

Antimicrobial Peptides(AMPs) Isolated from Coelomic Fluid, Cells and Tissues of Earthworms as a Source of Potential Therapeutic Agents.

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

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**DEPARTMENT OF BIOTECHNOLOGY
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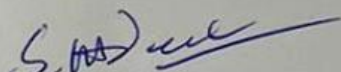
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This is to certify that this Project Report is the bonafide work of **Manasi K.A (40230024)** and **Amisha Baruah (40230002)** who carried out the project "**Antimicrobial Peptides(AMPs) Isolated from Coelomic Fluid, Cells and Tissues of Earthworms as a Source of Potential Therapeutic Agents.**" Under our supervision from **August 2023 to April 2024.**


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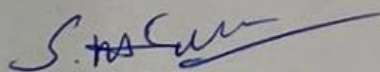

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DECLARATION

We, **Manasi K.A (40230024)** and **Amisha Baruah (40230002)** hereby declare that the Project Report entitled **“Antimicrobial Peptides(AMPs) Isolated from Coelomic Fluid, Cells and Tissues of Earthworms as a Source of Potential Therapeutic Agents”** done by us under the guidance of **Dr. S. Usha Nandhini** and **Dr. S Johnson Retnaraj Samuel** , at **International Research Centre, IRC** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in **Biotechnology**.

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ACKNOWLEDGEMENT

We are pleased to acknowledge our sincere thanks to the **Board of Management** of **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** for their kind encouragement in doing this project and for completing it successfully. We are grateful to them.

We convey our thanks to **Dr. V. RAMESH KUMAR, M. Phil., Ph.D.**, and Head of the Department of Biotechnology for providing us the necessary support and details at the right time during the progressive reviews.

We would like to express our sincere and deep sense of gratitude to our Internal Project Guide **Dr. S. USHA NANDHINI, Ph.D.**, for her constant support and encouragement during the advancement of our project work.

We are extremely grateful to **Dr. S JOHNSON RETNARAJ SAMUEL , Ph.D.**, for his valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of our final year project work.

We wish to express our thanks to all **Teaching and Non-teaching staff members** of the Department of Biotechnology who were helpful in many ways for the completion of the project.

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ABSTRACT

Earthworms possess remarkable antimicrobial properties that shield them from infections during the regeneration process, despite residing in the microbially diverse environment of soil. These properties are attributed to the production of a diverse array of antimicrobial peptides (AMPs), which have demonstrated effectiveness against a wide range of pathogens, including bacteria, fungi, and viruses. Antimicrobial peptides (AMPs) represent an essential component of the innate immune system in various organisms, including earthworms (Annelida: Oligochaeta). Earthworm-derived AMPs have gained significant attention in recent years due to their potent antimicrobial activity, broad-spectrum efficacy, and low propensity for inducing microbial resistance. The isolation of these AMPs from earthworms involves extracting them from the coelomic fluid, a fluid that circulates within the body cavity of the earthworm. The AMPs can be specifically isolated from coelomocytes, the cells present in the coelomic fluid. The isolation process entails further processing of active tissues to obtain the AMPs. Techniques such as liquid chromatography and gel electrophoresis are employed for separation and purification. The primary aim of this research is to explore the earthworm's innate immune response and identify AMPs with potent antimicrobial properties. By utilizing extraction and purification techniques, the study aims to isolate these peptides for subsequent functional assays. These functional assays assess the AMPs effectiveness against various pathogens, thereby evaluating their potential as therapeutic agents. Preliminary results indicate the successful isolation of several AMPs from the coelomic fluid of earthworms, with promising antimicrobial activity against a range of pathogens including bacteria and fungi. Further characterization and optimization of the isolated peptides will contribute to the development of novel antimicrobial therapies with potential applications in medicine and biotechnology.

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ABBREVIATION

APS	Ammonium persulphate
AMP	Antimicrobial peptide
CCF	Coelomocytes cytolytic factor
CF	Coelomic Fluid
AVPF	Antibacterial Vermipeptides Family
NAMP	Non-regenerated antimicrobial peptide
RAMP	Regenerated antimicrobial peptide
G-90	Glycolipoprotein Combination
EFE	Earthworm Fibrolytic Enzymes
PSR	Pattern Specific Recognition
PAMP	Pathogen Associated Molecular Patterns
TLR	Toll like receptors
LRR	Leucine-Rich Repeat
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
PBS	Phosphate buffered saline
HSP	Heat shock proteins
LC-MS	Liquid Chromatography- Mass Spectroscopy
ASP	Ammonium sulfate precipitation
CLSI	Clinical and Laboratory Standards Institute

APS	Ammonium persulphate
EUCAST	European Committee on Antimicrobial Susceptibility Testing
BSA	Bovine serum albumin
RGCB	Rajiv Gandhi Centre for Biotechnology
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight mass spectrometry
RP-HPLC	Reverse phase High-performance liquid chromatography
OD	Optical density
MIC	Minimum Inhibitory Concentration
PRV	Pseudorabies Virus
API	Active pharmaceutical ingredient
DIZ	Diameters of inhibition zone
CPE	Cytopathic Effect
HPLC	High-performance liquid chromatography

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1. INTRODUCTION

Earthworms are members of the Annelidae family of Lumbricidae, a class of oligochaetes that comprised more than 8000 species and emerged towards the end of the Precambrian. They are made up of coelomate organisms with many body compartments. The interior organs of each segment were enclosed by thin transverse walls, or septa, and were filled with a coelomic fluid; most body segments had a few small bristles, or setae, on the outside (Sims & Gerard, 1985).

As terrestrial creatures, earthworms were crucial for preserving the strength and health of soil ecosystems (Blouin et al., 2013). They played a pivotal role in decomposing organic matter, aerating soil, and cycling nutrients, all of which contributed to the fertility of terrestrial settings. Due to their segmented bodies and specialized features like muscle hydrostats and setae, earthworms were incredibly effective at burrowing through soil (Baskar et al., 2023). Their burrowing efforts improved the structure of the soil, facilitating water infiltration, root formation, and stimulating microbial activity. Additionally, organic matter was broken down into smaller particles by earthworm consumption, making it more accessible to other soil organisms (Le Bayon et al., 2021). Earthworms continuously produced channels that enhanced soil porosity, drainage, and overall quality through their digging and feeding activities. Earthworms and other invertebrates had different immunological defenses against infections. These mechanisms, similar to the immune system of vertebrates, were carried out by cellular and humoral immunity. Living in the soil made them vulnerable to various infections, against which earthworms had incorporated various defense mechanisms to keep themselves healthy. Earthworm's innate immunity was maintained by their cell components, namely several types of coelomocytes, which were found in the coelomic cavity and resembled leukocytes (Engelmann et al., 2018). Coelomocytes were freely mobile cells found in the

coelomic fluid of earthworms, and the potential of this compartment had not been evaluated for its utility in earthworm metabolomics (Griffith et al., 2013). Coelomic fluid, a clear, colorless liquid, was found in the coelomic cavity surrounding the internal organs of earthworms, providing protection and support. There were also many other immunologically active (microbial) compounds in the fluid of the xylem cavity (CF), including lysenin, fetidin, eiseniapor, coelom cytolytic factor (CCF-1), lumbricin I, etc. (Engelmann et al., 2018).

Earthworms possessed a diverse range of adaptations that enhanced their ability to withstand and overcome the various obstacles found in soil habitats. Despite being less sophisticated than those of vertebrates, their immune systems were nevertheless capable of fending off infections and preserving the health of their environment (Hendrix et al., 2006). Their thick, mucus-coated skin, which served as a physical barrier against dangerous pathogens and hazardous compounds found in the soil, was a crucial component of their immunity (Wotton, 2004). Furthermore, earthworms had a complex network of coelomic fluid that included pathogen-neutralizing enzymes and antimicrobial peptides. This liquid was essential for digestion as well as a vital line of defense against invasive microbes. In addition, earthworms had exceptional regeneration capabilities that enabled them to promptly restore any tissue damage brought on by infections or physical stress (Cremaldi & Bhushan, 2018). The various microbial communities found in the soil also had an impact on their immune responses; particular symbiotic bacteria offered defense against dangerous infections.

Antimicrobial peptides have become increasingly popular in recent times. They served as the first line of defense against microbial invasion, working in tandem with the host's humoral and immunological systems. Since earthworms and other invertebrates did not produce any specific antibodies, their host defense against microbial attacks was given by intrinsic processes. Because invading bacteria threatened the earthworm's existence, they had developed a robust defensive

mechanism. AMPs were elements of the innate immune system and served as the initial line of defense (de Paula & Valente, 2018). When AMPs interacted with molecules in the cytoplasm and membrane lipids, they were known to exhibit great selectivity and specificity, which influenced cellular metabolism. Antimicrobial peptides (AMPs) were small innate immune molecules that targeted bacteria, yeasts, fungi, and viruses with a broad spectrum of antibiotic actions. Additionally, they had anti-inflammatory and immunomodulatory effects, as well as cytotoxic effects on cancer cells (Zhang et al., 2023). Both lower and higher species generated antimicrobial peptides in reaction to pathogenic threats. These peptides had two functions: they suppressed invasive infections and altered the innate immune system. Their origins, compositions rich in amino acids, structural characteristics, and functional activities were frequently used to categorize them (Pasupuleti et al., 2011). Up until then, more than 3300 different AMPs had been discovered in a variety of species, such as humans, plants, insects, and other biological sources (Moretta et al., 2021).

1.1 EARTHWORM'S IMMUNE SYSTEM

Earthworms were subject to numerous studies. Their immunological response vastly relied on specialized cells called coelomocytes found in coelomic fluid (Tučková et al., 2018). Further research had revealed that these cells were crucial for the immune system, waste elimination, and homeostasis of these creatures (Hirayama et al., 2017). Coelomocytes were crucial for wound healing, regeneration, and repair. For the first time in earthworm history, research found that arginase activity in coelomocytes may have been up-regulated in response to immunological activation or infection, in the presence of Mn^{2+} ions, and during the healing process after segment amputation (Homa et al., 2007). It was also revealed that Calcium ions played an essential part in the activation of coelomocytes by cell signaling (Oppen et al., 2010). The cellular stress response, which involved the activation of metallothioneins (MTs), which are bound to hazardous compounds like metal, was

focused on coelomocytes. The combination of the heavy metal and the protein was then trapped inside lysosomes for elimination (Höckner et al., 2020). The segmented anatomy and touch-protective cuticle of earthworms enabled them to fend for themselves. Through the production of a protective mucous layer, the cuticle, a non-cellular layer covering the body, demonstrated the ability to endure even toxins like heavy metals (Pasupuleti et al., 2011).

Earthworm's hydrostatic skeleton included coelomic fluid, which served as a conduit between the interior and outside environments, maintained homoeostasis, and was home to a profusion of coelomocytes, which were immune-competent cells (Affar et al., 1998). Coelomic fluid (CF) was made up of several molecular constituents and cells that were involved in innate immunity. Glycoproteins with lectin characteristics attached to carbohydrates and immobilized and destroyed foreign material by lysis and agglutination (Kauschke et al., 2007). AMPs were crucial anti-infectious elements that had been maintained throughout evolution. Their effects on immune systems, however, varied according to the species, cells, and tissues. The strategic placement of AMPs in phagocytes, bodily fluids, and epithelial level, that is, at the interfaces where an organism meets its environment maintained their role in natural resistance to infection. This activity was reinforced in animals or plants confronted with bacteria by the quick expression of such AMP genes (Zasloff, 2002). In previous research conducted, earthworms were utilized to extract a new peptide with antioxidant properties that might protect cells from oxidative stress while also stimulating immunological lymphocyte development. As a result, it may have potential as a therapeutic agent for treating human diseases such as liver cancer (Wasunan et al., 2023). Antibiotics targeted specific biological processes, whereas AMPs targeted the lipopolysaccharide layer of the cell membrane, which was found in all bacteria. AMPs did not target eukaryotic cells because of their low anionic charge and large cholesterol content (Gao et al., 2022).

Earthworm's defense system against bacteria and other diseases required the presence of certain active antibacterial proteins and peptides, which differed from immunoglobulin(Ig) (Qiao et al., 2007). When exposed to pollutants, including nanoparticles, the earthworm immune system triggered a cellular signaling cascade by releasing a number of substances, including cytotoxic agents, antimicrobial peptides, and PAMPs (pathogen-associated molecular patterns) routes involving serine proteases.

1.2 AMPS

Earthworms are long, tubular, worm-like structures that range in length from 10 mm to 7 meters. They were classified under the class Oligochaeta under the phylum Annelida. Earthworms were essential for the formation of soil and the deterioration of soil organic matter. Around 80% of the diversity of soil fauna was created by earthworms (Kale & Karmegam, 2013). They were highly vulnerable to several different pathogens that could lead to infection and have a detrimental impact on the host organism. An important function of AMPs was to shield earthworms from microbial infection and contaminated soil. Lumbricin-1, the first annelid AMP, was identified in 1998 (Cho et al., 1998) from the earthworm *Lumbricus rubellus* and subsequently in leeches (Schikorski et al., 2008). Theromacin, the first member of the macin family, was initially identified in leeches in 2004 (Tasiemski et al., 2004). Among annelids, another class of AMPs rich in cysteine was identified as the first member of the BRICHOS AMP family (Bri2, CHOndromodulin, and proSurfactant protein C). It was arenicin, which was extracted from *Arenicola marina*'s bodily fluid in 2004 (Ovchinnikova et al., 2004). Hedistin, another novel antibacterial peptide, was discovered in *Nereis diversicolor* coelomocytes. Hedistin was the first antimicrobial peptide containing bromotryptophans shown in annelids and bore no clear similarity to other known peptides (Tasiemski et al., 2007). The earthworm *Pheretima guillelmi* (Michaelsen) skin secretions included a unique lumbricin-like antibacterial peptide known as lumbricin-PG (Li et al., 2011). A cDNA encoding a potential antibacterial

peptide (called PP-1) was produced via rapid amplification of cDNA ends from the Asian earthworm, *Pheretima tschiliensis* (Wang et al., 2003). EP5, a new class of antibacterial vermipeptides (Wang et al., 2007), and a novel peptide, OEP3121 derived from *Eisenia fetida* (Liu et al., 2004).

In this project, our objective was to identify earthworm-derived antimicrobial peptides (AMPs) with potent antibacterial activity against a range of diseases, including those resistant to conventional antibiotics. These AMPs would be extracted and characterized by us. Our investigation centered on the specific mechanisms via which these AMPs exerted their antimicrobial effects, including their interactions with microbial membranes, their ability to impair membrane integrity, and their influence on intracellular activity in pathogens. Our goal was to learn more about the potential therapeutic uses of these isolated AMPs, including their bioavailability and stability, their toxicity to mammalian cells, and how well they worked in animal infection models. Based on these findings, we wanted to synthesize novel antimicrobial medicines from earthworm AMPs that might serve as effective treatments.

2. LITERATURE SURVEY

The research paper explores the challenges of bacterial drug resistance and the potential of antimicrobial peptides (AMPs) in overcoming these challenges. It discusses the mechanisms of action of cationic AMPs, their broad-spectrum antimicrobial activities, and the need to develop synthetic and long-lasting AMP analogs for clinical applications. Cationic AMPs, with their positively charged and amphiphilic properties, are found to interact with bacterial cell membranes, leading to changes in the electrochemical potential, cell membrane damage, and ultimately cell death. These AMPs have shown advantages over traditional antibiotics, including broader antimicrobial activities, safety, and resistance to drug resistance. However, the paper also addresses the need to address potential issues such as stability, side effects, and immunogenicity. The challenge and prospective of antimicrobial peptides in clinical applications are discussed, emphasizing the importance of understanding the mechanism of action and the relationship of structure-activity in developing new cationic peptide drugs.

