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ISOLATION OF ANTI-MICROBIAL PEPTIDES FROM *EISENIA FETIDA*

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ABSTRACT

Antimicrobial peptides are potential alternatives to antibiotics. Antimicrobial peptides are there in Earthworms as they survive in environment with huge microorganisms. Novel antimicrobial peptides were isolated and characterized from earthworm *Eisenia fetida* coelomic fluid and tissue homogenate. The antimicrobial peptides were purified by Ammonium sulphate precipitation & Buffer exchange, anion exchange, gel filtration, HPLC upto single peptide level. The purified peptides were 6.5KD and 2.5KD in coelomic fluid and tissue homogenate respectively with good antimicrobial activity on gram negative (*Escherichia coli, Salmonella, Pseudomonas*), gram positive (*Staphylococcus aurues, Bacillus subtilis*) and fungi (*Aspergillus niger, Candida albicans, Aspergillus flavus*) without haemolytic activity.

Key words:

Antimicrobial peptides, Coelomic fluid, Tissue homogenate, *Eisenia fetida*, Earthworm

Introduction:

Earthworms are one of the important organisms in the soils of temperate region (Hussain et al. 2021).Their body is long, segmented and contain a true coelom which is filled with a fluid called coelomic fluid. The body wall contains a thin layer of mucopolysaccharide which comprises proteins, performing antimicrobial activity (Péter Engelmann et al., 2020). Earthworms are called "farmer's friend", as these worms enhance the fertility and productivity of soil with burrowing activity (Bhorgin and Uma, 2014). The soil fertility is improved due to air and water penetration through burrows (Katsvairo et al., 2007) and addition of organic and inorganic compounds in the form of nitrogenous waste in the soil through worm casting. In nature earthworms used to reduce pollutants by bioremediation (Wang et al., 2018; Selvi et al., 2019) and degrading toxic compounds with gut enzymes (Rudi et al., 2009; Byzov et al., 2015; Liu et al., 2018). Earthworms are also used good source of protein and widely used in poultry and fish industry (Sogbesan et al. 2007; Parolini et al. 2020). Earthworms have also been utilized in medicines for treatment against various diseases since 1340 AD (Cooper, 2009; Omar et al., 2012). Earthworm's tissue extracts, coelomic fluid and body paste possess various protein agents that have been well documented as antiulcer (Prakash et al., 2007), anti-coagulant (Popoviæ et al., 2001), antiviral (Liu et al., 2012), antibacterial (Aydoğdu and Çotuk, 2008; Balamurugan et al., 2010; Chauhan et al., 2014), antifungal (Vasanthi et al., 2013), antitumor (Chen et al., 2007; Hua et al., 2011; Augustine et al., 2018), anti-inflammatory (Balamurugan et al., 2007; Mathur et al., 2011), cytotoxic (Rudrammaji et al., 2008; Endharti et al., 2019), antipyretic and analgesic (Prakash and Gunasekaran, 2011). Earthworms living in the pathogen-abundant environment have anti-microbial peptides against the microbes to defend.Both anionic and cationic antimicrobial peptides were reported in earthworms which differ considerably in basic features, such as their size, the presence of disulfide bonds and structural motifs. These peptides have been shown to exert their antimicrobial activities through either the lipid bilayer of the cell membrane by the formation of multimeric pores or the interaction with DNA or RNA after penetrating into the cell membranes. The antimicrobial activity of these peptides depend upon the amino acid content and length (Péter Engelmann et al., 2020).The most intriguing feature of antimicrobial peptides is that they rarely induce bacterial resistance, which has become a serious problem with conventional antibiotics. Therefore, antimicrobial peptides have emerged as one of the most promising alternative candidates to antibiotics. Hence an attempt was made to isolate and characterize antimicrobial peptides from *Eisenia fetida*.

MATERIALS AND METHODS

Animals and bacterial strains

The experiments were conducted with earthworm *Eisenia fetida*. Earthworms (*Eisenia fetida*) were collected from culturing unit and were nearly at the same age. Microbial strains used for determining antimicrobial activity included *Escherichia coli*, *Salmonella*, *Pseudomonas*, *Staphylococcus aureus*, *Bacillus subtilis*, *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* which were isolated and maintained in our laboratory. For the bacteria Luria-Bertani (LB) medium and for fungi Potato dextrose agar were used as growth medium. HPLC grade solvents from Qualigens and AR grade chemicals from MERK were obtained.

