Changes in the Protein Profiling of Earthworms Cultured in Toxic Substrates

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ABSTRACT

In the present study, the coelomic fluid amino acids, protein profiling and antibacterial studies were performed under toxic plant and news paper ink stress condition. The amino acid profile of two species of earthworms namely, *Eudrilus eugeniae* and *Lampito mauritii* were evaluated using qualitative amino acid analysis to determine the both essential and non-essential amino acids present in the coelomic fluid of earthworms at different stress conditions. The changes in protein concentration and the number of proteins identified by SDS-PAGE. The number of proteins expressed gets varied between normal, toxic plant and news paper ink induced earthworms. A background protein profile has now been established for normal and stress condition. Future research will investigate the influence of other external agents on proteins involved in the immune response to infection, including identification of relevant bactericidal proteins .These results should provide important information about the effects of inorganic and organic toxicity, and expand the potential use of this experimental animal as a biomarker model organism.

Keywords:
Earthworms, News paper ink, Protein profile, *Ficus microcarpa*, *Strychnos nux-vomica*.

INTRODUCTION

Earthworms are very important soil animals that aerate the soil with their burrowing action and enrich the soil with their waste products. They enhance soil nutrient cycling, the activity of other beneficial soil organisms, and soil physical properties. Earthworms are very sensitive to anthropogenic contaminants and have been used as an indicator soil animal for estimating soil pollution (Nahmani and Rossi, 2003; Li and Cheng, 2006). Recently earthworm protein and its coelomic fluid were reported to have cytolytic, agglutinating, proteolytic, hemolytic, anti-pyritic, tumor static and antibacterial activities (Franken *et al*., 2006; Daniel *et al*., 2013).

Extensive literature on the basic biology and ecology of earthworms is available (Karmegam *et al.*, 2010). Earthworms have been used in laboratory and *in situ* acute toxicity and bioaccumulation studies. Standard laboratory exposure protocols have been developed

for earthworms. They are cost effective and socially no controversial research organ. They are relatively easy to conduct laboratory and field research with and to maintain and from hazardous waste sites. Their morphology and behavior enable direct exposure in the laboratory or *in situ* to complex mixtures of contaminants and matrices found in or near hazardous waste sites (Jeyanthi *et al.,* 2016; Yuvaraj *et al.,* 2020). Their high surface an area/volume ratio, feeding and behavior facilitates uptake of contaminants. Ability to collect immunocytes allows for *in vitro* exposure of immune cells to toxicants. Their tissue are readily compartmentalized and easily isolated for chemical characterization by conventional analytical techniques, enabling determination of actual tissue level dose and biological response profiles. They are sufficiently complex for use as surrogates in immune toxicity based research aimed at assessing the immune toxic potential of chemicals in higher animals because their immune active cells exhibit functions analogous or homologous to those of vertebrates. Even the earthworms are sensitive to nano-scale materials (Biruntha *et al.,* 2020a).

Earthworms are relatively long living organisms in an environment rich of microorganisms, fungi and other potential pathogens. Their successful survival under these conditions is supported by efficient innate immune mechanisms based on cellular activities of coelomycetes and humoral immune proteins, both components of the earthworm coelomic fluid. Coelomycetes are involved in eliminating foreign material by phagocytosis, encapsulation and NK-like activity (Kauschke *et al*., 2007). Glycoproteins of lectin character contribute to recognition of foreign material by binding to carbohydrates and cause its immobilization and destruction by agglutination and lysis. Analysing several earthworm species (*Allobophora chlorotica, Apporectodea caliginosa, Dendrodrillus rubidus, Eisenia fetida, Lumbricus rubellus* and *Lumbricus terrestris*) showed that wounding and injection of foreign material into the coelomic cavity increases coelomocyte numbers and the activity of easily measurable humoral immune factors like agglutinins, lysins and proteases (Eue *et al*., 1998).