2.1 HISTORY OF EARTHWORM'S HEALING PROPERTIES

Throughout history and across different cultures, earthworms were utilized in traditional healing practices. Their extract, known for its antioxidant and rheumatism-relieving properties, was employed in treating various ailments such as jaundice, bladder stones, fever, and smallpox. In Burma, earthworms played a significant role in managing medical conditions like "ye se kun byo," resembling symptoms of pyorrhea (Gates, 1925). These earthworms underwent heating in a sealed container until reduced to ashes, which were then either used alone as a dental powder or blended with roasted tamarind seeds and betel nuts for enhanced flavor (Wangkheimayum et al., 2022). For "meephwanoyeekhun thwaykhan," a condition often experienced by postpartum women causing weakness and hindering

breastfeeding, worms were boiled with salt and onions. The resulting clear liquid was separated and added to the patient's meals (Cooper et al., 2004). In the past, the Maori people cooked earthworms in water heated by hot stones, storing them in gourds, while Australian aborigines incorporated earthworms and insects into their diets as supplementary nutrition sources (Paoletti et al., 2000). Native American accounts, documented by Carr, described Cherokee Indian's use of earthworm poultices to extract thorns by finely chopping potent worms. Among the Nanticoke Indians of Delaware, earthworms were recognized as a remedy for relieving rheumatism-related pain. Despite the unpleasant odor, individuals suffering from this ailment would place earthworms in a container and apply them to stiff joints, resulting in some degree of pain relief (Jaafar et al., 2022). In Iran, earthworms were acknowledged as a potent therapeutic solution. Baked earthworms were consumed with bread to alleviate bladder stones, which were subsequently eliminated after the meal. Additionally, dried earthworms were ingested to treat jaundice-related skin yellowing. It was also reported that earthworm ashes, when applied to the scalp with rose oil, could promote hair regrowth in cases of alopecia (Bristowe, 1932).

2.2 EARTHWORM'S ANTIMICROBIAL ACTIVITY

Antimicrobial peptides (AMPs) are natural antibiotics produced by all living organisms, serving as host defense and aiding in the selection of colonizing bacterial symbionts. AMPs shared common structural characteristics but differed in coding sequences due to multiple parallel evolution. Most AMPs were specific to certain taxa, such as annelids, which had adapted to extreme environmental conditions. A review explored the diverse structures and functions of AMPs in marine annelids, highlighting their potential as antibiotics (Bruno et al., 2019). Earthworms had been recognized as a significant protein source and verified to contain a substantial protein content comprising 78-79 grams of free amino acids per liter (Zeibich et al., 2018). Additionally, they exhibited a notable concentration of essential vitamins and minerals, with a particular emphasis on iron (Fe) and calcium

(Ca) (Augustine et al., 2017). Regarding the underlying mechanisms, information was somewhat limited, but biochemists suggested that earthworm lipids containing fatty acids played a pivotal role in this therapeutic process. Furthermore, on a different note, other researchers identified a substance within earthworms that could dilate bronchial passages, aligning with reports of earthworm extracts capable of disintegrating blood clots (Cheng et al., 1996). Antimicrobial peptides were essential components of the innate immune defense mechanism used by both prokaryotic and eukaryotic organisms. They varied in amino acid composition, antimicrobial specificities, hemolysis, cytotoxicity, and mechanisms of action (Han et al., 2011). These peptides had been isolated from various animals, including vertebrates, invertebrates, plants, bacteria, and fungi. They initially interacted with microbes through lipid binding and exhibited broad-spectrum activity against various microorganisms. Some peptides had been found to be cytotoxic to sperm and tumor cells. A review discussed antimicrobial peptides isolated from marine invertebrates, which may have led to novel strategies for preventing bacterial infections in animals (Daphny, 2015).

2.3 ANTIMICROBIAL PEPTIDES PREVIOUSLY ISOLATED FROM EARTHWORMS

2.3.1 Lumbricin

The identification of lumbricin and a lumbricin-related mRNA sequence in *Eisenia andrei* earthworms has been reported. These peptides were discovered in the proximal part of the intestine, exhibiting the highest expression levels. The mRNA sequences for lumbricin and its related peptide in *E. andrei* consisted of 477 and 575 nucleotides, respectively. Phylogenetic analysis indicated a close relationship with other annelid *lumbricins*. The expression of these mRNAs increased continuously during embryo development. Following a 48-hour challenge

with Gram-positive bacteria, both mRNAs were significantly elevated in coelomocytes, while stimulation with Gram-negative bacteria or zymosan had no noticeable effects (Bodó et al., 2019).

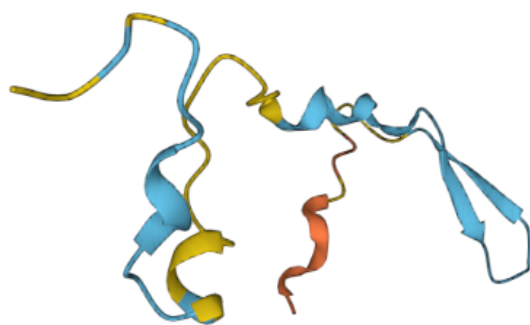


Fig 2.1. Lumbricin structure from uniprot database

2.3.2 AVPF

Six antimicrobial peptides were previously extracted and purified from earthworm tissue and coelomic fluid, collectively forming the antibacterial vermipeptides family (AVPF). Their preparation involved various methods, including ultrafiltration, ion-exchange chromatography, gel-filtration, and HPLC chromatography. AVPF exhibited broad-spectrum antimicrobial properties against both Gram-positive and Gram-negative bacteria, as well as fungi. Specific peptides such as EP3 and EP5 from AVPF were noted for their resistance to cancer cells

(HeLa and MGC803), leading to apoptosis in HeLa cells and inducing morphological changes in MGC803 cells. Short peptides consisting of 5–7 amino acid residues were observed to form multimers in the PB buffer, potentially elucidating their antibacterial mechanism. Additionally, EP5 demonstrated antiviral effects against the pseudorabies virus (PRV) by inhibiting viral DNA duplication, as evidenced in the cytopathic effect (CPE) inhibition test on BHK cells (Wang et al., 2007).

2.3.3 Perinerin

Perinerin, a novel antimicrobial peptide extracted from the Asian marine clamworm, *Perinereis aibuhitensis* Grube, was previously isolated and characterized. This peptide, consisting of 51 amino acid residues, was purified using heparin-affinity column and reverse-phase HPLC methods. Perinerin demonstrated significant antimicrobial activity against both Gram-negative and Gram-positive bacteria as well as fungi, indicating bactericidal properties. Its distinctive structure, characterized by high basicity and hydrophobicity, distinguished it from other known antimicrobial peptides. The novelty of Perinerin and its consistent presence suggested its potential as a valuable marker for studying host biochemistry changes in response to environmental variations. Additionally, the MTS-PMS colorimetric assay, employed to evaluate Perinerin's antimicrobial efficacy, appeared to be a promising method, potentially surpassing existing techniques and offering broader applications in the search for new antibiotic compounds (Pan, 2004).

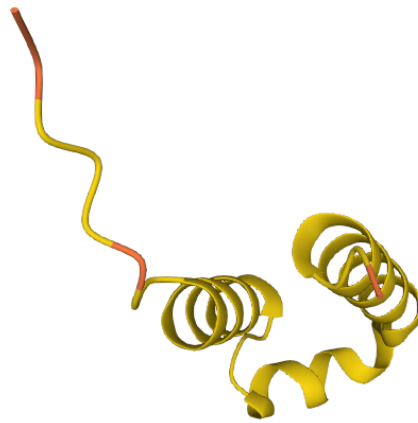


Fig 2.2. Perinerin structure from uniprot database

2.3.4 Lumbricin-PG

Lumbricin-PG, a newly discovered antimicrobial peptide similar to lumbricin, had been isolated from the skin secretions of the earthworm *Pheretima guillelmi*. The peptide was extracted using Sephadex G-50 gel filtration and C8 reverse-phase high-performance liquid chromatography (RP-HPLC). The cloned cDNA sequence revealed a protein of 73 amino acid residues, consisting of the mature lumbricin-PG and a predicted signal peptide, showing similarities to lumbricin from *Lumbricus rubellus* in a BLAST search. Lumbricin-PG exhibited antimicrobial activity against bacteria and fungi, with mild hemolytic effects on human and rabbit red blood cells (Li et al., 2011).

2.3.5 PP-1

PP-1, a putative antimicrobial peptide, was identified from the Asian earthworm, *Pheretima tschiliensis*, through cDNA cloning using rapid amplification of cDNA ends. PP-1 showed a 77.6% homology with lumbricin I, an antimicrobial peptide from *Lumbricus rubellus*, and notably lacked a distinct signal peptide sequence. Gene expression analysis revealed predominant expression of PP-1 in the body wall of the earthworm. PP-1 was successfully expressed in *Escherichia coli* as a fusion protein with a maltose-binding protein. Subsequently, a polyclonal antiserum against PP-1 was generated in mice using the recombinant fusion protein as an antigen. Immunohistochemical investigations indicated that PP-1 was specifically localized in the mucus of the epidermis (Wang et al., 2003).

2.3.6 EWAMP-R

In another study, a novel antimicrobial peptide named EWAMP-R was discovered through genome sequencing of earthworm species, predicting potential AMPs in *Eisenia andrei*'s genome and optimizing sequences. EWAMP-R showed potent antibacterial activity against various bacteria, including drug-resistant strains. Molecular simulations and wet-laboratory experiments revealed that EWAMP-R targeted bacterial membranes differently in Gram-negative and Gram-positive bacteria, disrupting membrane integrity. In *Escherichia coli*, EWAMP-R could penetrate the membrane at low concentrations without direct damage, suggesting a secondary response. Gene expression studies indicated activation of the apoptosis-like cell death (ALD) pathway in *E. coli* and upregulation of the MazEF pathway in *Staphylococcus aureus*. These findings shed light on the structure-activity relationship of AMPs and offered insights for designing AMPs with enhanced specificity. EWAMP-R represented a promising source of antibacterial agents to combat antibiotic resistance (Wu et al., 2023).

2.3.7 Hedistin

Hedistin, a novel antimicrobial peptide discovered in the coelomocytes of *Nereis diversicolor*, stood out for its uniqueness among known peptides and was the first annelid peptide to feature bromotryptophans. Upon bacterial challenge, hedistin was secreted post-processing from a precursor with a signal peptide and prosequences, as revealed by cDNA and mass spectrometry analysis. This peptide exhibited broad-spectrum antibacterial activity, including effectiveness against methicillin-resistant *Staphylococcus aureus* and *Vibrio alginolyticus*. Hedistin's gene expression was constitutive and exclusive to circulating NK cells, essential for the sand worm's immune response. This contrasted with leeches, where antimicrobial peptide genes were upregulated in specific tissues and peptides were rapidly released into the hemolymph post-septic injury (Tasiemski et al., 2007).

2.3.8 Theromacin and theromyzin

In previous studies, two novel antimicrobial peptides, theromacin and theromyzin, were discovered and isolated from the coelomic fluid of the leech *Theromyzon tessulatum*. Theromacin, a 75-amino acid cationic peptide with 10 cysteine residues, and theromyzin, an 86-amino acid linear peptide, both demonstrated activity against Gram-positive bacteria. They were expressed in large fat cells and were observed to be rapidly released after bacterial challenge (Tasiemski et al., 2004).



Fig 2.3. *Theromacin and theromyzin* from uniprot database

2.3.9 OEP3121

A novel antimicrobial short peptide, OEP3121, was purified from earthworm *Eisenia foetida* using a five-step protocol. The peptide was analyzed using MALDI-TOF MS and TOF MS-MS to determine its molecular mass and amino acid sequence. The resulting peptide, with a molecular mass of 510.8 Da and an amino acid sequence of "ACSAG", was identified (Liu *et al.*, 2004).

2.4 STUDIES CONDUCTED ON EARTHWORM IMMUNITY AND DEFENSE MECHANISM

Previous studies investigated the antibacterial and antifungal properties of earthworms, which play crucial roles in soil ecosystems by scavenging and recycling dead plant material. A paste derived from earthworm *Eudrilus eugeniae* underwent testing for its antimicrobial activity. Four species of bacterial isolates and two species of fungal isolates were chosen for screening. The minimum inhibitory concentration (MIC) was determined using the microdilution broth method. A dose of 100 µl of earthworm paste inhibited the growth of *S. aureus* bacteria to a maximum extent, while it moderately inhibited the growth of *Candida albicans*. The MIC results showed that a dose of 200 µl of earthworm paste inhibited bacterial growth, suggesting the potential for the development of a new antimicrobial drug. The antimicrobial activity of the paste was assessed using an agar diffusion method with wells and discs (Chauhan et al., 2014). A study conducted between May 2017 and November 2017 at the School of Environmental Science and College of Veterinary and Animal Science examined the antibacterial properties of coelomic fluid from local earthworms (*Pontoscolex corethrurus*, *Megascolex konkanensis*, *Drawida ghatensis*) against selected pathogens such as *Vibrio cholera*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*. The coelomic fluid showed maximum inhibition zones of 18 mm and 16 mm against *S. aureus*, while *Drawida ghatensis* exhibited the least antibacterial activity. *Staphylococcus aureus* showed the highest inhibition zone against coelomic fluid, with the least impact observed in the bacterial isolate *vibrio parahaemolyticus*. The study concluded that coelomic fluid activity is effective against pathogens like *salmonella typhi* and *Escherichia coli* and holds medicinal value (Prakash & Gunasekaran, 2011). The study aimed to explore the antibacterial properties of earthworm coelomic fluid, assess its antimicrobial activity, and determine its zone of

inhibition against various bacterial strains. The agar disc diffusion method was used to evaluate the fluid's antimicrobial activity. Significant inhibition of bacterial growth against the tested organisms was observed, suggesting that coelomic fluids could be utilized to discover bioactive natural products, potentially leading to new pharmaceutical research activities (Hatti & Ramkrishna B, 2011). Studies on earthworms led to the discovery of a defense system in their coelomic fluid, comprising different proteins and peptides responsible for their antibacterial and antifungal activities. A study tested the whole tissue extract of earthworm *Eudrilus eugeniae* using a well diffusion method. Nine species were selected for antimicrobial screening and two for antifungal screening. A dose of 10 mg/ml of earthworm paste inhibited bacterial growth, with the maximum antibacterial activity observed against *Pseudomonas aeruginosa*. The extract did not inhibit *Streptococcus pyrogenes*, *Enterococcus faecalis*, *Enterobacter aerogenes*, and *Escherichia coli*, and no antifungal activity was observed against *Candida albicans* and *Aspergillus niger*. The total protein concentration was 70 mg/100 gm (Chauhan et al., 2014). Another study analyzed the effects of earthworm antimicrobial peptides combined with probiotics on yellow-feathered broiler diets. The results indicated that the control group had a higher number of species, while the compound probiotic preparation group had a lower number of harmful bacteria. Firmicutes, Bacteroides, and Proteobacteria were the dominant bacterial groups in each group. The relative abundance of Firmicutes, Bacteroides, and *Lactobacillus* was higher in the compound probiotic preparation group, suggesting that earthworm antimicrobial peptides combined with probiotics could serve as new green antibiotics in animal production (Yan et al., 2022). A new tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), was used to assess antimicrobial activity in earthworms. The salt was purified from the coelomic fluid of the earthworm (ECF) using column chromatography techniques. The activity was tested against three Gram-negative strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and three Gram-positive strains of *Staphylococcus aureus*, *Bacillus megaterium*, and *Arthrobacter sp.* The pigment-free eluate of ECFPE showed activity against *Bacillus*

megaterium, while the anion exchange effluent of ECFPE had the strongest activity against *P. aeruginosa* (Dahiya & Pathak, 2007). Additionally, a polypeptide from the earthworm *Lumbricus terrestris*, similar to defensin, was synthesized to enhance neuronal cell proliferation. Lumbricusin treatment increased the proliferation of human neuroblastoma SH-SY5Y cells by approximately 51%, inhibited apoptosis, and decreased viability induced by 6-hydroxy dopamine, a Parkinson's disease-mimicking agent, while increasing ubiquitination of p27Kip1 protein. However, over-expression of p27Kip1 blocked the antiapoptotic effect of Lumbricusin in 6-hydroxy dopamine-treated SH-SY5Y cells. The study suggests that the promotion of p27Kip1 degradation may be the main mechanism underlying the neuroprotective and neurotropic effects of Lumbricusin (Kim et al., 2014).

3. AIM AND SCOPE

3.1 AIM

To isolate and identify the AMPs from earthworms and understand their Antimicrobial, Anti-inflammatory and cell cytotoxicity activities.

