acids. Notably, amino acids such as aspartic acid, glycine, alanine, leucine, tyrosine, lysine, and histidine likely contribute to the observed effects. For instance, glycine is known to modulate immune responses by suppressing pro-inflammatory cytokines,[44] while tyrosine and histidine may alleviate oxidative stress and inflammation.[45–47] Leucine, recognized for its ability to regulate macrophage activity, may synergize with other amino acids to enhance the overall anti-inflammatory effect.[45–47]

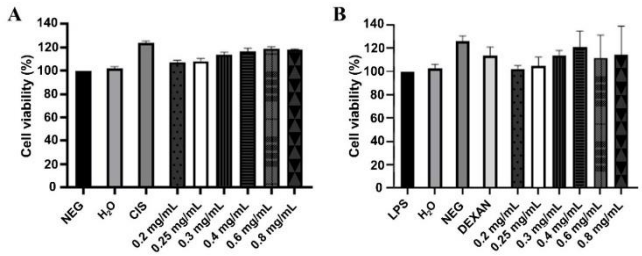

Figure 4: Effect of earthworm extract AC1:2 on the viability of RAW 264.7 macrophages before (A) and after (B) LPS-induced inflammatory stimulation.

The mechanism of NO inhibition by these extracts likely involves interference with the NF-κB signaling pathway, which plays a key role in regulating inducible nitric oxide synthase (iNOS) during inflammation.[43,46] Bioactive peptides and secondary metabolites present in the extracts may further amplify these effects.[46] In summary, acetone extracts of *P. excavatus* exhibit promising anti-inflammatory activity by effectively inhibiting NO production in LPS-stimulated RAW264.7 cells. Among the two extracts, AC1:4 demonstrated superior efficacy, highlighting the potential of earthworm-derived products as natural anti-inflammatory agents.

Anti-elastase activity of earthworm extracts

The anti-elastase activity of acetone extracts derived from earthworms was evaluated to assess their potential as natural inhibitors of elastase, an enzyme involved in tissue degradation and inflammatory processes.[48] Previous studies, such as by Azmi *et al.*,[26] have shown that extracts from species such as *Eisenia fetida*, *Lumbricus rubellus*, and *Eudrilus eugeniae* inhibit elastase activity by 40–52% at 10 mg/mL. This study extends these findings by investigating acetone extracts (AC1:2 and AC1:4) from *P. excavatus*. AC1:2 exhibited an IC_{50} value of 6.347 ± 0.198 mg/mL, while AC1:4 showed a stronger inhibitory effect with an IC_{50} of 5.372 ± 0.333 mg/mL (Figure 7). For comparison, the positive control, EGCG achieved an IC_{50} of 0.97 ± 0.013 mg/mL. The variation in IC_{50} values suggests differences in the bioactive compound profiles of the extracts. The enhanced activity of AC1:4 may stem from a higher concentration or better bioavailability of specific amino acids. These findings align with prior research, reinforcing the notion that earthworm extracts possess notable elastase-inhibitory potential.[26]

The observed anti-elastase activity is likely attributable to the extracts' rich amino acid content, including aspartic acid, glycine, alanine, leucine, tyrosine, lysine, and histidine.[49] These amino acids may interact with elastase, inhibiting its activity.

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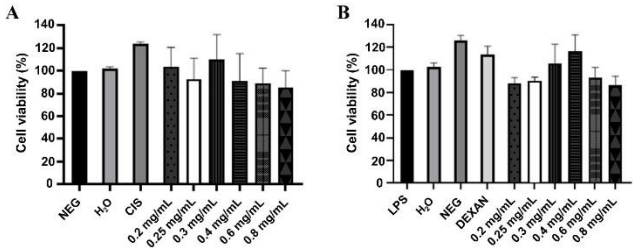

Figure 5: Effect of earthworm extract AC1:4 on the viability of RAW 264.7 macrophages before (A) and after (B) LPS-induced inflammatory stimulation.

Glycine and alanine are known for their roles in protein stabilization and enzymatic inhibition, while tyrosine and histidine may provide antioxidant properties that indirectly contribute to enzyme inhibition.[50,51] Additionally, leucine's hydrophobic interactions with elastase could enhance inhibitory effects.[52] Mechanistically, inhibition may occur through direct binding to the enzyme's active site or allosteric modulation, preventing substrate access.[26,49] Bioactive peptides and secondary metabolites in the extracts could also amplify these inhibitory effects.[49] In conclusion, acetone extracts of *P. excavatus* demonstrate significant anti-elastase activity, with AC1:4 outperforming AC1:2 in efficacy. Although less potent than EGCG, the natural origin and non-cytotoxic properties of these extracts underscore their potential as alternative elastase inhibitors.