The protein profile of earthworm coelomic fluid can be used to assay the proteomic response following exposure to external agents. This protein profile approach, in conjunction with the recognition of the gel locations and function of relevant proteins, has the possibility of increasing use of the earthworm model in scientific research and toxicological studies, Using this methodology, one can conceivably measure the effect of sub-lethal exposures to inorganic and organic toxicants in the normal coelomic fluid protein profiles and more importantly, the ability of the earthworm to take action to outside agents (Bio-Rad Bulletin#2651,2003). The coelomic fluid is generally secreted by the earthworms for maintaining moisture to help their physiological activities such as respiration and burrowing activities. It consists of watery matrix, the plasma and a large number of coelomocytes. These coelomocytes play a very important role in building innate immunity of earthworms, are differentiated into four different types of immune cells such as amoebocytes, mucocytes, circular cells and chloragogen cells, which have different shape, size and have wide variety of functions.

Ficus microcarpa, Moraceae family, (Tamil name: kallathi) was cited in the traditional medicine of many countries. Its dry leaves, aerial roots, and bark were used in folk medicine for reducing fever, relieving pain, and the treatment of liver diseases (Kalaskar and Surana, 2011; Phan *et al*., 2011). Likewise, this plant has other important medicinal potential such as, antipyretic, antioxidant, antibacterial, and analgesic properties (Sirisha *et al*., 2010). *Strychnos nux-vomica* belongs to the family Loganiaceae. It is an energetic poison affecting

the central nervous system. It's a medicine for paralysis and nervous debility generally (Chetan *et al*., 2010). Traditionally it is used for treating acute diarrhoea, mixed with lemon juice and made into pills and taken orally during dysentery, arthritis, rheumatism and piles (Dubey *et al*., 2012). *Strychnos nux-vomica* is such a plant described under the '*Upavisa Vargas*' (semi poisonous group) (Sharma *et al*., 2000) and its seeds have been used successfully in cure of many diseases after proper *Shodhana* (Gogte, 2000). Nux vomica was introduced in Europe in the sixteenth century, but was not used much in medicine, being chiefly employed to poison dogs, cats, crows, and etc. (Williams, 2009).

Newspapers and cardboard boxes used for packaged foods are made of recycled paper that may be contaminated with harmful chemicals like di-isobutyl phthalate and di-n-butyl phthalate which can cause digestive problems and also lead to severe toxicity. Recycled paper also has printing ink residues trapped from previous prints. These trapped residues have found to contain hormone disruptors like benzophenones and mineral oils.

During vermicomposting processes, in general, only physico-chemical parameters in vermicompost and reproductive behavior of the earthworms are analysed (Singh *et al.,* 2020; Biruntha *et al.,* 2020b). The changes accompanying in earthworms physiological systems are least studied. Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation; while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins, peptides based on their amino acid composition to support protein and peptide structure analysis to evaluate fragmentation strategies for peptide mapping and to detect atypical amino acids that might be present in a protein or peptide. The studies on protein profile of earthworms under stress are very limited. Hence the present study has been carried out to find out the protein profile of coelomic fluid of the earthworms, *Eudrilus eugeniae* and *Lampito mauritii* cultured in toxic substrates.

MATERIALS AND METHODS

Experimental animals

The pre-clitellate earthworms of *Eudrilus eugeniae (African species)*, *Lampito mauritii* were selected as the test animals for the present study. Earthworms were obtained from the Krishi Vigyan Kendra, Kundrakudi. Animals were carefully transported to the laboratory and mass multiplied in culture tanks by mixing cow dung powder with water and maintained at a temperature of 28 ± 2°C.

Cow dung and plant material

One week old cow dung was used in experiments because fresh cow dung may not be suitable for earthworms due to the decomposition process and heat generation during initial decomposition. Leaves of *Strychnos nux-vomica* plant were collected from Pudukkottai district. The leaves of *Ficus microcarpa* plant were collected from Sivagangai district. Leaves

was collected from the plant were shade dried. After drying the plant materials were powdered. Powders of the leaves were used for vermibed. Cow dung and plant leaves powder were mixed with 1:1 ratio following the method suggested by Biruntha et al. (2019) and Thangamani *et al.* (2020).