Earthworm Coelomic fluid collection

Earthworms were cleaned with distilled water in order to remove dirt from their body surface. Their guts were emptied for 2 days on moist filter paper in broad and deep plastic tray, covered with polythene sheet having tiny holes. Earthworms were again washed with water and placed on dry filter paper in order to remove excess water from body surface. 250g of earthworms were weighed and placed in plastic bag. For heat shock, 200ml warm water in a thick plastic bag having a temperature of 45^{O}C-50^{O}C was placed on the earthworms bags, for 5 minutes. The warm water stimulates the earthworms to secrete coelomic fluid from dorsal pores (Patil and Biradar, 2017). Cold shock was given to earthworms, 10 minutes after the heat shock. Ice pack was placed over the earthworms bag in order to give cold shock. Ice pack lowered the temperature of worms which made it to secrete coelomic fluid. Further the worms were excited with a 5V electric stimulation twice, to extrude the remaining coelomic fluid. Earthworms were removed from bag and coelomic fluid was transferred to sterilized eppendorfs and centrifuged at 5000rpm for 10 minutes to remove contaminants and debris. The supernatant was filtered with 0.2micron syringe filter. The filtered coelomic fluid was stored at -20C in sterile eppendorfs.

Earthworm tissue homogenation

Earthworms (*Eisenia fetida*) were collected from culturing unit and subjected to depuration on filter paper for 3 hours. Earthworms subjected to depuration on filter paper for 3 hours, tissue was homogenized in 0.1 M Phosphate Buffer (pH- 7.5) (10% W/V) using a homogenizer equipped with Teflon pestle. The homogenate obtained was centrifuged at 5000×g for 10 min (Beckman Coulter, TLX-361544). The supernatant obtained was further subjected to centrifugation at 5000×g for 10 min. Supernatant that was obtained at the end of the second round of centrifugation was used for Protein estimation, PAGE, anti-microbial activity and further purification.

Ammonium sulphate precipitation

The supernatants were precipitated with ammonium sulphate at a final saturation of 85% in a cooling bath on top of a magnetic stir plate and kept in a refrigerator overnight at 4 °C followed by centrifugation at 10,000 rpm for 30 min at 4 °C. The precipitate was collected and dissolved in pH 6.8 PBS (5 mM) and buffer exchanged with Phosphate buffer.

Purification of the peptide

Anion exchange chromatography

After ammonium sulphate precipitation and buffer exchange, samples were loaded onto a DE-52 column (1.6 cm×30 cm) previously equilibrated with PBS (Phosphate-buffered saline) (5 mM, pH

8.0) containing 10 μM EDTA. After washing with PBS (5 mM, pH 8.0) until the UV absorbance returned to baseline, the bound peptides were eluted with a linear gradient of NaCl (0–500 mM) in PBS (5 mM, pH 8.0) at a flow rate of 60 ml/h. The elution profile was verified at 220 nm. The fractions of each peak were collected in eppendorfs. The antimicrobial activity of these peptides was tested using well diffusion method.

Gel filtration

Fractions with antimicrobial activity after DE-52 anion exchange chromatography were further purified using Sephadex G-10 (2 cm×100 cm) column. Loaded the active fraction gently onto the surface of column. After loading, washed with 3 ml of 50% methanol (V/V) without disturbing the column bed. Then eluted with 50% methanol at a flow rate of 60 ml/h, monitored the absorbance at 220 nm, and collected the fractions 1ml/minute in eppendorfs. The antimicrobial activity of the fractions was tested using the well diffusion method.

HPLC purification of the peptide

The active fractions after the Sephadex G-10 gel filtration were further purified by reverse phase high-performance liquid chromatography (HPLC) on C18 column (Discovery) 4.6mm ×150 mm connected to an HPLC system with a simple linear gradient from 0 to 70% acetonitrile + 0.1% TFA(v/v) at a flow rate of 0.8ml/min at ambient temperature. The elution pattern was monitored at 220 nm. The peak obtained was collected and assayed for antimicrobial activity using well diffusion method.