3.2 SCOPE

- To isolate the AMPs from the coelomic fluid and the coelomocytes of the earthworms.
- To obtain low molecular weight AMPs upto 3kDa.
- To separate the various peptides using Tricine SDS-PAGE
- To identify and quantify the peptides using LS-MS technique.
- To show the Antibacterial action of AMPs against gram-negative and gram-positive bacteria.
- To check the Anti-inflammatory activity of the AMPs.
- To analyze the cytotoxicity of the AMPs

4. MATERIALS AND METHODS

4.1. MATERIALS REQUIRED

Earthworms of the species (*Perionyx excavatus*) bought from Chennai, were used in the project for the purpose of isolating the Antimicrobial Peptides. Tricine SDS PAGE apparatus by Medox was used. 3 kDa pierce protein concentrators were used as molecular cut-off filters for isolating the peptides of small sizes. Other than these materials, all chemicals used for the study were analytical grade purchased from Sigma-Aldrich, HiMedia, and Merck.

4.2. ISOLATION OF PROTEIN FROM EARTHWORMS

4.2.1. Collection of Earthworms

Perionyx excavatus are up to 2.75 inches in length and purple and red or brown in colour. These earthworms need to be properly identified from the storage bucket, to avoid mixing of other earthworms which might lead to an inaccurate protein sample. We collected two different types of samples from the earthworms. With the intention of understanding how the production of the AMPs during regeneration we used both regenerated and non-regenerated earthworms for our protein isolation.

While collecting the earthworms we need to make sure that the earthworms are long and are not under any sort of stress, for instance they should not have any injuries or cuts in their body which will stress them out too much and hinder their survival outside the soil. The earthworms need to be healthy and active. The collected worms should properly be washed with both tap water and distilled water.

4.2.1.1. Non-regenerated worms

For the purpose of isolating NAMP or Non-regenerated AMPs we need to collect about 50 earthworms and wash them properly to remove any dirt sticking onto the earthworms. Once the worms are thoroughly washed and cleaned, the worms are kept in a box of shredded and dampened tissues overnight for gut cleaning in order to empty their digestive tracts. This process encourages them to expel any remaining gut contents. After the purging process, the earthworms were rinsed again with clean water to remove any residue from the purging material. The earthworms were transferred to a beaker with fresh, clean water for a final rinse. This ensures that they are completely clean before proceeding with protein isolation.

4.2.1.2 Regenerated worms

Regeneration in earthworms can typically be initiated by amputating a portion of their body and monitoring the regrowth of tissues. Around 50 healthy earthworms are again collected. Ensure they are well-hydrated and free from any signs of stress or injury. A clean and sterile workspace is set to minimize the risk of contamination and infection. Any necessary equipment, such as a cutting board, scalpel blade, and 70% Ethanol is taken. In order to minimize their stress and discomfort during the amputation process the worms are immersed briefly in water to relax them.

Using a scalpel blade, the worm is amputated at the 10th segment right before the clitellum, typically regeneration is known to occur more effectively here. It is ensured that the amputation is performed cleanly and precisely to minimize trauma to the earthworm. The worms are kept in moist soil which composes of a mixture of organic matter (such as compost or leaf litter) and soil with good drainage and aeration is ideal, in a container with lid or cover to create a controlled environment for the earthworms. It is also ensured that the containers have adequate drainage holes to prevent waterlogging.

4.2.2. Preparation of protein sample

The worms are taken from their respective containers and rinsed once to remove tissue or soil. These worms are then gathered in a beaker or a pestle. It's important to ensure that they are healthy and free from any signs of stress or injury. The electrodes are positioned appropriately, ensuring that they contact the earthworm's body. Two electrodes are used, with one placed at the anterior end and the other at the posterior end of the earthworm. Electric current of about 9V is applied on the worms for a period of 3 secs. This is repeated for about 10-15 times until a yellow semi-viscous fluid is released. The worms are carefully taken out of the beaker and kept in a container with soil. These worms are further maintained until they revive back to their healthy state. Once the coelomic fluid is collected, acidic medium is added to adjust the pH of the solution to an acidic range (usually pH 4-5). This acidic medium is composed of 1% Trifluoroacetic acid, 1M HCl, 5% Formic Acid, 1% NaCl and 0.1% PMSF. The acidic conditions will cause proteins to denature and precipitate out of solution. The coelomic fluid is crushed to turn it in a semi- liquid state or by gently aspirating the fluid using a micropipette and then the fluid is carefully collected.

Once collected, the coelomic fluid is processed further for analysis or experimentation. This involves centrifugation to remove debris or cellular components, for about 60 mins at about 10,000 RPM. It needs to be noted that the centrifuge must be pre-cooled to 4°C before placing the coelomic fluid sample in it. The heavier components, such as cellular debris and organelles, will settle at the bottom, forming a pellet, while the lighter components remain in the supernatant. A pipette is used to collect the supernatant (the liquid portion) from the top of the tube, being careful not to disturb the pellet at the bottom. The supernatant is the part we want for our experiment, but the pellet can collected for further processing

Moving further protein or peptide can be isolated from the above sample by two methods:

4.2.2.1 Ammonium Sulfate Precipitation Method

Ammonium sulfate precipitation is a commonly used technique for protein isolation and purification. It involves the selective precipitation of proteins from solution ammonium sulfate. The appropriate level of ammonium sulfate saturation needed is determined for protein precipitation. This can be achieved through various methods, such as referring to protein solubility tables or conducting pilot experiments. A solution of Ammonium sulfate is prepared by estimating the saturation of ammonium sulfate, we took 10 ml of 75% Ammonium sulfate solution. It is kept in a magnetic stirrer until the solution gets completely dissolved without any residue. Once the solution gets completely dissolved the protein sample is added to it. And this is kept on a magnetic stirrer overnight at 4°C.

The proteins of interest will gradually begin to precipitate out of solution while other contaminants remain soluble. After complete saturation, the solution is centrifuged at a high speed, 4000 rpm for 30 mins to pellet the precipitated proteins. This separates the precipitated proteins from the rest of the solution. The supernatant (the liquid portion) is carefully removed and discarded.

The protein pellet is gently resuspended in 1X PBS or Phosphate Buffer Saline. Then to this solution equal volumes of a reducing sample buffer composed of 12% SDS, 6% mercaptoethanol, 30% Glucose, and 150mM Tris base is added.

Then the protein-sample buffer mixture is heated in a dry bath or heat block at 37°C for 15 minutes. This step denatures the proteins by disrupting their secondary and tertiary structures and reduces disulfide bonds, allowing the proteins to unfold and migrate uniformly during electrophoresis. After denaturation and reduction, the

samples are removed from the dry bath and stored in -20°C. It's important to avoid prolonged exposure to high temperatures, as this can lead to protein degradation.

4.2.2.2. Protein extraction without Ammonium sulfate precipitation

While ammonium sulfate precipitation remains a valuable tool for protein purification in many applications, using sample buffers for protein sample preparation can be opted when specific concerns regarding non-specificity, protein activity, scalability, or compatibility arise.

In this method the protein sample is directly mixed with an equal volume sample buffer. This ratio depends on the concentration of proteins in your sample and the desired protein-to-buffer ratio for SDS-PAGE. Generally, a higher concentration of sample buffer is used for more concentrated protein samples. Similar to the previous method this mixture is heated and then stored in -20°C.

4.2.3. Molecular cut-off filter

A 3 kDa cutoff filter can be utilized in the isolation of peptides from earthworms to separate peptides from larger proteins and other molecules that exceed the cutoff size. This method is commonly referred to as size-exclusion or molecular weight-based filtration. The coelomic fluid extracted from the earthworms can be taken.

The Thermo Scientific™ Pierce™ Protein Concentrators are single-use ultrafiltration centrifugal tools designed to deliver dependable and uniform outcomes for the concentration, diafiltration, and buffer exchange processes of biological samples, including proteins and nucleic acids, which were used for our experiment.

Assemble the 3 kDa cutoff filter according to the manufacturer's instructions. This typically involves attaching the filter membrane to a filtration device, such as a

centrifugal filter unit or a vacuum filtration setup. Concentrators may contain trace amounts of glycerin and sodium azide, in order to remove these trace additives, the concentrator can be pre-rinsed. 500 μ L of buffer solution or purified water is added to the sample chamber and the concentrator assembly is placed into the rotor with a proper counterbalance, then centrifuge at $12,000 \times g$ until at least 400 μ L of filtrate is produced. The filtrate and retentate is decanted to obtain clean concentrators.

After Pre-rinsing, the sample is loaded into the Concentrator sample chamber. It is capped and then inserted into the collection tube. It is then centrifuged at 12,000g until the desired concentration is achieved. The counterbalance must be another Concentrator or another centrifuge tube of equal weight with water in it, to ensure proper balance because of changing sample distribution during centrifugation. The centrifugation time is reduced by half for 100 μ L sample volumes. Then the sample is added to the original volume of the dilution buffer composed of 20 mM HEPES, 150 mM sodium chloride, pH 7.5. The last two steps involving centrifuging and adding dilution buffer is repeated until the desired solute removal has been achieved.

The filtrate that passes through the filter membrane is collected, since this fraction will contain peptides and other small molecules with molecular weights below 3 kDa, while larger proteins and contaminants will be retained on the membrane. The retentate is also collected for further analysis.

The isolated peptides are often stored frozen at -20°C or -80°C to prevent degradation, to maintain their stability until further use. However, it's important to note that this method may not provide complete separation of peptides from larger proteins, especially if the sample contains a complex mixture of molecules. Therefore, additional purification steps or complementary techniques may be required for more comprehensive peptide isolation and characterization.

4.3. SEPARATION OF PROTEINS

Tricine-SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) is a variation of the traditional SDS-PAGE method used for separating proteins based on their molecular weight. Tricine-SDS-PAGE is particularly useful for resolving low molecular weight proteins (less than 30 kDa) with higher resolution compared to conventional SDS-PAGE. Since our desired peptides and proteins are of extremely low molecular weight, it is more efficient to use Tricine SDS-PAGE instead of the conventional SDS-PAGE. Tricine is a buffer system used instead of the traditional glycine-SDS buffer in SDS-PAGE. Tricine has a lower buffering capacity compared to glycine, which is beneficial for separating small proteins effectively. Like conventional SDS-PAGE, Tricine-SDS-PAGE utilizes polyacrylamide gels for protein separation. These gels are poured between glass plates and contain a gradient or homogeneous concentration of acrylamide and bis-acrylamide. The gel concentration can be adjusted based on the size range of proteins to be separated.

The Tricine-SDS-PAGE plates are taken and held together using clamps keeping plastic strips between them to create a gap. The bottom strip is made to slightly pop out so that it can be taken out before keeping for electrophoresis. Sealing of Tricine-SDS-PAGE plates with agarose is a common technique used to prevent leaking during the pouring of the resolving gel. 1% agarose gel is prepared by heating the mixture until the agarose is completely dissolved. This can be done using a microwave or a hot plate while stirring intermittently. The agarose solution is allowed to cool slightly but remain in a liquid state.

Clean and dry Tricine-SDS-PAGE plates are taken. The clamps are positioned to create a mold for the gel. Using heat-resistant gloves, a thin layer of agarose is poured along the edges of the assembled gel plates with the help of a micropipette. This seals the plates together and prevents leakage during the pouring of the resolving gel. The agarose is allowed to solidify completely.

Table 4.1. Gel preparation for Tricine SDS-PAGE

	STACKING	INTERMEDIATE	SEPARATING
AB3	0.330 ml	2 ml	3.33 ml
GEL BUFFER	1 ml	3.33 ml	3.33 ml
WATER	2.670 ml	4.67 ml	2.34 ml
GLYCEROL	-	-	1 ml
	4 ml	10 ml	10 ml
APS	100 µl	200 µl	200 µl
TEMED	10 µl	20 µl	20 µl

The gel preparation requires AB3 Buffer and Gel buffer. AB3 buffer or acrylamide-bisacrylamide (AB-3) stock solution is prepared by dissolving 48 g of acrylamide and 1.5g of bisacrylamide in 100 ml of water. These gels are formed by polymerizing acrylamide and bis-acrylamide in the presence of a cross-linking agent. The concentration of acrylamide in the gel can be adjusted to optimize resolution based on the size range of proteins being analyzed. For example, a 10% separating buffer is used for separation of substances with a higher molecular weight. But for our project we require, 16% buffer which is much more capable of separating molecules with very low molecular weight. Gel buffer is composed of 3M Tris-Base, 3% SDS, in a pH of 8.45. 10% APS is also prepared to facilitate the solidification of the gel. Isopropanol is added to each layer of the gel in order to facilitate solidification as well as improving sharpness and resolution of the gel. Its inclusion in the buffer system can lead to clearer and more accurate separation of proteins during electrophoresis.

Ammonium persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED) are commonly used as initiators for polymerization of the polyacrylamide gel matrix. APS can be used as a source of free radicals to initiate the polymerization of acrylamide and bis-acrylamide monomers, forming the polyacrylamide gel matrix. In the presence of a catalyst like TEMED and under appropriate conditions such as pH and temperature, APS decomposes into sulfate radicals ($\text{SO}_4^{\bullet-}$) which initiates the polymerization reaction by initiating the formation of free radicals on acrylamide and bis-acrylamide molecules. APS is added to the gel solution along with TEMED just before gel casting to initiate polymerization.

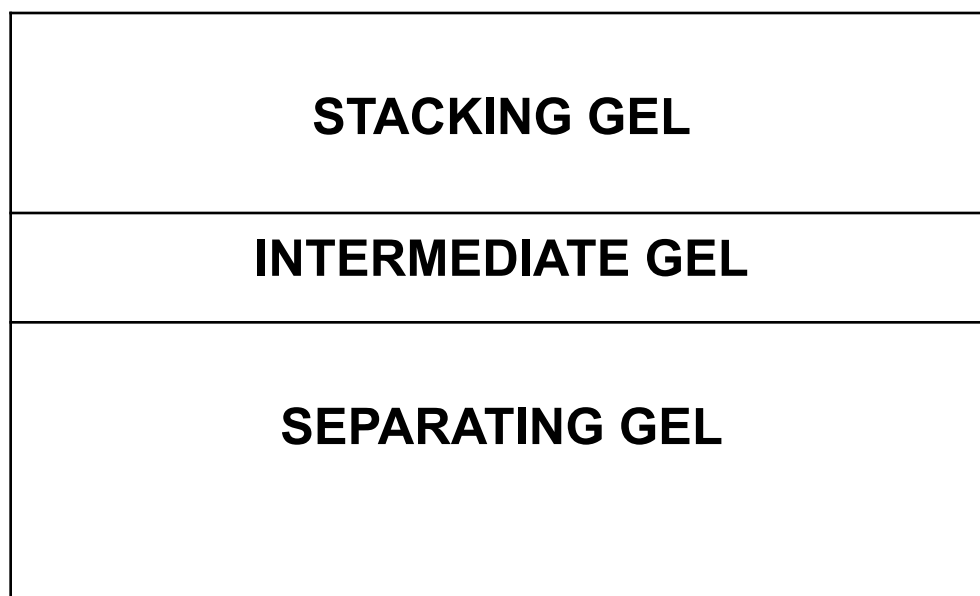


Figure 4.1. Set up of the gel layers

Once the stacking buffer is added into the plate, a grid like strip or a “comb” is inserted into the gel to make wells. Once the buffer solidifies, the comb can be taken out making sure that there are no air bubbles. In its place wells would have been formed onto which the sample to be analyzed or separated can be loaded. Furthermore, the gel electrophoresis apparatus is assembled by placing the solidified gel into the electrophoresis tank.

Protein samples mixed with loading dye are loaded into wells at the top of the polyacrylamide gel and subjected to an electric field. We used various types of protein samples we made. NAMP samples with and without ammonium sulfate, RAMP samples with and without ammonium precipitation, as well as the retentate and the filtrate from molecular cut-off filters of both NAMP and RAMP samples.

The anode buffer is placed in the lower compartment of the electrophoresis chamber where the electrode connected to the positive terminal of the power supply (the anode) is located. The cathode buffer is placed in the upper compartment of the electrophoresis chamber where the electrode connected to the negative terminal of the power supply (the cathode) is located. The negatively charged SDS-protein complexes migrate through the gel matrix towards the positive electrode. Smaller proteins move more rapidly through the gel pores than larger ones, resulting in separation based on size.

Once the proteins get separated, the gel is stained for an hour and then de-stained overnight to help visualize the bands.