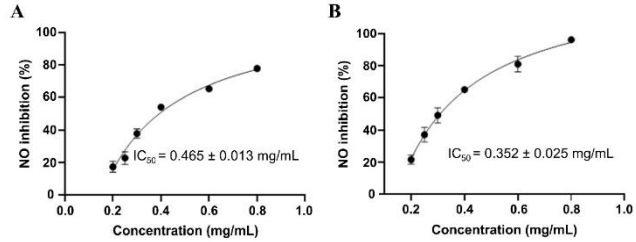

Figure 6: Inhibitory effect of earthworm extracts AC1:2 (A) and AC1:4 (B) on nitric oxide production in LPS-stimulated RAW 264.7 macrophages.

Anti-tyrosinase activity of earthworm extracts

Tyrosinase is a key enzyme in melanin biosynthesis, and its inhibitors hold potential applications in both the cosmetic and medical industries.[53] This study evaluated the tyrosinase inhibitory activity of two acetone extracts of *P. excavatus* (AC1:2 and AC1:4) compared to L-cysteine as the positive control. The AC1:2 extract exhibited tyrosinase inhibitory activity with an IC_{50} value of 0.27 ± 0.014 mg/mL, whereas AC1:4 demonstrated significantly stronger inhibition, with an IC_{50} value of 0.12 ± 0.013 mg/mL (Figure 8). L-cysteine, the positive control, achieved an IC_{50} of 0.10 ± 0.012 mg/mL. These results suggest that AC1:4 possesses a higher tyrosinase inhibitory potential than AC1:2 and approaches the efficacy of L-cysteine. When compared to findings by Azmi *et al.*,[26] where *Eisenia fetida* extracts achieved 80.12% inhibition at 0.25 mg/mL, the tyrosinase inhibitory activity of *P. excavatus* extracts in this study was relatively lower. The higher inhibition observed in *Eisenia fetida* extracts could reflect differences in protein composition, enzyme cofactors, or specific active compounds that may be absent or present in lower concentrations in *P. excavatus*.

The amino acids abundant in the protein-rich extracts of *P. excavatus* are likely contributors to their tyrosinase inhibitory effects. Specific amino acids, such as tyrosine, lysine, and histidine, may bind to the copper ions within the tyrosinase active site, disrupting catalytic activity.[54,55] Other amino acids, such as glycine and alanine, might act as competitive inhibitors or stabilize active compounds, while aspartic acid and leucine could enhance structural interactions between the extract and the enzyme, further improving inhibition.[54,55] The tyrosinase inhibitory mechanism of *P. excavatus* extracts likely involves copper ion chelation, competitive inhibition at the substrate-binding site, or allosteric modulation.[26,53] The combined effects of amino acid composition, peptides, and other bioactive molecules contribute synergistically to inhibition.[54] In summary, the findings highlight the potential of *P. excavatus* extracts as natural tyrosinase inhibitors, though their activity is lower than that of *Eisenia fetida* extracts.

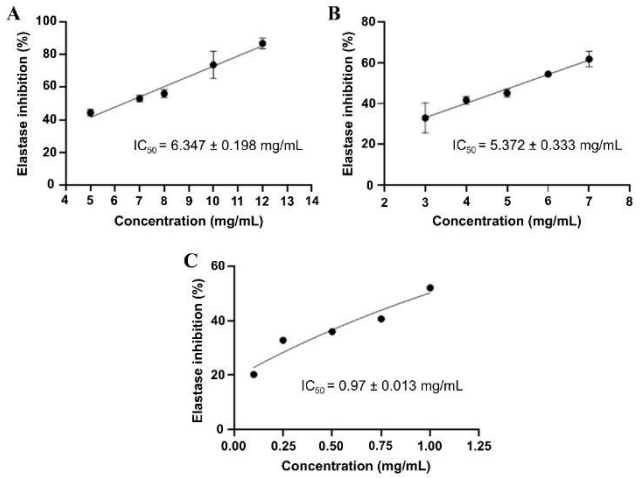

Figure 7: Anti-elastase activity of earthworm extracts AC1:2 (A) and AC1:4 (B), compared to positive control EGCG (C).

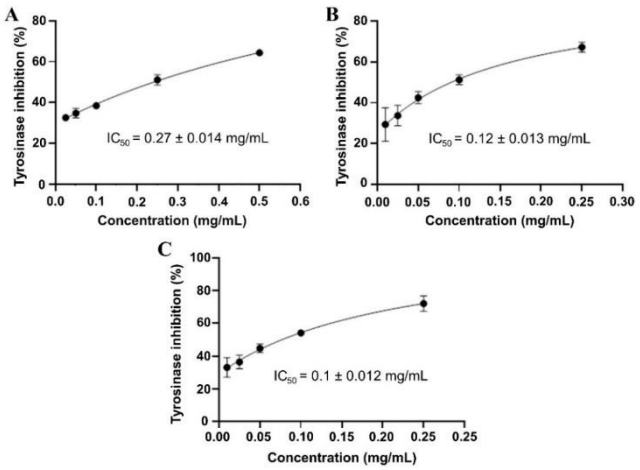

Figure 8: Anti-tyrosinase activity of earthworm extracts AC1:2 (A) and AC1:4 (B), compared to positive control L-cysteine (C).