Tris-Tricine SDS Page:

Purified peptides were separated by Tris Tricine SDS PAGE whereas crude extracts were separated by normal Tris Glycine SDS PAGE. Protein preparation was done by mixing 40microlitres of protein sample with 10microlitres of disruption buffer, and then boiled the mixture for 3minutes at 99°C. SDS-PAGE disruption buffer contained 10% (w/v) SDS, 1M tris/ Hcl, pH 6.8, Glycerol, B-Mercaptoethanol, Bromophenol blue. In the polyacrylamide gel preparation, firstly, separation gel was prepared, and then stacking gel was prepared. Running buffer used here was tris trycine buffer (25mM Tris, 25mM Tricine and 0.05% SDS, pH 8.8). Gel was stained using staining buffer which contained Glacial acetic acid, Methanol, Coomassie brilliant blue 250-R, destained using De-staining buffer which consisted of Glacial acetic acid and methanol.

Protein estimation:

The protein concentration of the extract was estimated by the Lowry method, BSA was used for plotting standard curve (Lowry et al., 1951).

Antimicrobial activity assay:

Antimicrobial activity assay was done by well diffusion method. Pure cultures of *E.coli*, *Salmonella*, *Pseudomonas*, *S.aureus*, *B.subtilis*, *A.niger*, *A.flavus and* C.albicans were spread on nutrient agar/PDA plates. Different peptide samples (crude peptides, an anion exchange chromatography purified peptides, gel filtration purified peptides and HPLC purified peptides of both coelomic fluid and tissue homogenate) of 50μg were loaded in the wells and incubated at 37°C for 24hr.

Haemolytic activity assay:

The blood with citrate was added to 0.5M, pH 7 PBS and centrifuged at 5000rpm for 10min. Then sedimented RBC was washed with PBS till clear supernatant is seen. Silmultaneously, test samples were prepared in PBS to give 100μg/ml. The blood solution is then added to sample solution in different tubes and incubated at 37°C for 24 hrs. Then they were again centrifuged and supernatants were subjected to absorbance at 415nm.

Statistical analysis:

Experiments were repeated thrice in triplicates (n = 9) and average values with standard deviation was provided.

Results:

Purification of the peptide

Anion exchange chromatography:

Protein was found in wash and eluted fractions of anion exchange chromatography. Antimicrobial activity was observed only in eluted fraction of coelomic fluid and washed fraction of tissue homogenate. Hence only active fraction was used for further studies. Active peptide was eluted with retention time of 24 minutes in coelomic fluid. The active peptide was extracted from wash in tissue homogenate (Fig.1).