Preparation of earthworm beds

The experimental setup of the present study is given in Fig. 1. All the experiments were carried out under laboratory conditions. For each beds containing, bedding material separately were prepared in plastic container by mixing cow dung and organic waste with water at a ratio of 1:1(W/V) in plastic container, covered with nylon net to prevent escape of earthworms. Four types of experimental beds were prepared as follows: Test 1. Control bed (cow dung powder + 10 numbers of earthworms); Test 2. Toxic plant (*Ficus microcarpa*) treated bed (cow dung powder + leaf litter +10 numbers of earthworms); Test 3. Toxic plant (*Strychnos nux-vomica*) treated bed (cow dung powder + leaf litter + 10 numbers of earthworms); Test 4. News paper bed (cow dung powder + news paper + 10 numbers of earthworms) (Chaudhuri *et al*., 2002).

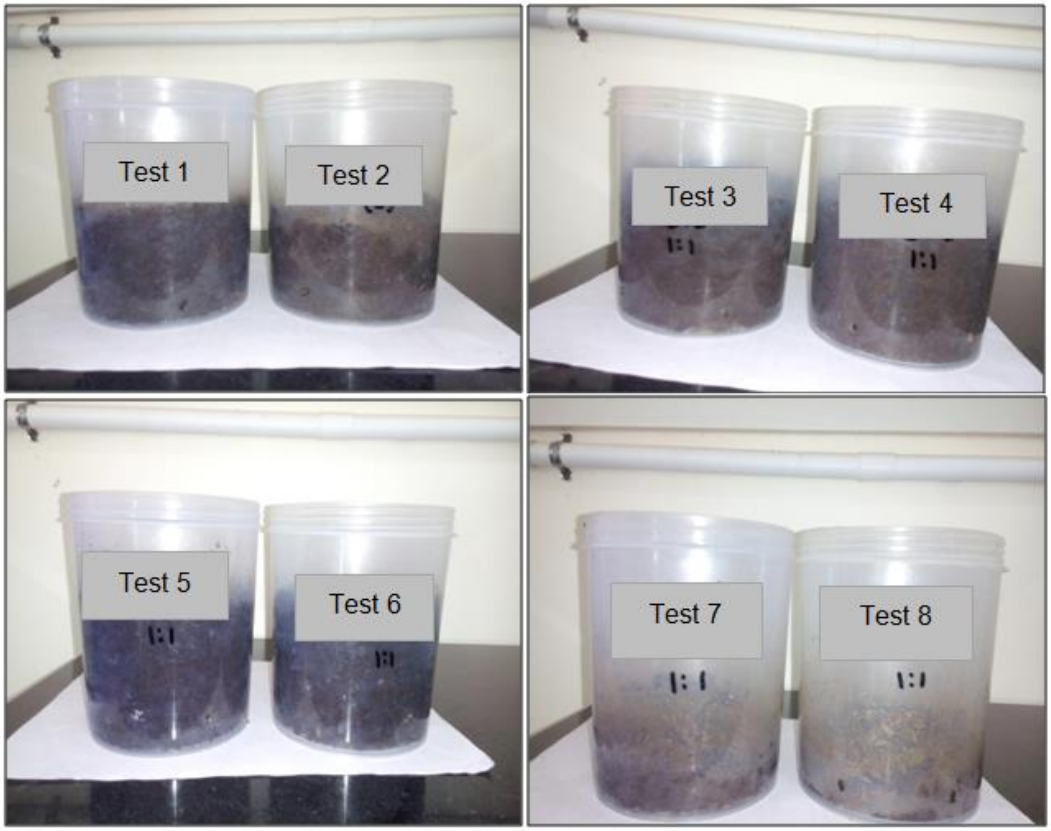

Fig. 1: Experimental setup of the study.