Anti-MMP-1 activity of earthworm extracts

MMP-1 is a critical enzyme involved in collagen degradation within the extracellular matrix, and its overexpression is associated with skin aging and inflammatory conditions.[56] This study investigated the MMP-1 inhibitory activity of acetone extracts from *P. excavatus*. Extracts AC1:2 and AC1:4, prepared using different solvent-to-sample ratios, were tested at concentrations ranging from 1 to 9 mg/mL. The IC_{50} values for AC1:2 and AC1:4 were 5.877 ± 0.222 mg/mL and 4.885 ± 0.228 mg/mL, respectively, indicating stronger inhibition by AC1:4 (Figure 9). In comparison, the positive control EGCG demonstrated an IC_{50} of 0.620 ± 0.042 mg/mL.

The MMP-1 inhibitory activity of *P. excavatus* extracts surpassed that of *Eisenia fetida*, *Lumbricus rubellus*, and *Eudrilus eugeniae*, as reported by Azmi *et al.*,[26] with inhibition rates of 75.47%, 72.9%, and 81.42% at 10 mg/mL, respectively. The superior activity observed in *P. excavatus* extracts may be attributed to their rich protein content and the presence of bioactive amino acids such as aspartic acid, glycine, alanine, leucine, tyrosine, lysine, and histidine. Aspartic acid and glycine, for instance, are known to influence collagen synthesis and stability, while tyrosine and lysine, with their functional side chains, may interact directly with the MMP-1 active site or modify its conformation to reduce activity.[57–59] Histidine, with its metal-coordination properties, could disrupt the zinc-dependent catalytic mechanism of MMP-1, further enhancing inhibition.[60]

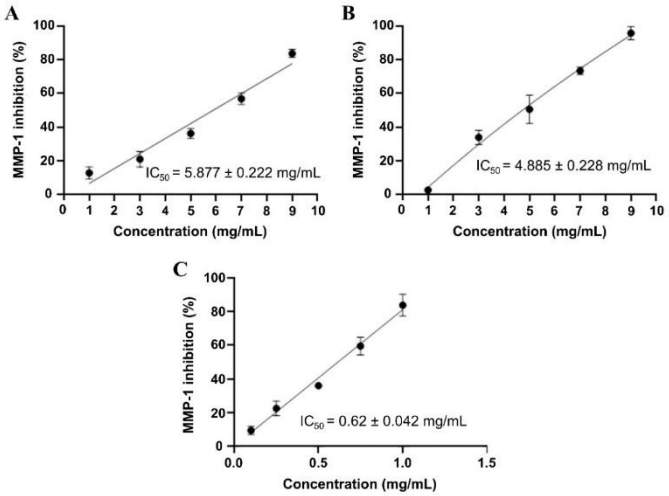

Figure 9: Anti-MMP-1 activity of earthworm extracts AC1:2 (A) and AC1:4 (B), compared to positive control EGCG (C).

The mechanism of MMP-1 inhibition by *P. excavatus* extracts likely involves the chelation of the enzyme's catalytic zinc ion, interference with the substrate-binding region, or modulation of enzyme conformation.[26] The diverse array of amino acids and other bioactive molecules within the extracts likely acts synergistically to inhibit MMP-1.[26] The differences in inhibitory efficacy between AC1:2 and AC1:4 may reflect variations in the concentration or bioavailability of amino acid components resulting from extraction parameters. Overall, the acetone extracts of *P. excavatus* demonstrate significant MMP-1 inhibitory activity, outperforming other earthworm species studied previously.

Conclusion

The acetone extracts of *P. excavatus*, particularly AC1:4, exhibit significant potential for diverse applications due to their rich protein and amino acid composition, including key components such as aspartic acid, glycine, alanine, leucine, tyrosine, lysine, and histidine. AC1:4 demonstrated superior antioxidant, anti-inflammatory, and enzyme-inhibitory activities, effectively mitigating oxidative stress, inflammation, and age-related skin conditions. Notably, its strong inhibition of elastase, tyrosinase, and MMP-1 highlights its potential as a valuable bioactive ingredient in skincare and anti-aging formulations. These findings support the feasibility of utilizing *P. excavatus* extracts as a sustainable and natural alternative to synthetic compounds in pharmaceuticals, nutraceuticals, and cosmetics. This study provides a foundation for further research into the industrial applications of *P. excavatus*, reinforcing its role as a multifunctional and eco-friendly bioactive resource.

Conflict of Interest

The authors declare no conflicts of interest.

Author's Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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