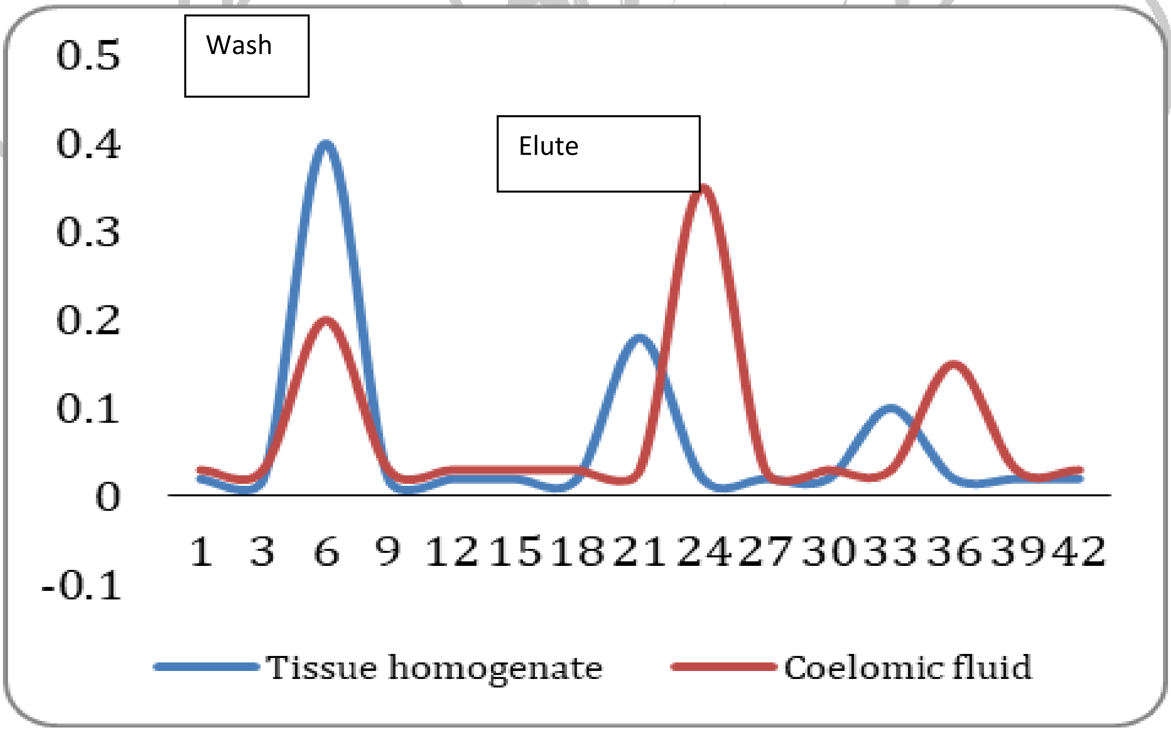


Fig.1: Anion exchange chromatography.

Gel filtration:

The active peptides obtained from coelomic fluid and wash of tissue homogenate in ion exchange chromatography were further purified by Sephadex G-10 gel filtration separately. In both the samples major fractions were eluted in later time of chromatography indicating presence of small peptides whereas few proteins with early elution with high molecular weight were also observed (Fig.2). Coelomic peptide eluted with retention time of 18 minutes and tissue homogenate protein eluted with retention time of 21 minutes.