Test 1=cow dung+10 earth worm (*Eudrilus eugeniae*)
Test 2=cow dung+10 earth worm (*Lampito mauritii*)
Test 3=cow dung+10 earth worm *(Eudrilus eugeniae)+ Strychnos nux-vomica*
Test 4=cow dung+10 earth worm *(Lampito mauritii)+ Strychnos nux-vomica*
Test 5=cow dung+10 earth worm *(Eudrilus eugeniae)+Ficus microcarpa*
Test 6=cow dung+10 earth worm *(Lampito mauritii)+ Ficus microcarpa*
Test7 =cow dung+10 earth worm *(Eudrilus eugeniae)*+Newspaper
Test 8=cow dung+10 earth worm *(Lampito mauritii*)+Newspaper

Harvesting of coelomic fluid

Collection of coelomic fluid by cold shock method

Coelomic fluid is obtained by actual washing of earthworms. Earthworms release the coelomic fluid along with mucus through the pores present on the dorsal surface of their body called dorsal pores. This is a natural process to keep their body surface moist that acts as respiratory organ. As the pores remain open all the time, slight stimulation by either cold or hot shock makes them to release the coelomic fluid can be directly collected from the body cavity of earthworms without causing any harm to them. In this method of collecting the fluid, ten earthworms are taken in an approximately 10cm diameter Petri plate and holding the plates in a slanting position and keep earthworms pointing downwards. Cold shock is given to earthworm by gently moving a small beaker containing a few ice cubes. The coelomic fluid released due to cold shock drips and gets collected at the lower side of the Petri plate. This fluid can be pipette out using a sterilized pipette with fine nozzle. This is the pure coelomic fluid that can be used for different biological investigations (Kale, 2006).

Whole body dissection method

Following incubation, worms were removed, placed on a dissection tray, cut into segments post-clitellum, and the segments placed in 15-ml centrifuge tubes. Samples were centrifuged for ten minutes at 13 x g and the supernatant removed and re-centrifuged for ten minutes at 13 x g to remove any remaining particulates contaminating the coelomic fluid. A final five-minute centrifugation at 16 x g was performed to ensure samples contained no solid matter. Samples were stored at –20° C if examined at a later date.

Qualitative amino acid analysis

Several chemical tests were performed to identify the both essential and non essential amino acids present in the coelomic fluid of earthworms. **(**Jayaraman 1968**)**They include Ninhydrin test (for all amino acids), Nitroprusside test (for cysteine and cystine), Xanthoproteic test (for tryptophan), Millon's test (tyrosine) and Ehrlich test.

Determination of coelomic fluid protein concentration

Total crude coelomic fluid protein concentration for samples collected from control and experimental organisms was determined according to the Lowry's method (Lowry *et al*., 1951).

SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

Polyacrylamide gels are the mostly widely and routinely used gel system in labs for analysis of proteins. The protein separation was accomplished using 13% SDS run at 100V for 2 hours. Following this separation, gels were removed from the plate carrier and placed in a plastic tray containing staining solution (0.3g Coomasie blue, 80% methanol, 20% glacial acetic acid) for 2 hours. The staining solution was decanted and 100ml of destaining solution (40% methanol, 10% glacial acetic acid) was added to gel tray. The destaining solution was changed 2-3 times until the bands get clearly visible. The destain was decanted and the gels were washed with distilled water to remove any remaining destaining solution. Then the gel is stored in 10%aceticacid and photographed (Plummer, 1977).

Biological activity of coelomic fluid

The biological activities like antibacterial and hemolytic activity was studied with agar well diffusion method.