4.4. LIQUID CHROMATOGRAPHY - MASS SPECTROSCOPY

The samples were given to Rajiv Gandhi Centre for Biotechnology (RGCB) for LC-MS. Protein identification was carried out using ESI-nanoLC-MS/MS. The sample was injected in duplicate (2 injections per sample (technical replicates)).

The protocol they employed is given as follows:

4.3.1. In-Gel Sample Digestion Protocol

For the in-gel sample digestion protocol, the following reagents are required: Ammonium bicarbonate (Sigma A6141), Dithiothreitol (Sigma D5545), Iodoacetamide

(Sigma I1149), MS grade Trypsin (Sigma T6567), Formic acid (Merck 5.33002.0050), and Acetonitrile (Merck 1.00029.2500).

Reagent setup (prepare freshly before use): Destaining solution: 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v), Formic acid 5% in MilliQ water (v/v), Extraction buffer: 5% formic acid/acetonitrile (1:2, v/v), Trypsin buffer: 13 ng/μl trypsin in 50 mM ammonium bicarbonate.

These reagents and solutions are utilized in the digestion and processing of protein samples for subsequent analysis, such as mass spectrometry. It's essential to prepare them fresh before use to ensure optimal performance and reliability of the assay.

After wiping the glass slides and scalpel with methanol, the protein band was excised from the acrylamide gel with the scalpel onto a glass slide, avoiding the unstained gel region. The excised bands were chopped into small cubes of 1 x 1 mm size and transferred into duly labeled 1.5 ml centrifuge tubes. Following this, the gel bands underwent rinsing with MilliQ water for 1 hour by vortexing at 1500 rpm. Subsequently, the water was discarded, and the rinsing step was repeated for another 1 hour with 1 ml of fresh MilliQ water. The gel cubes were then spun at 3000 rpm for 20 seconds, and the water was removed completely. Following this, 300 μl of destaining solution was added, and the mixture was vortexed for 30 minutes at 1500 rpm, adjusting the duration based on the staining intensity. Next, 500 μl of 100% acetonitrile was introduced, and the mixture was incubated at room temperature with vortexing for 10-15 minutes until the gel pieces became white and shrunk. The acetonitrile was then completely removed, and the vials were left open for 20 minutes for the solvent to evaporate fully. Afterward, 30-50 μl of 13 ng/μl trypsin buffer was added in a manner that covered the dry gel pieces, and the vials were placed in an ice bucket or a fridge for gel saturation. Following a 30-minute incubation period, the level of trypsin buffer was checked, and additional buffer was added as needed for complete coverage of the gel pieces. The gel pieces were then left for an additional

90 minutes for saturation, periodically checking and adjusting the levels of trypsin buffer if necessary. Subsequently, the vials were incubated at 37°C at 300 rpm overnight with gentle vortexing. Once complete, the trypsin buffer was removed from the sample vials and transferred to freshly labeled 0.6 ml microcentrifuge tubes. Extraction buffer (usually 100µl, twice the volume of trypsin buffer used initially) was added to the sample tubes containing gel pieces, and the mixture was vortexed vigorously for 20 minutes. Following this, the extraction buffer was aspirated and combined with the 0.6 ml microcentrifuge tubes. The entire samples were then dried using a Speed Vacuum concentrator and stored at -20°C until use. Finally, the samples were reconstituted in 30 µl of 3% acetonitrile before LC/MS analysis.

4.3.2. Liquid Chromatography Conditions

The liquid chromatography setup involved using the nanoACQUITY UPLC® chromatographic system by Waters with MassLynx4.1 SCN781 software. The system utilized a binary solvent system comprising Solvent A, which was 0.1% formic acid in water, and Solvent B, consisting of 0.1% formic acid in acetonitrile. It employed a Symmetry® C18 trap column and an HSS T3 C18 analytical column. The chromatography was operated in reverse phase mode with a solvent flow rate of 300 nL/min and a column temperature set at 35°C. The auto sampler was maintained at 4°C, and injections of 3 µl were made. The chromatography gradient spanned a total run time of 60 minutes.

4.3.3. Mass Spectrometry Conditions

The mass spectrometry conditions for the MS runs were executed using ion mobility enabled separation. The MS system employed was the Synapt G2 High-Definition MS™ System (HDMSE System) by Waters. Calibration was performed using sodium iodide. The acquisition mode utilized was ESI positive. Online mass correction was conducted using *Leucine encephalin* in positive ion mode ($m/z = 556.2766$). The nano ESI capillary voltage was set at 3.4 kV. Sample cone

voltage was maintained at 40 V, while extraction cone voltage was set to 4 V. The IMS gas (N₂) flow rate was 90 mL/min. IMS T-Wave™ pulse height and velocity were set to 40 V and 800 m/s, respectively. IMS voltage was applied at 8 V and 20 V. The mode of operation was resolution mode, and data acquisition was in continuum format. Collision energy ranged from 20 eV to 45 eV during the acquisition process.

4.3.4. Data Analysis

For data analysis, Progenesis QI for Proteomics v4.2 software by Non Linear Dynamics, Waters, was utilized. The organisms studied for comparison were "*Capitella teleta*" and "*Eisenia Fetida*." The database source used for analysis was UniProt. Parameters set for analysis included a false positive rate of 1, allowing for a maximum of 1 missed cleavage. Each peptide was required to have at least 1 fragment, while each protein needed to have a minimum of 3 fragments. Additionally, each protein was expected to have at least 1 peptide. Variable modification considered was the oxidation of methionine, while cysteine carbamidomethylation was set as a fixed modification. These parameters were chosen to ensure accurate identification and quantification of proteins in the samples, facilitating comprehensive analysis of the proteomic data obtained from the experiments.

4.5. ANTI-INFLAMMATORY ASSAY

The basic principle behind the Anti-Inflammatory Assay is inducing an inflammatory reaction or introducing some sort of inflammatory stimulus to the sample. This will lead to the sample turning turbid due to protein aggregation, which mimics the inflammatory conditions. Now this turbidity is measured using spectroscopy to understand the inflammatory activity of the protein.

In order to perform the assay we need to make 1% Aspirin and 1% BSA. For the preparation of a 1% aspirin solution in 75 mL of water, Initially, 0.75 grams of aspirin was weighed out accurately using a balance. Subsequently, the aspirin was

added to the 75 mL of water in a clean and dry beaker. The container was securely covered to prevent any spillage during the sonication process. The mixture was then subjected to sonication for a predetermined duration to ensure complete dissolution of the aspirin particles in the water. After sonication, the solution was visually inspected to confirm the absence of any undissolved particles. Once confirmed, the solution was labeled appropriately with the concentration, date, and contents. Similarly for the preparation of a 1% BSA solution in 2 mL of water, a stock solution of BSA was prepared by accurately weighing 20 milligrams of BSA powder using a balance. The BSA powder was then dissolved in 2 mL of distilled water in a clean and dry vial. The vial was securely capped, and the solution was mixed thoroughly by gentle agitation until the BSA powder was completely dissolved.

The sample was prepared using dilution. We took the protein sample with PBS in it and pipette out 5 μ l of the sample and dissolved it in 45 μ l of water to dilute it. This will be our protein sample stock for the assay. For this assay protein samples which have undergone ammonium sulfate were used since it has higher concentration of the protein. The samples are taken in different concentrations to make an effective analysis of the relationship.

We make the following samples for the assay:

Positive Control:

Aspirin is a very common anti-inflammatory compound and it is used as a positive control in varying concentrations.

Table 4.2 Positive control sample preparation

	Concentration 1	Concentration 2	Concentration 3	Concentration 4
Aspirin	5 μ l	10 μ l	15 μ l	20 μ l
1% BSA	100 μ l	100 μ l	100 μ l	100 μ l

Sample 1:**Table 4.3 NAMP sample preparation**

	Concentration 1	Concentration 2	Concentration 3	Concentration 4
NAMP	5 µl	10 µl	15 µl	20 µl
1% BSA	100 µl	100 µl	100 µl	100 µl

Sample 2:**Table 4.4. RAMP sample preparation**

	Concentration 1	Concentration 2	Concentration 3	Concentration 4
RAMP	5 µl	10 µl	15 µl	20 µl
1% BSA	100 µl	100 µl	100 µl	100 µl

Controls:

100 µl 1X PBS with 100 µl distilled water.

100 µl of 1%BSA with 100 µl distilled water.

Blanks:

1X PBS

Distilled water

1% Aspirin

These samples were prepared in 1.5 ml vials and then left at room temperature for 10 minutes. Then they are kept at 51°C for 15 mins. This denatures the protein, making it turbid. Equal volumes of all these samples are added into the 96 well plate. We generally use about 100 µl of the samples into the wells. These samples are read in a BioTek Epoch Microplate Spectrophotometer. UV-Vis spectroscopy method is used to monitor changes in turbidity of the BSA solution over time. Turbidity, measured as the optical density (OD) at a specific wavelength, increases with the formation of protein aggregates, providing a quantitative measure of inflammation-induced protein aggregation. The anti-inflammatory activity of the test compounds is evaluated based on their ability to inhibit or reduce BSA aggregation and turbidity compared to control samples. Parameters such as percentage inhibition of turbidity or IC50 values are also calculated to assess the potency of the compounds as anti-inflammatory agents.

Formula for the calculation of inhibition percentage is given as:

$$\text{Inhibition percentage} = 100 * (\text{Control OD} - \text{Test sample})$$

Using this formula the inhibition percentage is calculated and graph is plotted to better understand the activity.

4.6. KIRBY-BAUER DISK DIFFUSION SUSCEPTIBILITY TEST FOR ANTIBACTERIAL ACTIVITY

The Kirby-Bauer disk diffusion susceptibility test aims to ascertain whether pathogenic aerobic and facultative anaerobic bacteria are sensitive or resistant to different antimicrobial compounds. This aids physicians in choosing appropriate treatment for their patients. The test involves cultivating the pathogenic organism on Mueller-Hinton agar alongside antimicrobial-impregnated filter paper disks. The growth or lack thereof around the disks serves as an indicator of the compound's ability to inhibit the organism (Hudzicki, J.,2009).

4.6.1 Autoclaving of Substances

All the substances required for the experiment, such as nutrient broth, agar plates, and sample discs, were autoclaved to ensure they were free from any microbial contaminants that could interfere with the experiment.

4.6.2 Preparation of Nutrient Broth

Nutrient broth is a liquid medium containing essential nutrients required for bacterial growth. To prepare the nutrient broth, appropriate quantities of nutrient powder were dissolved in distilled water and sterilized by autoclaving. This ensured that the medium was free from any contaminating microorganisms.

4.6.3 Inoculation of Bacteria

After preparing the nutrient broth, it was divided into two separate flasks, with 25 mL allocated to each. One flask was designated for inoculation with *Escherichia coli* (*E. coli*) bacteria, while the other flask was designated for inoculation with *Bacillus* spp. bacteria. The inoculation process involved transferring a small amount of bacterial culture (obtained from a pure culture or stock culture) into the respective flasks containing the nutrient broth using a sterile pipette or inoculation loop.. This step introduced the bacteria into the nutrient-rich environment of the broth, allowing them to proliferate and grow.

4.6.4 Sterile Techniques

During the inoculation process, it was crucial to maintain sterility to prevent contamination of the nutrient broth with other microorganisms. Sterile techniques, such as flame sterilization of the inoculation loop or pipette, were employed to

minimize the risk of introducing unwanted contaminants into the bacterial cultures. It was performed in a laminar air flow.

4.6.5 Incubation in Shaking Incubator

Following inoculation, the flasks containing the nutrient broth and bacteria were placed in a shaking incubator. The shaking incubator provides a controlled environment with constant agitation and optimal temperature for bacterial growth. The flasks were incubated at 25°C and agitated at a 9 rpm speed to ensure uniform distribution of nutrients and oxygen throughout the broth. Incubating the flasks in a shaking incubator promotes rapid and uniform bacterial growth, leading to the attainment of a suitable bacterial density for subsequent experiments. By following these steps, the bacterial strains were provided with a nutrient-rich environment conducive to growth, ensuring their viability and activity prior to conducting the disc diffusion assay to assess their susceptibility to the samples being tested for antibacterial activity.

Turbidity is an indirect measure of bacterial growth and is caused by the scattering of light by bacterial cells suspended in the liquid medium. When bacteria are inoculated into the nutrient broth and allowed to grow under suitable conditions (such as in a shaking incubator), they utilize the nutrients present in the broth for metabolic activities and cell division. As the bacterial population increases, the number of bacterial cells suspended in the broth also increases, leading to an increase in turbidity. The degree of turbidity observed in the broth is correlated with the density of bacterial cells present in the medium. Higher turbidity indicates a higher concentration of bacterial cells and thus a higher level of bacterial growth.

4.6.6 Preparation of Mueller-Hinton Plates:

MH agar plates were allowed to reach room temperature. It's best to keep the plates in their plastic sleeve during this time to minimize condensation.

If any visible liquid is present on the agar surface, the plate were and set slightly ajar on its lid to allow excess liquid to drain and evaporate. This can be done in a 35°C incubator or a laminar flow hood at room temperature for about 10 to 30 minutes until the agar surface is dry. Each MH agar plate is labelled appropriately for the organism to be tested.

4.6.7 Preparation of Inoculum:

Using a sterile inoculating loop or needle, four or five isolated colonies of the organism is picked up to be tested from a pure culture plate. The collected colonies are suspended in 2 mL of sterile saline solution. This step helps to create a smooth suspension of bacterial cells in the liquid medium. The saline tube containing the bacterial suspension is vortexed to ensure thorough mixing and homogenization of the cells. This step helps to achieve a consistent concentration of bacteria in the suspension, which is crucial for accurate antimicrobial susceptibility testing. If the suspension appears too light, more organism is added to increase turbidity. Conversely, if the suspension is too heavy, it is diluted with sterile saline solution until the desired turbidity is achieved.

4.6.8 Inoculation of the MH Plate:

The bacterial suspension was prepared by dipping a sterile swab into the prepared bacterial suspension. The swab was rotated against the inner side of the tube, using firm pressure to remove excess fluid without allowing it to drip. Subsequently, the dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab three times over the entire agar surface, ensuring even distribution of the inoculum by rotating the plate approximately 60 degrees each time. After inoculation, the edge of the plate was rimmed with the swab to absorb any excess liquid. The used swab was disposed of in an appropriate container. The lid of the plate was left slightly ajar, allowing it to sit at room temperature for at least 3 to 5 minutes

(but no more than 15 minutes) to allow the agar surface to dry before proceeding to the next step in the antimicrobial susceptibility testing procedure.

4.6.9 Placement of the Antibiotic discs

After the agar plates were inoculated with the test bacteria, paper discs containing the antimicrobial peptides were placed on the agar surface. 4 discs were taken with the NAMP, RAMP, NAMP filtered sample, and RAMP filtered samples. For positive control, Gentamicin was used. The placement of antibiotic disks on the agar surface was a critical step in the disc diffusion method for antimicrobial susceptibility testing. The Mueller-Hinton agar plate was placed on a flat surface, and the lid was removed. Then, either a multidisk dispenser or forceps were used to place the antibiotic disks on the agar surface, ensuring complete contact with the agar. After all disks were in place, the lid was replaced, and the plates were inverted and incubated for the specified duration before reading the results. The antimicrobial agents diffused from the discs into the surrounding agar, creating a concentration gradient. If the bacteria were susceptible to the antimicrobial agent, growth inhibition occurred around the disc, forming a zone of inhibition. The diameter of this zone was measured using a ruler (Bauer et al. 1966).

4.6.10 Interpretation of the results

The interpretation of the results was based on standardized criteria established by organizations such as the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The size of the zone of inhibition was compared to reference values provided by these organizations to determine whether the bacterial strain was susceptible, intermediate, or resistant to the tested antimicrobial agent.

4.7 CELL CYTOTOXICITY ANALYSIS USING MTT ASSAY

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a commonly used colorimetric method to assess cell viability, proliferation, and cytotoxicity in cell culture experiments. When MTT is added to cell cultures, viable cells with active mitochondria reduce the yellow tetrazolium salt MTT into purple formazan crystals through the action of mitochondrial dehydrogenase enzymes. This reduction reaction occurs primarily in metabolically active cells with intact mitochondrial function. (tolosa et al, 2015). We used C2C12 cells, which are mouse myoblast cells for our experiment. For performing the MTT assay with C2C12 cells and antimicrobial peptide samples in triple concentrations of 2 μ l, 4 μ l, and 8 μ l:

4.7.1 Cell Culture Preparation

C2C12 cells were prepared by thawing a frozen vial and culturing them in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-amphotericin. The cells were incubated in a humidified incubator at 37°C with 5% CO₂ until they reached 70-80% confluency.