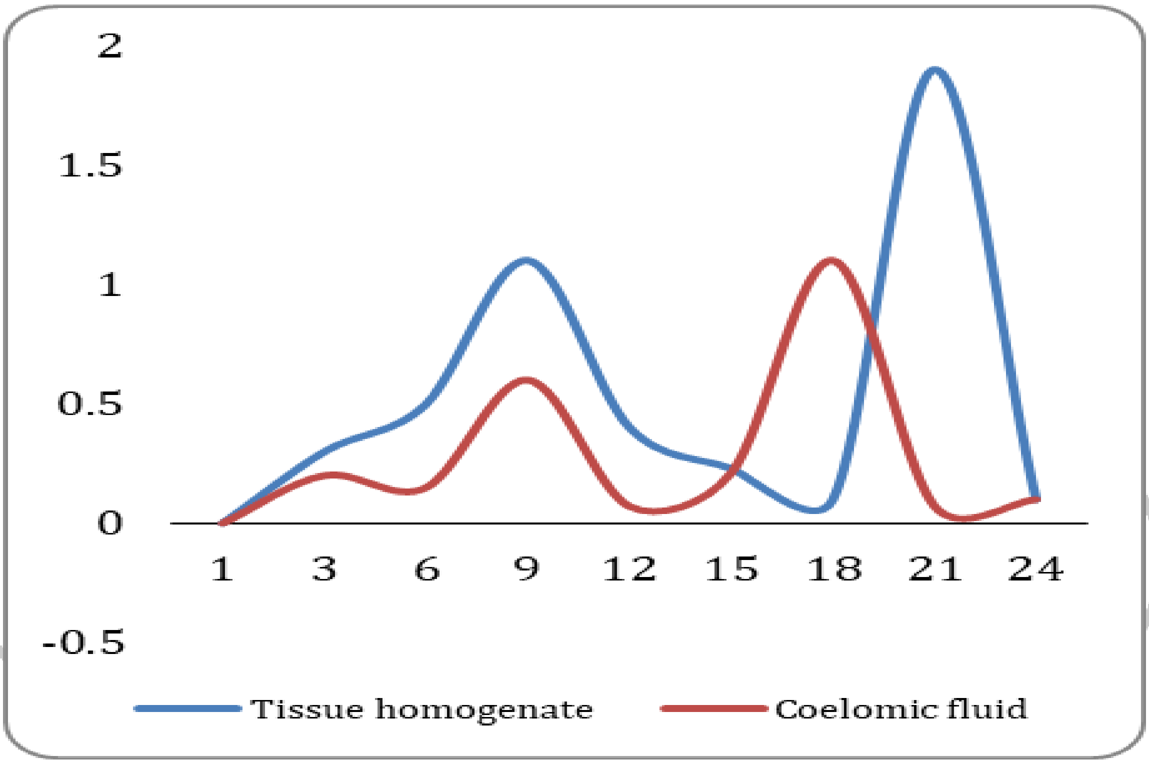


Fig.2: Gel filtration chromatography.

HPLC purification of the peptide:

The most active peptides obtained in coelomic fluid and tissue homogenate gel filtration were further subjected to C18 reversed-phase HPLC. Eluted single peptide fraction with retention time of 12 minutes in tissue homogenate and 6 minutes in coelomic fluid (Fig.3).

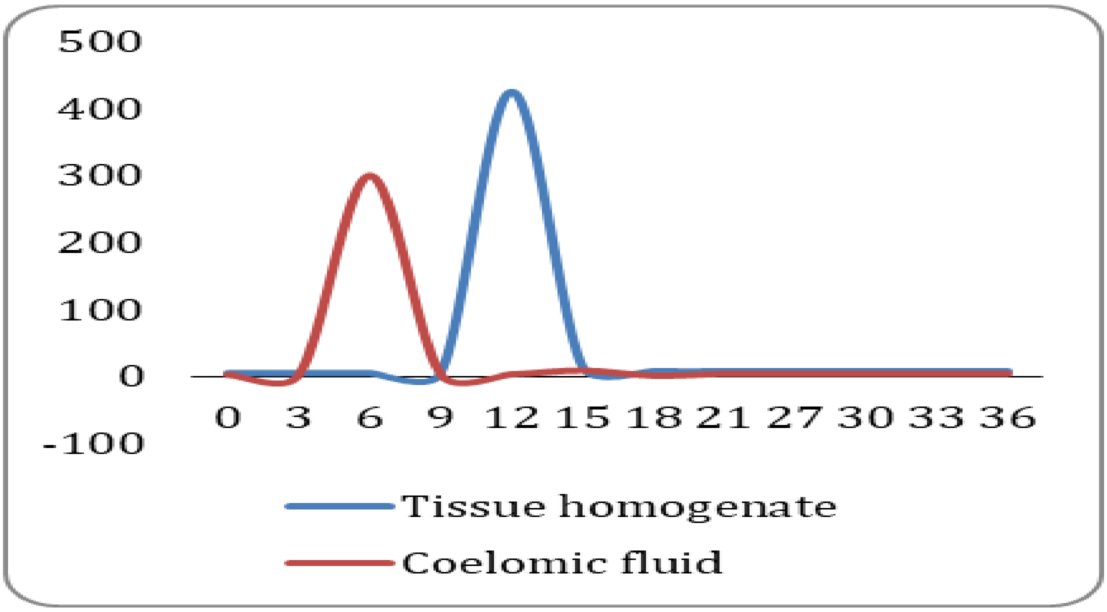
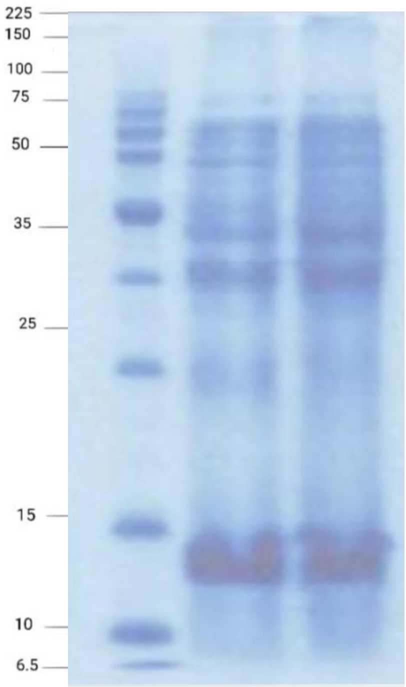