Antibacterial activity

The agar well diffusion method (Thomas *et al*., 2014) was followed Gram negative bacteria were used for the study. During the bioactivity method, the Muller Hinton Agar media was prepared and they were sterilized using autoclave. After sterilization, the media was poured in the sterile Petri plates. After solidification the overnight culture *Klebsiella* sp. swabbed in sterile condition A sterile cotton swab was dipped into the broth culture. The cotton swab was then rotated pressing against the inside wall of the tube, above the fluid level to remove the excess inoculums. The agar surface of the plate was inoculated by swabbing three times. Four wells of the 8mm diameter were punched into the agar on each plate using a sterile well cutter. Into each well, 50 mg/ml of different substrate coelomic fluid were added and the solutions were allowed to diffuse for 2 h. The plates were incubated at 37°C for 24-48 h. The antibacterial activity was evaluated by measuring the zone of inhibition around the well.

RESULTS

Earthworm survival and growth

During the present investigation, no mortality and avoidance behavior were noted in the earthworms exposed to toxic plant and newspaper ink treated beds, but there was no significant alteration in body weight of earthworms with respect to the control for the specific period of exposure. The changes in the body weight of two species of earthworms after exposure to toxic plant and newspaper ink stress. Compared with control, the rate of body weight of earthworms was reduced under toxic plant and newspaper ink stress after 7 and 14 days. A number of studies have been conducted on the earthworm *Eudrilus eugeniae.* Some of the responses of earthworms to sub lethal concentrations of toxic plant have reported that the weight of the earthworms was a most sensitive index compared to the mortality in indicating toxic effects.

The qualitative analysis of amino acids

The qualitative analysis of amino acids was carried out to identify the both essential and non-essential amino acids present in the earthworm coelomic fluid at different stress conditions. A total of twenty amino acids consisting of ten essential namely, Arginine, Valine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and ten non-essential amino acids namely Aspartic acid, Serine, Glutamic acid, Proline, Glycine, Tyrosine, Aspargine, Cysteine, Cystine were recorded in this study from each species of earthworm. Notable changes in the concentrations of amino acids were observed between the control and stress conditions (Table 1). Similar observations was made for two species of earthworms namely *Eudrilus eugeniae*, *Lampito mauritii* through high performance of amino acids (Dedeke *et al.*, 2010).

Determination of coelomic fluid protein concentration

The coelomic fluid of protein concentration was determined by method of lowry *et al*. (1951). The 7th day coelomic fluid concentration was slightly decreased in 14 day coelomic fluid concentration (Fig. 2).