4.7.2 Plate Seeding

The growth medium was aspirated from the C2C12 cell culture flask. The cells were washed once with phosphate-buffered saline (PBS) to remove any residual medium. The cells were detached using trypsin-EDTA and neutralized with fresh growth medium. Cell counting was performed using a hemocytometer, and the cells were seeded into a 96-well plate at a density of X cells per well, ensuring each well received the same number of cells.

4.7.3 Treatment of Cells with AMP

The antimicrobial peptide samples were diluted to the desired concentrations of 2µl, 4µl, and 8µl using sterile cell culture medium. Proper mixing of the peptides was ensured to achieve homogeneity. The diluted antimicrobial peptide samples were added to the appropriate wells of the 96-well plate containing the seeded C2C12 cells. Wells with medium only and wells with cells treated with a known cytotoxic agent were included for comparison. The plate was incubated at 37°C with 5% CO₂ for a predetermined period, typically 24-72 hours, to allow the cells to be exposed to the peptides.

4.7.4 MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was prepared according to the manufacturer's instructions. After the incubation period, the medium was carefully removed from each well without disturbing the cells. The MTT reagent was added to each well, ensuring all wells were evenly covered, and the plate was incubated at 37°C for 2-4 hours to allow the formazan crystals to form. After the incubation, the MTT reagent was carefully removed from each well, and a suitable solvent (e.g., DMSO) was added to dissolve the formazan crystals.

4.7.5 Data Analysis

The plate was gently mixed to ensure complete solubilization of the formazan crystals. The absorbance of each well was measured at the 570nm or 590nm using a microplate reader. The absorbance data were analyzed to calculate cell viability and determine the effects of the antimicrobial peptides on the C2C12 cells.

4.7.6 Results Interpretation

The absorbance values of the treated wells were compared to the negative and positive controls to assess the cytotoxicity or cytostatic effects of the antimicrobial peptides. The results were interpreted to determine the optimal concentration of antimicrobial peptides for further experiments or applications.

5. RESULT AND DISCUSSIONS

5.1 COLLECTION OF PROTEIN SAMPLES

Two types of samples, one from regenerated and the other from non-regenerated earthworms, were collected to explore the production of antimicrobial peptides (AMPs) during regeneration.

5.1.1 Non regenerated worms

Approximately 50 healthy earthworms were collected, and gut cleaned to expel remaining gut contents. and subjected to thorough washing to eliminate any adhering dirt.



Fig 5.1: Normal gut cleaned earthworms

5.1.2 Regenerated worms

Regeneration in earthworms was induced by carefully amputating a segment of their bodies and monitoring tissue regrowth. Approximately 50 healthy earthworms were collected, ensuring they were well-hydrated and devoid of any signs of stress.



Fig 5.2 : Amputated earthworms

5.2 COELOMIC FLUID EXTRACTION

Applying an electric current of approximately 10V for 3 seconds, repeated 10-15 times, prompted the release of a yellow semi-viscous fluid. This fluid was used for the preparation of protein sample.



Fig 5.3 : Earthworms before being subjected to electric current

5.2.1 Ammonium sulfate precipitation

The Ammonium Sulfate Precipitation Method produces about 2-3 ml samples which compose PBS in it as well. This sample is subjected to dry bath and used for various assays like Inflammatory assay, Anti-bacterial assay, and cytotoxicity. The sample produced by this method was found to have a higher concentration of protein content. Therefore, making it an ideal method, although the low quantity is a disadvantage.

5.2.2 Without ammonium sulfate precipitation

Alternatively, the Protein extraction without Ammonium sulfate precipitation method produces low concentration protein in higher amounts. The supernatant from the centrifugation of coelomic fluid was directly taken and mixed with a sample buffer which contains mercaptoethanol which is responsible for cleaving di-sulfide bonds and provide native protein structures making the protein sample stable.

5.2.3 Molecular cut-off filter

Peptides were filtered from protein samples using a 3 kDa cutoff filter.



Fig 5.4 : Molecular cut-off filter with coelomic fluid collected from normal earthworms

5.3 SDS PAGE

SDS-PAGE was performed on various samples of protein like NAMP with ASP, without ASP, RAMP with ASP, without ASP, retentate and filtrates of the molecular cut-off filters etc. The concentration of the protein in the sample with ASP is very less therefore the samples without AS was used. The Coelomic fluid and the Pellet samples were collected for the SDS PAGE. The tricine-SDS PAGE gives

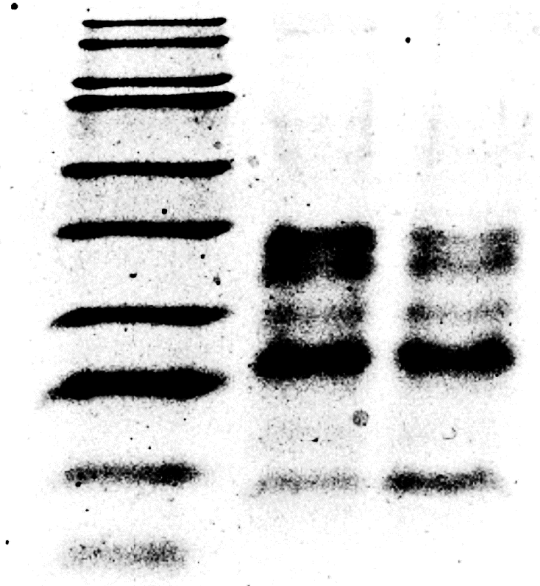


Fig 5.5 .Tricine SDS-PAGE

The bands were separated which was sent for LC-MS, for identification of the peptides.

5.4 LIQUID CHROMATOGRAPHY - MASS SPECTROSCOPY

The protein samples of *Perionyx excavatus* was compared with *Capitella teleta* and *Eisenia Fetida*.

The peptides and proteins were identified in the databases of these two species. Some unknown peptides were identified, some known peptides were also identified. The protein and peptides lists is given below:

Table 5.1. NAMP peptide list from *Capitella teleta* database

Sequence	Description
VAPEEHPVLLTEAPLNPK	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_160805 PE=3 SV=1
TTGIVLDSGDGVTHTVPIYE GYALPHAILR	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_160805 PE=3 SV=1
AGFAGDDAPR	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1
DLTDYLMK	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1
IIAPPER	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1
EDYVYK	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
SSDSKIEMIR	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
NLLSVAYK	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
EAAENSLVAYK	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
YLAEFATGNDRK	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
LSVAYK	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
LAEQAER	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1

DSTLIMQLLR	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
TLSDYNIQK	Ubiquitin-like domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_162204 PE=4 SV=1
MQIFVK	Ubiquitin-like domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_162204 PE=4 SV=1
TITLEVEPSDTIENVK	Ubiquitin-like domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_162204 PE=4 SV=1
EQREEVAISK	Uncharacterized protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_196376 PE=4 SV=1
EQREEVAISK	Uncharacterized protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_196376 PE=4 SV=1
QWMIHIDYLEK	Uncharacterized protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_45117 PE=4 SV=1
YSIFEGELSDTIPVVHSSIA GCR	Eukaryotic translation initiation factor 6 (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_156578 PE=4 SV=1

Table 5.2. NAMP protein list from *Capitella teleta* database

Accession	Mass	Description
R7TEK5	18270.98	Uncharacterized protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_196376 PE=4 SV=1
R7TJR2;R7US7 1;R7V2K8;R7U5 54	51427.18	Ubiquitin-like domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_162204 PE=4 SV=1
R7TMG3	42009.94	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1
R7TPD2;R7VCX 4	40470.26	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_160805 PE=3 SV=1

R7U7Q6	10893.26	Eukaryotic translation initiation factor 6 (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_156578 PE=4 SV=1
R7UMQ5;R7V3 X6	29383.94	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
R7VL83	6584.554	Uncharacterized protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_45117 PE=4 SV=1

Table 5.3. RAMP peptide list from *Capitella teleta* database

Sequence	Description
VAPEEHPVLLTEAPLNPK	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1
DLTDYLMK	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1
VVDLLAPYAK	ATP synthase subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_157862 PE=3 SV=1
LVPLKETIK	ATP synthase subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_157862 PE=3 SV=1
DLLAPYAK	ATP synthase subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_157862 PE=3 SV=1
DQEGQDVLLFIDNIFR	ATP synthase subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_157862 PE=3 SV=1
AGLQFPVGR	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198869 PE=3 SV=1
LQFPVGR	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198869 PE=3 SV=1
LLLPGELAK	Histone H3 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198870 PE=3 SV=1
STELLIR	Histone H3 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198870 PE=3 SV=1
RVTI	Histone H3 OS= <i>Capitella teleta</i> OX=283909

	GN=CAPTEDRAFT_198870 PE=3 SV=1
NLLSVAYK	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
AASDIAMTELPPTHPIR	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
LAVNMVPFPR	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_156132 PE=3 SV=1
PFPR	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_147894 PE=3 SV=1
LAVNMVPFPR	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
LHFFMPGFAPLTSR	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
LLLPGELAK	Histone H4 OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
DNIQGITKPAIR	Histone H4 OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
HQGVMMVGMGQK	Poly [ADP-ribose] polymerase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_220539 PE=3 SV=1
STELLIR	Histone H3 (Fragment) OS= <i>Capitella aff. teleta</i> ST-2016 OX=1789173 GN=H3 PE=3 SV=1
ALR	Histone H3 (Fragment) OS= <i>Capitella aff. teleta</i> ST-2016 OX=1789173 GN=H3 PE=3 SV=1
LGDVYVNDAFGTAHR	Phosphoglycerate kinase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_222818 PE=3 SV=1
NEKFSLKPVAAELK	Phosphoglycerate kinase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_222818 PE=3 SV=1
NEKFSLKPVAAELK	Phosphoglycerate kinase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_222818 PE=3 SV=1
NLDIGRPSYTNLNR	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198644 PE=3 SV=1
IHFPLATYAPVISA EK	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198644 PE=3 SV=1
LHFFMPGFAPLTSR	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909

	GN=CAPTEDRAFT_214108 PE=3 SV=1
LENVIR	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_103539 PE=3 SV=1
FDGALNVDLTEFQTNLV PYPR	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149167 PE=3 SV=1
VGINYQPPTVVPGGDLA K	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149167 PE=3 SV=1
EIVDLVLDRIR	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149167 PE=3 SV=1
DVNAAIATIKTK	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149167 PE=3 SV=1
STAGDTHLGGEDFDNR	Heat shock protein 70 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149823 PE=3 SV=1
SIHPDEAVAYGAAVQAAV LTGSTDSAVK	Heat shock protein 70 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149823 PE=3 SV=1
YLGPAVIMQAYRWIMDS R	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit_ mitochondrial OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_21987 PE=3 SV=1
GLNPGAAGVEMKK	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit_ mitochondrial OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_21987 PE=3 SV=1
HFSVEGQLEFR	Histidine kinase/HSP90-like ATPase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159573 PE=3 SV=1
VAVLGASGGIGQPLSLLL K	Malate dehydrogenase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159451 PE=3 SV=1
IIDDK	Thioredoxin domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_180369 PE=4 SV=1
FIIDDK	Thioredoxin domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_180369 PE=4 SV=1
DATDDKVTVDCAEAMLK	Isocitrate dehydrogenase [NADP] OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_183927 PE=3 SV=1
MSALHGSERAQASGFY VK	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_125228 PE=3 SV=1

VVGIK	Uncharacterized protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_171547 PE=3 SV=1
GTHVEVNTNMLLQSPQY FHDAHRFIPER	Uncharacterized protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_171547 PE=3 SV=1
IHHLRDEEGLVENFSNEI K	Uncharacterized protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_171547 PE=3 SV=1
LLLPGELAK	Histone H2A OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_2179 PE=3 SV=1
LISWYDNEYGYSNR	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159125 PE=3 SV=1
MAFR	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159125 PE=3 SV=1
VLTSgifetkftvek	Guanine nucleotide-binding protein G(S) subunit alpha OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_164626 PE=3 SV=1
STELLRL	Uncharacterized protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_205469 PE=4 SV=1
KRPGTGVNAEGAAGVM R	Uncharacterized protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_205469 PE=4 SV=1
SACLDLPNPESNGLNR	Uncharacterized protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_205469 PE=4 SV=1
LSILYPATTGR	Thioredoxin domain-containing protein OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
SVNSINLQELNPEWK	BTB domain-containing protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_63514 PE=4 SV=1
FPFCSQR	BTB domain-containing protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_63514 PE=4 SV=1
LQIWDTAGQER	Ras-related protein Rab-1A OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_177262 PE=4 SV=1
TITLEVEPSDTIENVK	Ubiquitin-like domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_162204 PE=4 SV=1
IINGLSVEGKMIGLNLDL HLK	BTB domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_227326 PE=4 SV=1

LLIK	BTB domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_227326 PE=4 SV=1
TPAQVILR	NADP-dependent oxidoreductase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_163211 PE=4 SV=1
EELFVTSK	NADP-dependent oxidoreductase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_163211 PE=4 SV=1
VTGADVPMPLYAK	Pyruvate dehydrogenase E1 component subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_161753 PE=4 SV=1
LLIIGNSSVGKTSFLFR	Ras-related protein Rab-3 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149846 PE=3 SV=1
LQIWDTAGQER	Ras-related protein Rab-3 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149846 PE=3 SV=1
AAVDAGFVPNDMQVGQ TGK	Electron transfer flavoprotein subunit alpha OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152823 PE=3 SV=1
SDRPELTGAK	Electron transfer flavoprotein subunit alpha OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152823 PE=3 SV=1
DITK	FMN hydroxy acid dehydrogenase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_212622 PE=3 SV=1
FFQLYIFKQR	FMN hydroxy acid dehydrogenase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_212622 PE=3 SV=1
AQIWDTAGQER	Ras-related protein Rab-11A OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_226009 PE=4 SV=1
GAVGALLVYDIAK	Ras-related protein Rab-11A OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_226009 PE=4 SV=1
ELIDLVLDR	Tubulin/FtsZ GTPase domain-containing protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_39468 PE=3 SV=1

Table 5.4. RAMP protein list from *Capitella teleta* database

Accession	Mass	Description
A0A3Q9X820	12038.98	Histone H3 (Fragment) OS= <i>Capitella</i> aff. <i>teleta</i> ST-2016 OX=1789173 GN=H3 PE=3 SV=1
R7T3B8	72796.73	BTB domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_227326 PE=4 SV=1
R7T3U1;R7TI15;R7TJ82; R7TNC5;R7TPJ5;R7V9E9 ;R7VD40;R7VKM8;G1BGI 6;A0A3T1BAF7;R7VE63; R7UBI0;R7UD78;R7UF98 ;R7USC4;R7V1D3;R7VAJ 0;R7VG38;R7VHW6	27941.57	Histone H3 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198870 PE=3 SV=1
R7T3W2	11367.36	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_103539 PE=3 SV=1
R7T5U3;R7TAR2	51016.97	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_147894 PE=3 SV=1
R7T6Z8;R7TAC7;R7TUJ6 ;R7U9Q3;R7V1Q6;R7TKK 8;R7U008;R7UVW6	50780.02	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149167 PE=3 SV=1
R7T426;R7UGD5;R7TUN 6;R7VGG5;R7UGC9;R7T JA7;R7TNM4;R7TW75;R7 UAU0;R7UD67;R7VKN6; R7TTA3;R7TTY4;R7UHB 3;R7V2P2	25272.63	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198869 PE=3 SV=1
R7TCD0;R7UX62	57132.54	Uncharacterized protein

		OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_171547 PE=3 SV=1
R7TD52	25213.19	Ras-related protein Rab-3 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149846 PE=3 SV=1
R7THX4;R7U7V7;R7UZH 1;R7V212;R7V748;R7U46 5;R7V3D4	49193.14	Tubulin alpha chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_198644 PE=3 SV=1
R7TJM0	45059.76	Phosphoglycerate kinase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_222818 PE=3 SV=1
R7TJR2;R7US71;R7V2K8 ;R7U554	51427.18	Ubiquitin-like domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_162204 PE=4 SV=1
R7TKU3	50216.3	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_214108 PE=3 SV=1
R7TL07;R7TCP3;R7V0T4 ;X1Z7A7;R7TL47;R7UPT 5;R7UR96;R7VCQ9;R7VJ W8	23020.97	Ras-related protein Rab-1A OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_177262 PE=4 SV=1
R7TMG3	42009.94	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_158679 PE=3 SV=1

R7TPD2	40470.26	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_160805 PE=3 SV=1
R7TPV7;R7TVV4	36974.23	Pyruvate dehydrogenase E1 component subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_161753 PE=4 SV=1
R7TSV0	36864.03	Malate dehydrogenase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159451 PE=3 SV=1
R7TU16	18833.04	Histone H2A OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_2179 PE=3 SV=1
R7U4A0	35184.15	Electron transfer flavoprotein subunit alpha OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152823 PE=3 SV=1
R7U480	36520.93	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159125 PE=3 SV=1
R7U507	8284.525	Histone H4 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_125228 PE=3 SV=1
R7UJC6	238628.8	Poly [ADP-ribose] polymerase OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_220539 PE=3 SV=1

R7UMQ5;R7V3X6	29383.94	14-3-3 domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_152326 PE=3 SV=1
R7UPQ7	73226.08	Uncharacterized protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_205469 PE=4 SV=1
R7URD2	47210.78	Isocitrate dehydrogenase [NADP] OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_183927 PE=3 SV=1
R7UXL6	26488.01	Thioredoxin domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_180369 PE=4 SV=1
R7UXP1	44799.65	Guanine nucleotide-binding protein G(S) subunit alpha OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_164626 PE=3 SV=1
R7V1K7	39677.5	FMN hydroxy acid dehydrogenase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_212622 PE=3 SV=1
R7V2V4	14133.66	Tubulin/FtsZ GTPase domain-containing protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_39468 PE=3 SV=1
R7V3K1	82856.85	Histidine kinase/HSP90-like

		ATPase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_159573 PE=3 SV=1
R7V9M3;R7TK96;R7TYJ4 ;R7V522;R7VCW6;R7TS X9;R7TKU8;R7TRL1;R7T JX4	50480.56	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_156132 PE=3 SV=1
R7V071	24877.45	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit_ mitochondrial OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_21987 PE=3 SV=1
R7VBB9;R7T757;R7TCX0 ;R7TNR2;R7TP15;R7UA6 4;R7UD54;R7UPN7;R7U VZ5;X1ZKS3	49930.76	BTB domain-containing protein (Fragment) OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_63514 PE=4 SV=1
R7VBM0	50615.67	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_150647 PE=3 SV=1
R7VCX4	37504.87	Actin_ cytoplasmic OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_176468 PE=3 SV=1
R7VE96;R7UME6;R7TM W8;R7V1F3	69629.23	Heat shock protein 70 OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_149823 PE=3 SV=1
R7VFI5	24163.01	Ras-related protein Rab-11A OS= <i>Capitella teleta</i>

		OX=283909 GN=CAPTEDRAFT_226009 PE=4 SV=1
R7VGL7	56677.81	ATP synthase subunit beta OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_157862 PE=3 SV=1
R7VIM1	36887.16	NADP-dependent oxidoreductase domain-containing protein OS= <i>Capitella teleta</i> OX=283909 GN=CAPTEDRAFT_163211 PE=4 SV=1
X1ZF31	22233.59	Thioredoxin domain-containing protein OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
X2A9S4;R7UJF6	24189.23	Histone H4 OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1
X2AX40	48815.08	Tubulin beta chain OS= <i>Capitella teleta</i> OX=283909 PE=3 SV=1

Table 5.5. NAMP peptide list from *Eisenia fetida* database

Sequence	Description
VAPEEHPVLLTEAPLNPK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TTGIVLDSGDGVTHTVPIYEGYA LPHAILR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TTGIVLDSGDGVTHTVPIYEGYA LPHAILR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
MQKEITALAPPTMK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
FRCPEAMFQPSFLGMESVGIHE TTYNSIMK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1

QEYDESGPSIVHR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
GYSFTTTAER	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
AGFAGDDAPR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
DLTDYLMK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
IIAPPER	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
FRCPEAMFQPSFLGMESVGIHE TTYNSIMK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
DSYVGDEAQSKR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
QEYDESGPSIVHR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
HQGVMMVGMGQK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
ATKHSDLYVFPNLNAISK	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
TLEK	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
LEK	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
DYGISELK	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
FLLMNGAR	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
IIDGTPLAR	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
SLASLIALEDIDYR	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
RENFYLINER	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i>

	OX=2064768 GN=CYL18_06125 PE=4 SV=1
YRSVWYDR	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
VVDLIK	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
VVDLIK	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
YDPSHHVISNASCTTNCLAPLAK	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TAAQNIIPSSTGAAK	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TLSDYNIQK	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TITLEVEPSDTIENVK	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1

Table 5.6. NAMP protein list from *Eisenia fetida* database

Accession	Mass	Description
A0A2I7YV10	36387.19	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
A0A2I7YV73	42261.24	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
A0A2S7N2G5	45576.49	Aminoglycoside phosphotransferase domain-containing protein OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=CYL18_06125 PE=4 SV=1
Q2I6A1	13682.7	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1

Table 5.7. RAMP peptide list from *Eisenia fetida* database

Sequence	Description
VAPEEHPVLLTEAPLNPK	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TTGIVLDSGDGVTHTVPIYE GYALPHAILR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
PGIADR	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
STELLIR	Histone H3 (Fragment) OS= <i>Eisenia andrei</i> x <i>Eisenia fetida</i> OX=2079789 GN=H3 PE=3 SV=1
TELLIR	Histone H3 (Fragment) OS= <i>Eisenia andrei</i> x <i>Eisenia fetida</i> OX=2079789 GN=H3 PE=3 SV=1
FTQAGSEVSALLGR	ATP synthase subunit beta OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=atpD PE=3 SV=1
IGLFGGAGVGK	ATP synthase subunit beta OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=atpD PE=3 SV=1
AIMMQPTDGLVR	ATP synthase subunit beta OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=atpD PE=3 SV=1
KAPNFDQLESK	ATP synthase subunit beta OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=atpD PE=3 SV=1
ANAKELGVS	ATP synthase subunit beta OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=atpD PE=3 SV=1
ALR	Histone H3 (Fragment) OS= <i>Eisenia fetida</i> OX=6396 GN=H3 PE=3 SV=1
IVLAK	L-ectoine synthase OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=ectC PE=3 SV=1
ETGETHWIEPGTMYLLNGH EK	L-ectoine synthase OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=ectC PE=3 SV=1
SFKDIENTDR	L-ectoine synthase OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=ectC PE=3 SV=1
TLSDYNIQKESTLHLV	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
QLEDGRTLSDYNIQK	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1

TLSDYNIQK	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
TITLEVEPSDTIENVK	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1

Table 5.8. RAMP protein list from *Eisenia fetida* database

Accession	Mass	Description
A0A2I7YV73	42261.24	Actin OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1
A0A2N3JW49	15305.05	L-ectoine synthase OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=ectC PE=3 SV=1
A0A2N3K457;A0A348FVH2	52277.39	ATP synthase subunit beta OS= <i>Streptomyces</i> sp. EAG2 OX=2056495 GN=atpD PE=3 SV=1
A0A2S7N2A8	51626.68	ATP synthase subunit beta OS= <i>Pradoshia eiseniae</i> OX=2064768 GN=atpD PE=3 SV=1
A0A481S8R7	12419.52	Histone H3 (Fragment) OS= <i>Eisenia fetida</i> OX=6396 GN=H3 PE=3 SV=1
A0A481S8S7;B6E9G3;A0A481S9U9;A0A481SAC9	12351.36	Histone H3 (Fragment) OS= <i>Eisenia andrei</i> x <i>Eisenia fetida</i> OX=2079789 GN=H3 PE=3 SV=1
Q2I6A1	13682.7	Ubiquitin (Fragment) OS= <i>Eisenia fetida</i> OX=6396 PE=2 SV=1

Some of the major peptide sequences found in the LC/MS results were that resembling Actin_ cytoplasmic, 14-3-3 domain-containing protein, Ubiquitin-like domain-containing protein, Eukaryotic translation initiation factor 6 (Fragment) and certain uncharacterized proteins. The NAMP samples. The Actin_ cytoplasmic found in the sample has a match with Actin proteins seen in *Lumbricus terrestris* and *L. rubellus*. It also has wound healing, immune system processing and programmed cell death (Nowak *et al*, 2009). The 14-3-3 domain-containing protein also exhibits

similar wound healing properties. Apart from that there are domains which are similar to Ubiquitin. Ubiquitin is responsible for wound healing, cell death and inflammatory responses. Ubiquitin can affect macrophage functioning which plays a very important role in wound healing. In treating several diseases, plasma ubiquitin levels are increased, including heart diseases (Shook *et al*, 2023). Apart from these there are sequences from a number of uncharacterised proteins.

The regenerated AMP sample seemed to contain enzymes like ATP synthase subunit beta, Histone H4 and H3, Histidine kinase/HSP90-like ATPase domain-containing protein, Thioredoxin domain-containing protein, etc. ATP synthase subunits not only function as pattern recognition receptors but they were also found to inhibit the growth of certain gram-positive and gram-negative bacteria (Ni *et al*, 2019). The sequences found in this peptide were similar to that in *L. terrestris*. Research has indicated that certain histones possess antimicrobial properties, capable of inhibiting the growth of various microorganisms including bacteria, fungi, and even some viruses (Kawasaki & Iwamura, 2008). Histones can interact with microbial membranes, causing destabilization and disruption, leading to cell death. Moreover, they can also bind to microbial DNA or RNA, inhibiting essential cellular processes and ultimately impeding microbial growth. Additionally, more research is needed to fully elucidate the mechanisms underlying histone-mediated antimicrobial activity and to explore the potential applications of histones or histone-derived peptides as antimicrobial agents (Tanaka *et al*, 2022). These histones have a match with the histones in *L. terrestris* and *L. rubellus*.

Heat shock proteins (HSPs) are vital for skin wound healing by regulating inflammation, cellular debris clearance, proliferation, migration, and collagen synthesis, ensuring proper tissue repair and integrity (Atalay *et al*, 2009). It exhibits activity against bacteria as well (Lv *et al*, 2023) There are certain Histidine

kinase/HSP90-like ATPase domain-containing proteins which is a part of a family which includes HSPs as well. These sequences match with *L.terrestris*, *L.rubellus*, and *Arenicola marina*. Thioredoxin domain-containing protein-like sequences was also found in the sample which has shown some anti-inflammatory properties (Masutani, 2022).

5.5 Anti-inflammatory assay results

Samples are prepared in 1.5 ml vials. Blanks are 1X PBS(F1) ,Distilled water(F3) ,1% Aspirin(F4). Controls used are 100 µl 1X PBS with 100 µl distilled water (F2) and 100 µl of 1%BSA with 100 µl distilled water (F5). Aspirin is used as a positive control in varying concentrations along with 1% BSA(100µl) in four different concentrations. The samples were collected from normal gut cleaned earthworms(NAMP) and regenerated earthworms(RAMP). NAMP are added in four different concentrations ; SPL1(G2) ,SPL2(G3) ,SPL3(G4) ,SPL4(G5) and RAMP are also added in four different concentrations SPL5(G6) ,SPL6(G7) ,SPL7(G8) ,SPL8(G9). Equal volumes of all these samples were added into the 96 well plate. We used about 100 µl of the samples into the wells. The samples were diluted by 10% and the experiment was performed.

The anti-inflammatory activity of the test compounds was evaluated by measure of turbidity in the samples and by observing the OD values

$$\text{Percentage of Inhibition} = ((\text{Test OD} - \text{Control OD})/\text{Control OD}) * 100$$

Inhibition percentage is calculated and graph is plotted to better understand the activity. The average of the four different concentrations of the samples are taken and then the formula is applied to calculate the percentage inhibition.

Table 5.9. Multiplate reader OD value results

Name	660 nm
	0.053
Control	0.037
NAMP 5µl	0.034
NAMP 10µl	0.042
NAMP 15 µl	0.056
NAMP 20 µl	0.098
RAMP 5 µl	0.038
RAMP 10 µl	0.045
RAMP 15 µl	0.078
RAMP 20 µl	0.094
ASPIRIN 5 µl	0.044
ASPIRIN 10 µl	0.039
ASPIRIN 15 µl	0.04
ASPIRIN 20 µl	0.047

Table 5.10 Anti-inflammatory assay calculation

SAMPLES	CONTROL	NAMP	RAMP
		0.034	0.038
		0.042	0.045
		0.056	0.078
		0.098	0.094
AVERAGE	0.037	0.0575	0.06375
PERCENTAGE INHIBITION		55.40540541	72.2972973

Once the percentage of inhibition is calculated the graph is plotted. It shows that the the NAMP and the RAMP samples show anti-inflammatory activity.

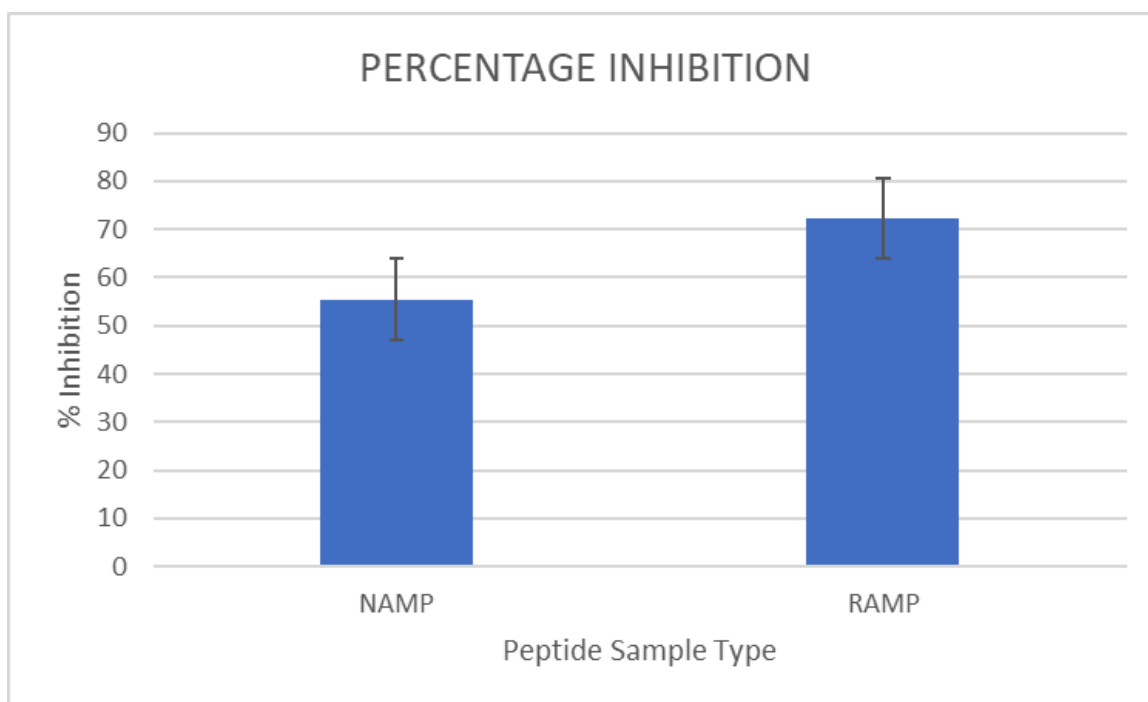


Fig 5.6 . Graphical representation of percentage of anti-inflammatory assay

Antimicrobial Peptides are known to have anti-inflammatory activity. The Inhibition % for the normal AMP sample is 55.405 and regenerated AMP sample is 72.297. This indicates that anti-inflammatory activity is more prominent in regenerated worm AMPs. Inflammatory activities are higher during regeneration (Meschler *et al*, 2017). Previously anti-inflammatory peptides were isolated from *Eisenia Foetida*, which was also found to have anti-analgesic properties (Seo *et al*, 2017) and also from annelids (Tasiemski, 2008). The earthworm peptide Lumbricin is a prominent regular of neuroinflammation (Li *et al*, 2017).