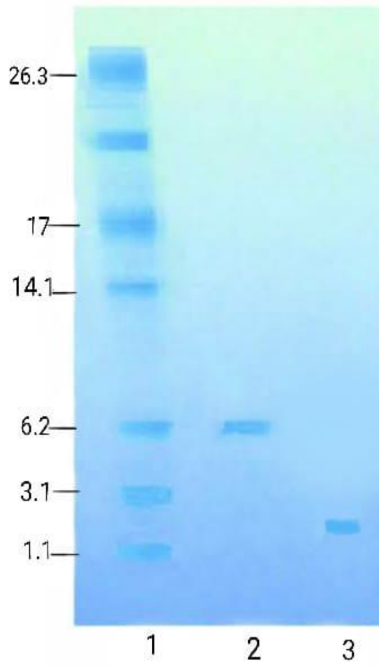
Fig 3: Purification of peptides by HPLC.

Tris-Tricine SDS PAGE:

Low molecular weight proteins or peptides were separated by this method. Active peptide of coelomic fluid was found to be 6.5KD and active peptide of tissue homogenate was found to be 2.5KD, as confirmed by Tris-Tricine PAGE of HPLC samples. Fig 4(a) displays the band patterns of crude peptide samples of both coelomic fluid and tissue homogenate. Fig 4(b) displays the band patterns of HPLC purified peptides of both coelomic fluid and tissue homogenate.



a



b

Fig 4 (a): Band patterns of crude peptides in SDS PAGE

Fig 4 (b): Band patterns of HPLC purified peptides in Tris-tricine SDS PAGE.

Protein purification efficiency:

The protein concentration, protein volume, total protein and recovery percentage at each step of both coelomic fluid and tissue homogenate were given in table 1(a) and table 1(b) respectively. The total recovery in the purification processes of coelomic fluid is 45% and the total recovery in the purification processes of tissue homogenate was 29%.

Table 1(a): Protein purification efficiency in Coelomic fluid

Coelomic fluid	Protein concentration	Protein solution volume	Total protein	Recovery %
Crude	111µg/ml	25	2775	100%
Ammonium sulphate and buffer extraction	122µg/ml	20	2440	87.9%
Ion exchange chromatography	190µg/ml	10	1900	77.8%
Gel filtration	173µg/ml	9	1557	81.9%
HPLC	209µg/ml	6	1254	80.5%

Table 1(b): Protein purification efficiency in tissue homogenate

Tissue homogenate	Protein concentration	Protein volume	Total protein	Recovery %
Crude	182 µg/ml	25	4550	100%
Ammonium sulphate and buffer extraction	205 µg/ml	20	4100	90.1%
Ion exchange chromatography	200 µg/ml	14	2800	68.2%
Gel filtration	185 µg/ml	9	1665	59.4%
HPLC	220 µg/ml	6	1320	79.2%

Antimicrobial activity assay:

The antimicrobial activity assay of all the peptide samples (crude, ion exchange purified, gel filtration purified, HPLC purified) of both coelomic fluid and tissue homogenate was done using *E.coli, Salmonella, Pseudomonas, S.aureus, B.subtilis, A.niger, A.flavus and C.albicans*. The inhibition zones were measured in (mm). Coelomic fluid was found to form larger inhibition zones than tissue homogenate. The antimicrobial activity assay of peptides at different purification stages was given in table 2.

Table 2: The Antimicrobial activity assay of peptides at different purification stages.