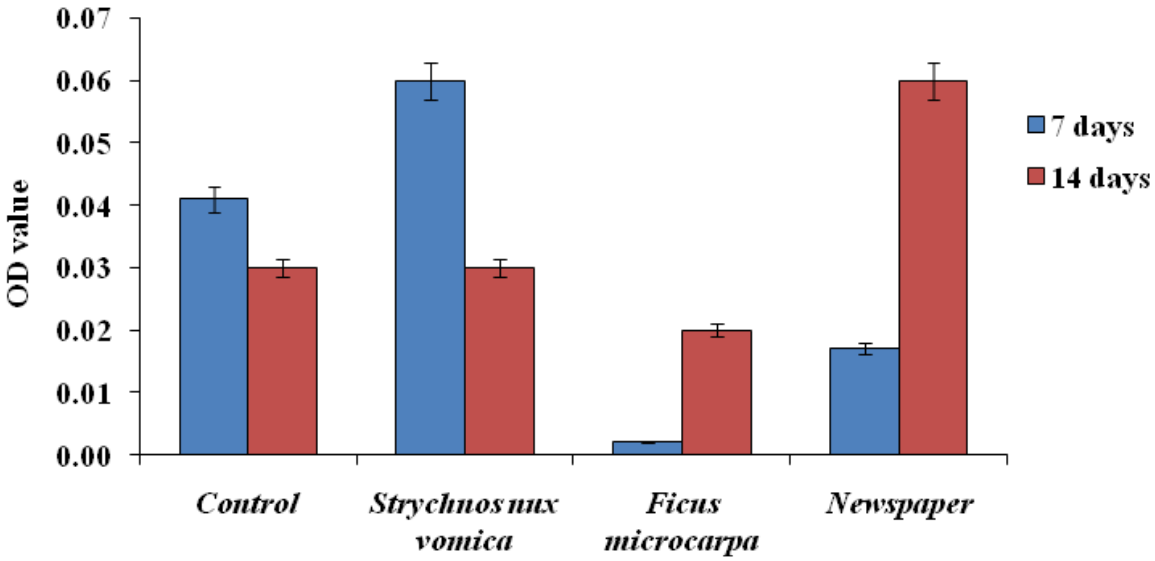

Fig. 2(A): Concentration of coelomic fluid mediated protein (*Eudrilus eugeniae*).

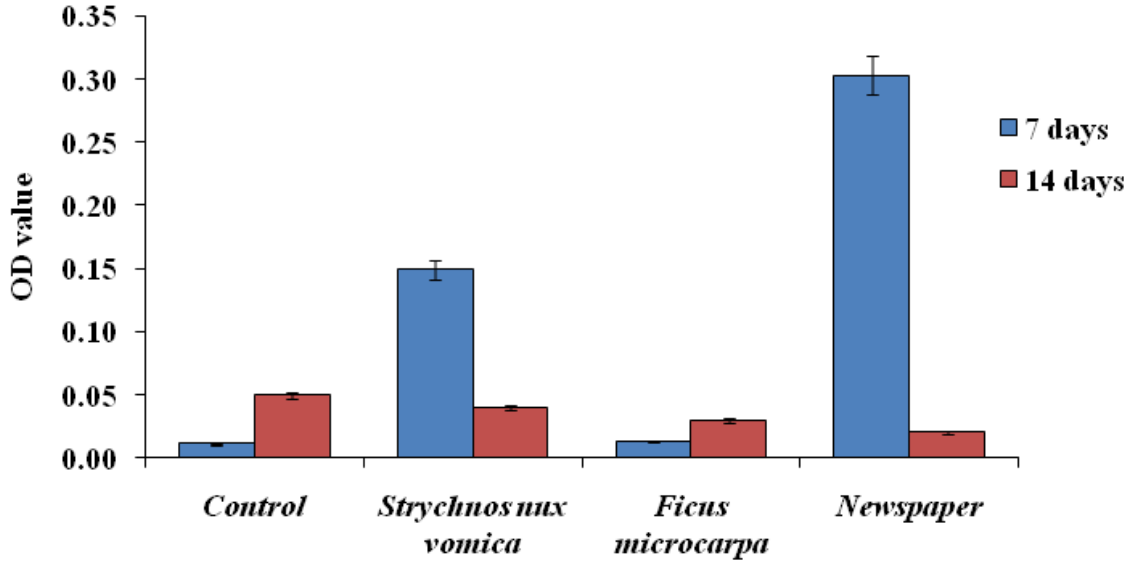

Fig. 2(B): Concentration of coelomic fluid mediated protein (*Lampito mauritii*).

SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

The number of proteins expressed gets varied between normal and stress induced earthworms. The proteomic analysis was done on earthworm *Eudrilus eugeniae* and *Lampito mauritii* during toxic plant and newspaper ink stress. The molecular weight of the coelomic fluid in earthworm species the size of the protein in the fluid was determined as more than 40

KDa in both samples. It showed that the protein was very large in size. *Lampito mauritii* coelomic fluid was determined more than 30 KDa.

Antibacterial activity

The toxic plant treated coelomic fluid was found to have low antibacterial activity against Gram negative bacteria *Klebsiella pneumoniae*. The very low zone of inhibition was measured for *Strychnos nux-vomica* treated coelomic fluid. News paper treated coelomic fluid did not show antibacterial activity. It was found that the diameter of the clear zones were proportional to the volume of the coelomic fluid.

Table1. Qualitative analysis of amino acids in the earthworm coelomic fluid under different type's substratum.