5.6 MTT Assay

The principle behind the MTT assay is that only viable cells have active metabolism and can convert MTT to purple formazan, which has an absorbance maximum at 570nm. The amount of formazan produced can be measured using a spectrophotometer and is proportional to the number of viable cells.

Percentage of cytotoxicity = 100 (Control OD -Test sample)

This percentage is calculated and graph is plotted to better understand the activity.

Table 5.11 . MTT assay calculation for NAMP without ammonium precipitation

	Control	2µl	4µl	8µl
	0.767	0.322	0.131	0.127
	0.463	0.248	0.116	0.193
	0.392	0.141	0.072	0.151
Average	0.540667	0.237	0.106333	0.157
Percentage of cytotoxicity		30.367	43.434	38.367

Table 5.12 MTT assay calculation for RAMP without ammonium precipitation

	Control	2µl	4µl	8µl
	0.767	0.162	0.106	0.165
	0.463	0.181	0.096	0.133
	0.392	0.102	0.085	0.108
Average	0.540667	0.148333	0.095667	0.135333
Percentage of cytotoxicity		39.23333	44.5	40.53333

Table 5.13 MTT assay calculation for NAMP filtrate

	Control	2µl	4µl	8µl
	0.767	0.167	0.071	0.055
	0.463	0.14	0.057	0.057
	0.392	0.086	0.057	0.08
Average	0.540667	0.131	0.061667	0.064
Percentage of cytotoxicity		40.96667	47.9	47.66667

Table 5.14 MTT assay calculation for RAMP filtrate

	Control	2µl	4µl	8µl
	0.767	0.061	0.057	0.052
	0.463	0.054	0.051	0.052
	0.392	0.076	0.053	0.055
Average	0.540667	0.063667	0.053667	0.053
Percentage of cytotoxicity		47.7	48.7	48.76667

Table 5.15 MTT assay results with Percentage of cytotoxicity for different concentrations of the sample

	2µl	4µl	8µl
NAMP	30.367	43.434	38.367
RAMP	39.234	44.5	40.534
NAMP FILTERED	40.967	47.9	47.667
RAMP FILTERED	47.7	48.7	48.767

The cytotoxicity for the different samples were calculated with the help of MS Excel. The average of OD values for every concentration of the samples were calculated to be used as the test sample OD value in the cytotoxicity percentage formula. A chart was formed using this data with the Percentage of cytotoxicity at the Y-axis and the various concentrations on the X- Axis. The NAMP sample shows a nonlinear curve with the cytotoxicity decreasing after 4 μ l. There is significant cytotoxicity in the protein samples, with the values generally increasing with concentration. Furthermore it was observed that the filtered samples were more cytotoxic compared to the non-filtered ammonium sulfate precipitated samples because of its highly concentrated nature.

The highest values of cytotoxicity were exhibited by the filtered RAMP sample with an average of 48.38889 and the lowest was exhibited by the NAMP sample with an average of 37.389.

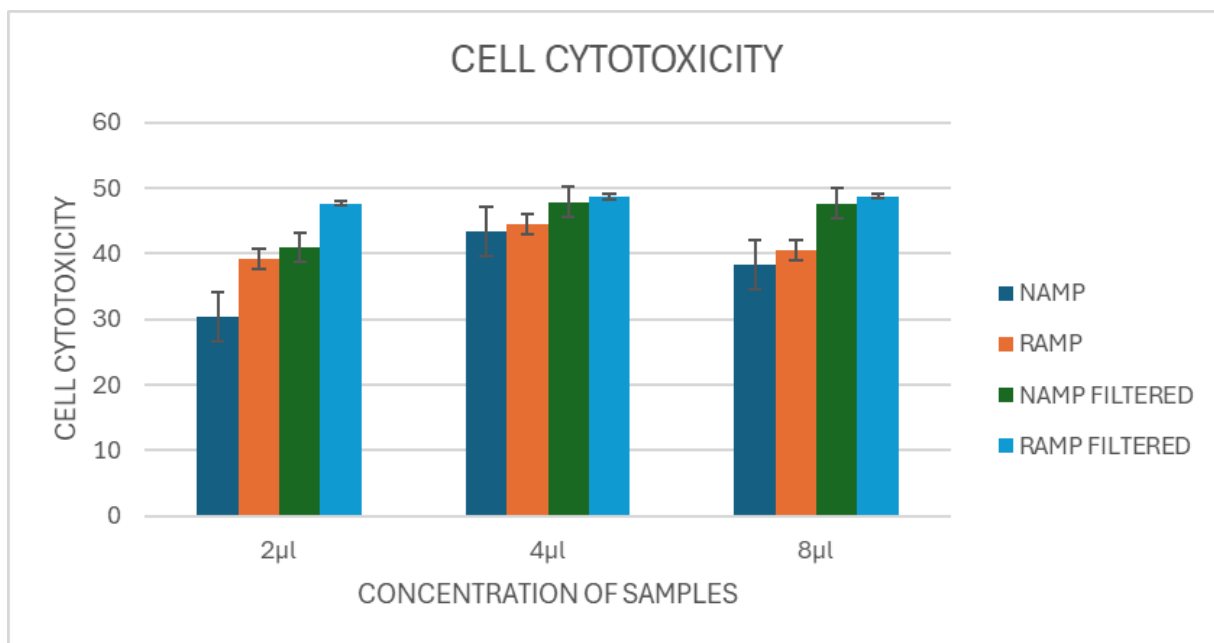


Fig 5.7 . Graphical representation of percentage of cytotoxicity

One-way anova was performed using Graphpad Prism application to better analyze the data produced.

NULL HYPOTHESIS: If there is no significant difference in means, null value is accepted. This would indicate that there is no relationship between the concentration and the cytotoxicity of the samples

ALTERNATE HYPOTHESIS: If there is significant difference in the means, null value is rejected and alternate hypothesis is accepted. This would indicate a relationship between concentration and cytotoxicity.

Table Analyzed	one way anova				
Data sets analyzed	A-D				
ANOVA summary					
F	4.144				
P value	0.0479				
P value summary	*				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.6084				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	207.6	3	69.19	F (3, 8) = 4.144	P=0.0479
Residual (within columns)	133.6	8	16.70		
Total	341.1	11			
Data summary					
Number of treatments (columns)	4				
Number of values (total)	12				

Fig 5.8 Anova analysis for the cytotoxicity data of AMP samples Since the *P* value is less than 0.05, the null hypothesis is rejected and the alternative hypothesis is accepted. Establishing a relationship between the concentration of samples and the cytotoxicity.

Previously coelomic fluid when treated with different cancer cell showed cytotoxic effect up to a concentration of 0.5 mg/ml, during MTT assay showcasing its cytotoxic effects (Dinesh *et al*, 2013). Similarly there was inhibition of HeLa cells at higher concentrations of coelomic fluid in the case of *Eisenia eugeniae* (Yan *et al*, 2011). It can be noted that this is similar to our experiment results too, where it can be seen that the cytotoxic activity of the peptides increases with an increase in the concentration.

EP3 which is a peptide isolated from the AVPF family also played a role in the antitumor activity of MGC803 which was analyzed through MTT assay (Wang *et al*, 2007). ECFP, which is another protein isolated from *E. foetida* also exhibited cytotoxic activity against chicken red blood cells (CRBC) (Hua *et al*, 2011).

Therefore, the cytotoxic properties of the Antimicrobial peptides have been hence proved and the cytotoxicity generally increases with increase in concentration.

5.7 KIRBY-BAUER DISK DIFFUSION SUSCEPTIBILITY TEST RESULTS FOR ANTIBACTERIAL ACTIVITY.

The Kirby-Bauer test, also known as the disk diffusion antibiotic sensitivity test, was performed by applying a thin layer of two species of bacteria species that is *Bacillus sp* bacteria and *Escherichia coli* bacteria to two petri plates and exposing it to two distinct protein samples that are extracted from earthworms: those from normal gut-cleaned worms and those that have undergone regeneration. The antibiotic used was Gentamicin (10 µg) which indicates resistance >12, intermediate 13-14 and

susceptibility ≥ 15 range. The antibiotic's "zone of inhibition" is the circular region surrounding the antibiotic site where bacterial colonies are unable to proliferate. The assessment of the zone of inhibition serves as a valuable method to gauge the susceptibility of bacteria to the samples under study. Furthermore, this research utilizes both the calculated zone of inhibition and established standard benchmarks to evaluate whether a particular bacterium exhibits susceptibility or resistance to the administered antibiotic (Konate *et al*, 2012).

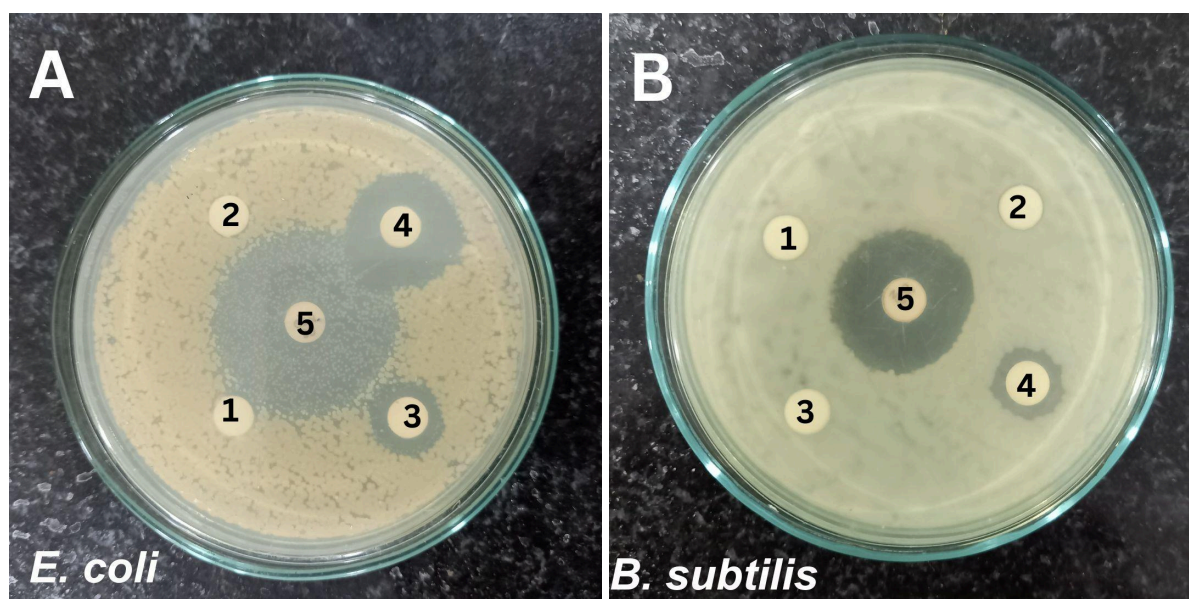


Fig 5.9 : Antibacterial activity with protein samples in during disk diffusion method. A) Activity in *Escherichia coli* in 1) NAMP sample 2) RAMP sample 3) NAMP filtrate sample 4) RAMP filtrate sample 5) Gentamicin control B) Activity in *Bacillus subtilis* in 1) NAMP sample 2) RAMP sample 3) NAMP filtrate sample 4) RAMP filtrate sample 5) Gentamicin control

The present study reports the antibacterial potency of antimicrobial peptides and control antibiotic gentamicin. These AMPs were tested for antibacterial activity against 2 clinically isolated bacterial strains like that of *Bacillus subtilis* and *E.coli*. One can notice that the susceptibility of the bacteria to the AMP as well as the antibiotic on the basis of inhibition zone diameters varied according to microorganism, the results are

reported in (Figure 5.12 and Figure 5.13). There is a significant variation in the diameters of inhibition zone values (DIZ) of the various AMP samples and the gentamicin.

Table 5.16 Zone of inhibition of two strains of bacteria

Sample	Bacterial species	Zone of inhibition (mm)
Control	Gentamicin	25 mm
NAMP	<i>Bacillus subtilis</i>	18 mm
RAMP	<i>Bacillus subtilis</i>	21 mm
Control	Gentamicin	14 mm
NAMP	<i>Escherichia coli</i>	1 mm
RAMP	<i>Escherichia coli</i>	9 mm

Zone of inhibition was then measured using a ruler and then compared to zone diameter interpretative standards for *B. subtilis* bacteria and *Escherichia coli* bacteria when gentamicin is introduced as an antibiotic. It was noticed that the *Bacillus* strains were susceptible to the AMP samples, especially the filtered RAMP sample exhibited noticeable antibacterial activity with Zone of Inhibition more than 15 mm . Whereas the unfiltered samples of NAMP and RAMP showed 0 mm or no Zone of Inhibition, due to its lower concentration. The *E. coli* strains were quite resistant to the AMP samples except a considerably small Zone of Inhibition observed for the unfiltered RAMP sample at 9 mm, which is still resistant.

Indigenous earthworms *P. excavatus* has shown a strong antimicrobial activity in previous studies as well, with the formation of clear disks for the bacterial strains (Prakash *et al*, 2011). Another research also observed that for protein samples from *Pheretima posthuma* the peptide showed more activity towards gram-positive strains like that of *B. cereus* followed by *S. aureus*. Whereas the gram-negative proteins showed less sensitivity with *E. coli* having the least antibacterial impact, similar to our

study (Hussain *et al*, 1868). It was also noted that the antibacterial was directly proportional to the concentration of the samples.

Therefore, it can be concluded as the samples we used have more antibacterial activity towards gram-positive bacteria such as *B. subtilis* bacteria.

6. SUMMARY AND CONCLUSION

The utilization of earthworms as a potential source of active pharmaceutical ingredients (APIs) represents an innovative and promising area of exploration within modern pharmaceutical research. In an era marked by a growing interest in natural products and complementary therapies, the investigation into earthworm-derived substances offers fresh opportunities for drug discovery and development. Of particular interest are antimicrobial peptides (AMPs) obtained from species such as *Perionyx excavatus*, which have exhibited a wide range of bioactive properties including anti-inflammatory and antimicrobial. However, to fully harness the therapeutic potential of these AMPs, it is imperative to delve deeper into their characterization, expression patterns, and physiological roles within earthworms. This involves comprehensive studies encompassing biochemical and biophysical analyses, expression profiling under various physiological conditions, rigorous bioactivity assays, safety evaluations, formulation optimization, mechanistic elucidation, and progression to preclinical and clinical trials. Through such multidisciplinary investigations, researchers aim to unlock the therapeutic potential of earthworm-derived substances, contributing to the development of novel pharmaceutical interventions and advancing our understanding of natural product-based therapies.