Peptide samples		Inhibition zones(mm)							
		E.coli	*Salmonella*	*Pseudomonas*	*S.aureus*	*B.subtilis*	*A.niger*	*A.flavus*	*C.albicans*
Crude	Coelomic fluid	14 ± 0.2	12 ± 0.8	11 ± 0.5	14 ± 0.2	23 ± 0.3	25 ± 0.8	12 ± 0.8	16 ± 0.7
	Tissue homogenate	10 ±0.4	9 ± 1.2	9 ± 0.4	12 ± 1.1	21 ± 0.8	16 ± 1.2	8 ± 0.7	12 ± 1.2
Anionexchange chromatography	Coelomic fluid	18 ±0.5	15 ± 1.1	15 ± 0.1	17 ± 0.8	27 ± 1.1	29 ± 1.1	15 ± 1.2	19 ± 1.1
	Tissue homogenate	12 ±0.1	13 ± 0.8	14 ± 0.9	16 ± 0.7	26 ± 0.9	20 ± 0.7	13 ± 1.1	15 ± 0.9
Gel filtration	Coelomic fluid	16 ±0.8	13 ± 0.7	13 ± 0.6	15 ± 1.2	24 ± 0.8	27 ± 0.6	13 ± 1.2	17 ± 1.1
	Tissue homogenate	11 ±0.9	11 ± 0.6	12 ± 1.1	13 ± 0.7	22 ± 0.7	17 ± 0.4	10 ± 0.7	13 ± 1.2
HPLC	Coelomic fluid	23 ±0.5	19 ± 1.1	20 ± 1.2	22 ± 0.8	32 ± 0.6	33 ± 0.8	19 ± 0.6	23 ± 0.8
	Tissue homogenate	16 ±0.6	15 ± 1.2	17 ± 1.1	20 ± 1.2	31 ± 0.7	24 ± 0.5	18 ± 0.5	20 ± 1.2

Haemolytic activity assay:

All samples tested (crudes, ion exchange purified peptides, gel filtration purified peptides and HPLC purified peptides) didn't show any change in O.D as there was no lysis of RBC's, hence doesn't have haemolytic activity.

Discussion:

Antimicrobial peptides comprises key component of protection in living organisms (Zasloff M., 2002). All living organisms are found to produce anti microbial peptides, which play a significant role in protection from microbial invasion. Earthworms live in an environment which is contaminated by microbes, to defend them, they produce antimicrobial peptides in their body (Prakash and Gunasekaran, 2011). In present study, the isolation and purification of antimicrobial peptides from coelomic fluid and tissue homogenate of earthworm *Eisenia fetida* is reported. Purification and partial characterization of a novel peptide from coelomic fluid of *Eisenia fetida* was reported by Yan-Qin Liu et al. (2004). The antimicrobial potential of Coelomic fluid and tissue homogenate was determined in this study, *E.coli*, *S.aureus* and *A.niger* were sensitive to Coelomic fluid and Tissue homogenate. The findings of Bansal et al. (2015) report that *S.aureus, P. aeruginosa* and *E.coli* were resistant to coelomic fluid and tissue homogenate. The antimicrobial potential was because of presence of number of bioactive compounds (proteins and peptides) in *Eisenia fetida*, which act as antimicrobial agent (Patil and Biradar, 2017). Coelomic fluid of *Eisenia fetida* reported to have 0.51KD anionic antimicrobial peptides ACSAG (Yan-Qin Liu et al., 2004). In the present study, anionic 6.5KD peptide in coelomic fluid and cationic 2.5KD peptide in tissue homogenate were observed with antimicrobial, antifungal, non-haemolytic activities. Coelomic fluid and body paste were found almost similar antimicrobial activity of *Pheretima posthuma* (Hussain et al., 2023). *Lumbricidin* 1, a proline rich antimicrobial peptide of *Lumbricus* is 7.5KD antimicrobial peptide (Cho et al., 1998). In the present finding, coelomic fluid contains anionic antimicrobial peptides whereas tissue homogenate contains cationic antimicrobial peptides.

This paper describes short antimicrobial peptides 2.5KD and 6.5KD in *Eisenia fetida* with possible exploring them for treating antibiotic resistant microbes.

Conclusion:

Novel antimicrobial peptides were isolated and characterized from earthworm *Eisenia fetida* coelomic fluid and tissue homogenate. The antimicrobial peptides were purified by, Ammonium sulphate precipitation & Buffer exchange, anion exchange, gel filtration, HPLC upto single peptide level. The purified peptides were 6.5KD anionic peptide in coelomic fluid and 2.5KD cationic peptide in tissue homogenate with good antimicrobial activity against gram negative, gram positive bacteria and fungi without haemolytic activity.

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