AMINO ACIDS	*Eudrilus eugeniae*				*Lampito mauritii*			
Essential amino acids	Control	*Strychnos nux vomica*	*Ficus microcarpa*	News paper	control	*Strychnos nux-vomica*	*Ficus microcarpa*	News paper
Ariginine	++	++	++	+++	+	++	+	+
Valine	+++	++	+++	+	++	++	+	+
Histidine	+	+	+	++	++	+++	++	+
Isoleucine	+	+++	++	+	+	+	+++	++
Leucine	++	+	++	+++	+++	+	+	+++
Lysine	+	+	+	++	++	+++	++	++
Methionine	+	++	++	+++	+++	+	+	+++
Phenylalanine	+++	+	++	+	++	+++	+	+
Threonine	+	++	++	+++	++	++	+	++
Tryptophan	+++	++	+	++	+	++	+++	+
Non essential amino acids								
Aspartic acid	+++	+	++	+	+	++	+++	++
Serine	+	+	+	++	++	++	+	+
Glutamic acid	+	++	+	+	++	+++	+	++
Proline	+	++	+	+	+++	+	++	+++
Glycine	+++	+++	++	+++	+	+	++	++
Alanine	+	+	+++	+	+	+	+++	++
Cystine	++	++	++	++	++	++	+	+
Tyrosine	+++	+++	+	+++	++	++	+	+
Aspargine	+	++	++	+	+	+	+	+
Cysteine	++	+	++	++	+	+++	++	++

Here, (+ + +) = highly present; (+ +) = moderately present; (+) = less present

DISCUSSION

Earthworm's immunity consists of humoral and cellular components and works as specific and non-specific mechanisms which occur in coelomic fluid (Roch and Cooper, 1991). The coelomic fluid harvested in different methods reveals that the earthworms were punctured in the post clitellum segments of the coelomic cavity for the release of fluid (Prochazkova *et al*., 2006). In the present study, ice cold treatment was selected. In this method the earthworm did not die and the worm was alive. So the particular method was used for this study. The qualitative analysis of amino acids to identify the both essential and non-essential amino acids presents in the earthworm coelomic fluid at different stress conditions. A total of twenty amino acids consisting of ten essential namely Arginine, Valine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and ten non-essential amino acids namely Aspartic acid, Serine, Glutamic acid, Proline, Glycine, Tyrosine, Aspargine, Cysteine, Cystine were recorded in this study from each species of earthworm. Notable changes in the concentrations of amino acids were observed between the

control and stress conditions. The molecular size analysis determined was more than 50 KDa by SDS- PAGE. The study of Hanusova *et al*. (1999) reported the protein of more than 210 KDa which is in line with the present study.

Earthworms contain amazing antimicrobial activity (Cotuk and Dales, 1984) showed that coelomic fluid of *Eudrilus eugeniae* and *Lampito mauritii* was ineffective against the test bacterium, *Klebsiella pneumoniae*. In this present study, the coelomic fluid of *Lampito mauritii* and *Eudrilus eugeniae* was ineffective against the other tested pathogens. *Klebsiella sp.* was highly inhibited than other organisms. The zone of inhibition found for *Lampito mauritii* was 7 mm in diameter and the fluid of *Eudrilus eugeniae* showed 6 mm zone of inhibition.

CONCLUSION

In the present study, measurements of different parameters, such as the earthworm survival, growth rate, protein concentration and antibacterial activity of coelomic fluid, The changes in protein concentration and the number of proteins identified by SDS-PAGE. The number of proteins expressed gets varied between normal and toxic induced earthworms were used to evaluate the toxicity in two different earthworm species, *Eudrilus eugeniae* and *Lampito mauritii.*

ACKNOWLEDGEMENT

The authors are thankful to RUSA scheme Phase 2.0 grant [F-24-51/2014–U, Policy (TNMulti-Gen), Dept of Edn., Govt. of India, dated 09.10.2018] for the financial support.

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