7. REFERENCES

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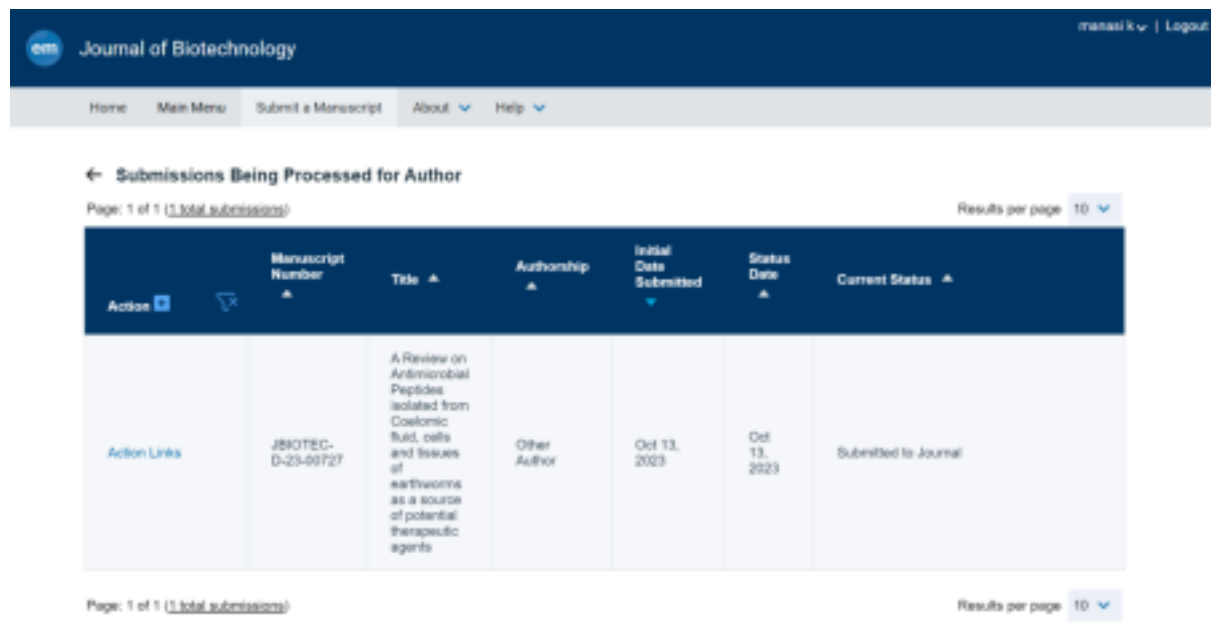
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ANNEXURE

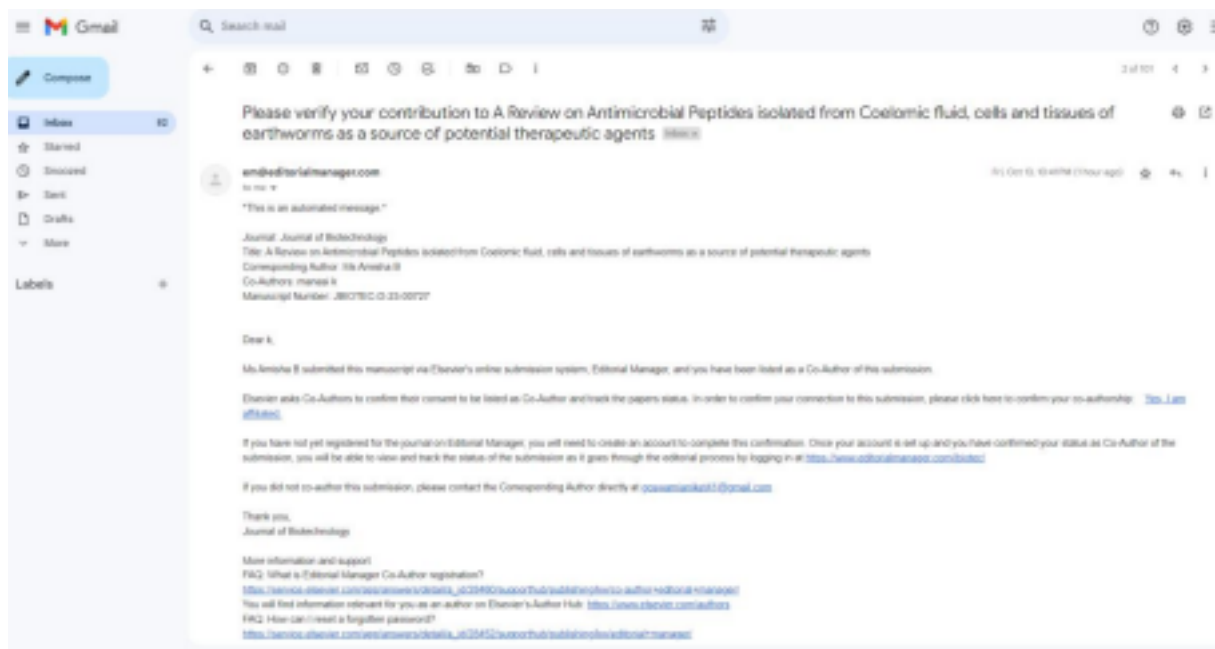
i) PROOF OF SUBMISSION OF REVIEW ARTICLE



The screenshot shows the 'Submissions Being Processed for Author' page in the Editorial Manager system for the Journal of Biotechnology. The page displays a table with one submission entry. The submission details are as follows:

Action	Manuscript Number	Title	Authorship	Initial Date Submitted	Status Date	Current Status
Action Links	JBOTEC-D-23-00727	A Review on Antimicrobial Peptides isolated from Coelomic fluid, cells and tissues of earthworms as a source of potential therapeutic agents	Other Author	Oct 13, 2023	Oct 13, 2023	Submitted to Journal

ii) EMAIL FOR SUBMISSION OF REVIEW ARTICLE



iii) GATE SCORE CARD



GRADUATE APTITUDE TEST IN ENGINEERING 2024

अभियांत्रिकी स्नातक अभिक्षमता परीक्षा २०२४

ORGANISING INSTITUTE: INDIAN INSTITUTE OF SCIENCE, BENGALURU

SCORE CARD

Name of the Candidate MANASI K A			
Name of the Parent/Guardian ANUP KUMAR MADHAVAN			
Registration No.		BT24S57407035	
Test Paper Biotechnology (BT)			
Date of Examination		February 10, 2024	
GATE Score	188	Marks out of 100	28.0
All India Rank (AIR) in the test paper	6943	Qualifying Marks	
		General	38.9
		EWS/OBC-NCL	35.0
Number of candidates appeared for the test paper	17078	SC/ST/PwD	25.9

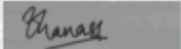


Prof. Chandra Sekhar Seelamantula
Organising Chairperson, GATE 2024
On behalf of NCB-GATE
Ministry of Education (MoE)



A candidate is considered **qualified** if the marks secured are greater than or equal to the qualifying marks mentioned for the category, for which a valid category certificate, if applicable, must be produced along with this Score Card.

This Score Card is valid up to 31st March 2027.

GATE SCORE COMPUTATION

The GATE 2024 score is calculated using the formula

$$\text{GATE Score} = S_0 + (S_1 - S_0) \frac{(M - M_0)}{(M_t - M_0)}$$

where

M is the marks obtained by the candidate in the paper mentioned on the GATE 2024 Score Card

M₀ is the qualifying marks for general category candidates in the paper

M_t is the mean of marks of top 0.1% or top 10 (whichever is larger) of all the candidates who appeared for the test paper

S₀ = 350, is the score assigned to M₀

S₁ = 900, is the score assigned to M_t

M_q is 25 marks (out of 100) or $\mu + \sigma$, whichever is greater. Here, μ is the mean and σ is the standard deviation of marks of all the candidates who appeared for the test paper.

Qualifying in GATE 2024 does not guarantee admission to a postgraduate program or scholarship/financial assistance. Admitting institutes may conduct additional tests or interviews for final selection of candidates.

Graduate Aptitude Test in Engineering (GATE) 2024 was organised by Indian Institute of Science, Bengaluru, on behalf of National Coordination Board (NCB) - GATE for the Department of Higher Education, Ministry of Education (MoE), Government of India.

iv) IELTS score card

IELTS™**Test Report Form**

ACADEMIC

NOTE Admission to undergraduate and post graduate courses should be based on the ACADEMIC Reading and Writing Modules.
GENERAL TRAINING Reading and Writing Modules are **not** designed to test the full range of language skills required for academic purposes.
It is recommended that the candidate's language ability as indicated in this Test Report Form be re-assessed **after two years** from the date of the test.

Centre Number

IN855

Date

23/SEP/2023

Candidate Number

361411

Candidate Details

Family Name

KULATHINGAL ANUP KUMAR

First Name

MANASI

Candidate ID

V5076593



Date of Birth

02/08/2002

Sex (M/F)

F

Scheme Code

Private Candidate

Country or Region
of OriginCountry of
Nationality

INDIA

First Language

MALAYALAM

Test Results

Listening

9.0

Reading

9.0

Writing

7.0

Speaking

7.5

Overall
Band
Score

8.0

CEFR
Level

C1

Administrator Comments**Centre stamp****Validation stamp**Administrator's
Signature

Date

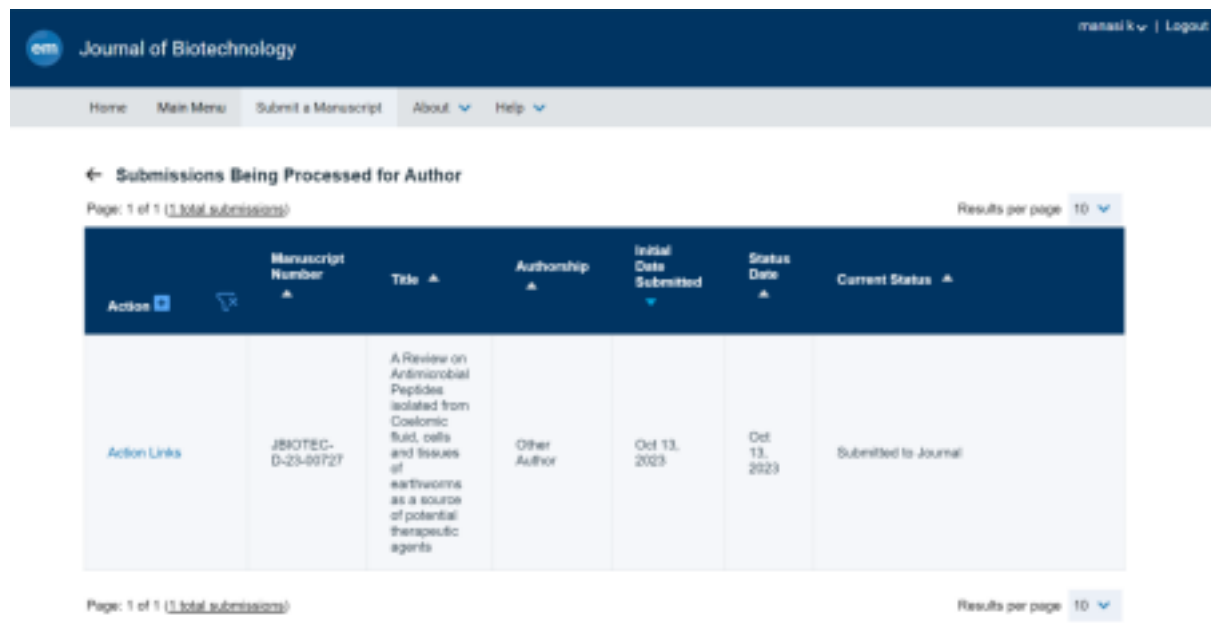
05/10/2023

Test Report Form
Number

23IN361411KULM855A

ANNEXURE

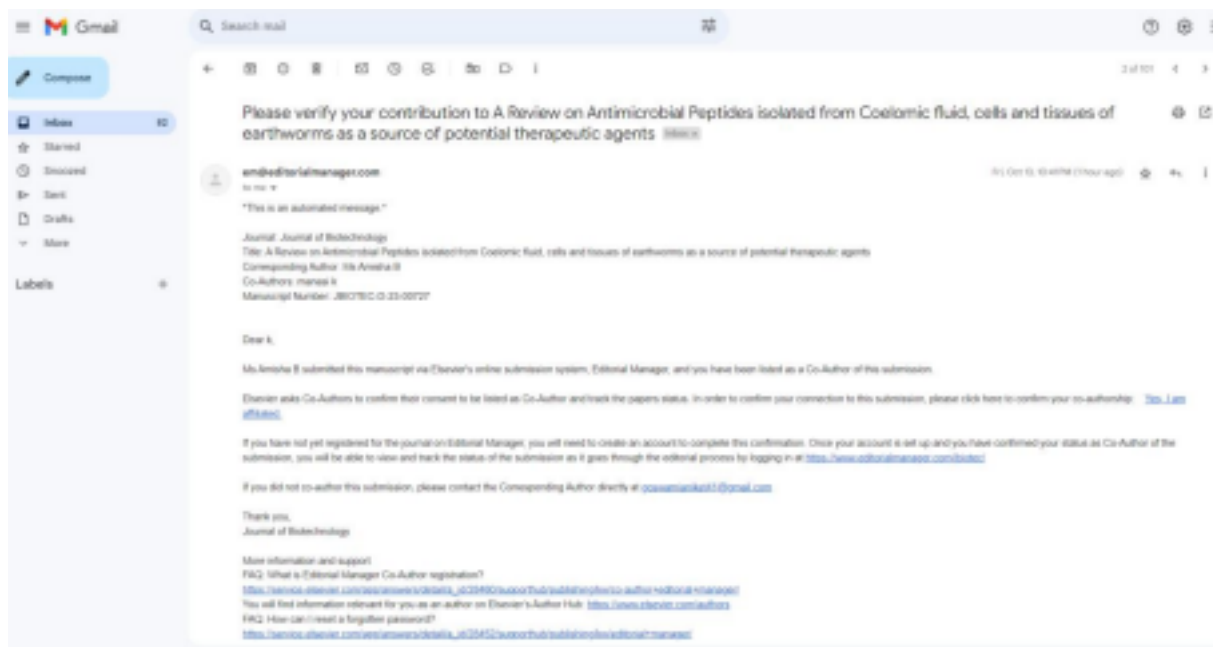
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GRADUATE APTITUDE TEST IN ENGINEERING 2024

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ORGANISING INSTITUTE: INDIAN INSTITUTE OF SCIENCE, BENGALURU

SCORE CARD

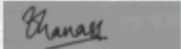
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05/10/2023

Test Report Form
Number

23IN361411KULM855A



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

[DEEMED TO BE UNIVERSITY]

Accredited "A++" Grade by NAAC | 12B Status by UGC | Approved by AICTE

www.sathyabama.ac.in

Name of the student : Manasi K.A

Register Number : 40230024

Title of the Thesis : "Antimicrobial Peptides(AMPs) Isolated from Coelomic Fluid, Cells and Tissues of Earthworms as a Source of Potential Therapeutic Agents

Internal Guide Name : Dr. S. Usha Nandhini

External Guide Name : Dr. S Johnson Retnaraj Samuel

PO / PSO / PEO / SDG	Justification
PEO 1 & PSO1:	The project directly relates to excelling globally with strong fundamentals and domain knowledge by addressing technological problems in the healthcare domain. By exploring earthworm-derived AMPs and their potential as therapeutic agents, the project aims to provide effective solutions for combating infections, which is crucial in healthcare.
PEO 2:	The project demonstrates commitment to research aptitude, lifelong learning, and investigative approach by delving into the earthworm's innate immune response and isolating AMPs with potent antimicrobial properties. This involves continuous learning and investigation to understand and harness the potential of these peptides.
PEO 3 & PEO 9:	The project involves conducting investigations of complex technological problems (such as isolating and characterizing AMPs) and working efficiently in teams (e.g., collaborating with experts in biology, biochemistry, and biotechnology). Strong managerial and communication skills are essential for coordinating research efforts and disseminating findings effectively.
PEO 6 & PEO 7:	Understanding the societal impact and the need for sustainable development are critical aspects addressed by this project. Developing novel antimicrobial therapies contributes to societal well-being and addresses the global challenge of antimicrobial resistance. Moreover, exploring natural sources like earthworms aligns with sustainability goals.



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PEO 8:	The project underscores the importance of professional ethics and responsibilities by seeking to develop therapies that are effective against pathogens while minimizing the risk of inducing microbial resistance. Adhering to ethical standards is crucial in the research and development of biotechnological solutions.
PEO 12:	The project exemplifies the need for lifelong learning by engaging in independent research on technological changes and advancements. As new insights are gained from this project, researchers must continuously update their knowledge and skills to stay abreast of developments in the field.
PO2 & PO4:	Performing tests and assays to establish antibacterial and antimicrobial properties involves problem analysis, experimentation, data analysis, and interpretation. These activities contribute to the investigation of complex technological problems and the synthesis of information to provide valid conclusions.
PO5:	Utilizing modern tools and techniques for testing and characterizing the antimicrobial properties of AMPs demonstrates the application of appropriate techniques and resources to complex engineering activities. This includes the use of prediction models and experimental assays to assess the efficacy of the isolated peptides.
PO6:	Contributing to societal health and safety by evaluating the antimicrobial properties of AMPs aligns with the responsibility of engineers to address societal issues. Understanding the impact of engineering solutions, such as novel antimicrobial therapies, in a societal context is crucial for ethical and effective engineering practice.
PO10:	Communicating the results of tests and assays effectively to the engineering community and society at large is essential. This involves comprehending and conveying technical information through reports, presentations, and instructions. Clear communication ensures that the significance and potential applications of the research findings are understood by relevant stakeholders.



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PO11:	Project management aspects are also evident in conducting tests and assays, including planning experimental protocols, managing resources, and overseeing the progress of research activities. Understanding management practices and applying them to research projects is essential for successful project outcomes.
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