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Research Article

COELOMIC FLUID EXTRACTION AND COELOMOCYTES COUNT IN DIFFERENT EPIGEIC EARTHWORMS

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ABSTRACT
The cavity of earthworm is filled with transparent fluid called coelomic fluid which is derived from the mesenchymal lining, exhibits a wide variety of biological activities. The coleomic fluid consists of various cells and many biomolecular components involved in innate immunity. This study aims to highlight the use of various standard methods for the extraction of coelomic fluid and also to calculate the differential coelomocytes count of three epigeic earthworm species such as *Eudrilus eugeniae* (EE), *Eisenia fetida* (EF) and *Perioynx excavatus* (PE). In present study, cold shock method was used for extraction of coelomic fluid from different epigeic earthworm species as it is more advantages than the other methods, especially for earthworms. During the study, four different kinds of coelomocytes were witnessed such as amoebocytes, chloragogen cells, mucocytes and circular cells. The total coelomocytes count was somewhat equal in all three species with no significant difference, whereas a significant difference was noticed in differential count of coelomocytes *i.e.* Among all the coelomocytes amoebocytes, chloragogen cell and mucocytes were more in EE (56.33±0.33, 37.00±0.57 and 3.66±0.33) followed by PE (31.00±0.57, 16.33±0.33 and 2.00±0.57) and less in EF (24.00±0.32, 8.00±0.21 and 2.00±0.33) whereas circular cells were more in EF (66.00±0.57) followed by PE (50.66±0.33) and less in EE (2.66±0.33).

Keywords: Epigeic earthworms, Coelomic fluid, Coelomocytes, Extraction methods.

1. INTRODUCTION
Earthworms are protostomian animals possessing true coelom cavity filled with a transparent yellowish fluid called coelomic fluid that forms not only a stable hydrostatic skeleton in earthworms but it also includes many cells called coelomocytes and other humoral factors involved in the working of immune system [1, 2]. In the course of evolution, earthworms have developed efficient defensive mechanisms against microbes, virus and other microorganisms, although they face the invasion of pathogenic microorganisms in their environment. Earthworms have; cell mediated and humoral immune mechanisms, cell mediated immunity works through different types of coelomocytes present in the body fluid. Coelomic fluid comprises of different types of coelomic cells derived from mesenchyma lining, the coelomocytes can be classified based on their morphology and functions, there are usually four kinds of coelomocytes such as amoebocytes, mucocytes, granulocytes and eleocytes have been observed by various workers [3, 4]. Coelomocytes play an important role in maintaining physiological activities like body moisture, transportation of nutrients, burrowing, respiration etc. [5-9]. Besides this, coelomocytes major immune cells of earthworms and have been attributed several functions to these cells such as ability to recognise and eliminate foreign materials primarily by phagocytosis encapsulation of pathogenic microorganisms coagulation of coelomic fluid [10, 11], through humoral immune response, secretion of lysosomes, which includes lectin, preforming proteins, phenoloxidase & protease nullifies antigenecity by agglutination and cytotoxicity [12-14]. These cells are known to be involved in some aspects of nutrition, excretion, regeneration and they take part in graft rejection in the phenomenon of antigen recognition [15].

The earthworm coelomic fluid consists of many proteins, lysozymes, fetidins, lysine protease and some variety of specialized coelomocytes [2]. Many studies have been performed regarding the composition of coelmoic fluid at an enzymatic level and demonstrated the presence of haemolytic, proteolytic and cytotoxic enzymes that are active against foreign cell organisms and peptides [16]. Literature revealed that many studies in relation to earthworms coelomic fluid have been worked out such as composition of proteins, enzymes and their immunological aspects, biological activities, but very little has been known about the composition of coelomic fluid with respect to different types of coelomocytes and their count/number in different epigeic earthworms. Therefore, in the present study an attempt has been made to extract coelomic fluid so as to study different coelomocytes and their total and differential count present in the coelomic fluid of different epigeic earthworms such as *Eudrilus eugeniae*, *Eisenia fetida* and *Perionyx excavatus*.

2. MATERIAL AND METHODS

2.1. Collection of epigeic earthworms

Adult epigeic earthworms such as *Eudrilus eugeniae* (EE), *Eisenia fetida* (EF) and *Perionyx excavates* (PE) were obtained from separate stock cultures maintained at Department of Zoology, Karnatak University, Dharwad (Karnataka), India.

2.2. Cleaning of earthworm gut

Some white papers were collected and cut into small pieces; they were put into beaker containing 100ml of distilled water and then boiled for 20-30mins followed by cooling. ten adult earthworms of each species (EE, EF & PE) were added into the beaker containing sterilized paper waste for about 24hrs so that they could release all content and cleaned up all waste materials present in the gut of earthworms [17]. Soon after 24hrs, the worms were removed from the beaker and cleaned in distilled water, soon after cleaning; worms were transferred to a clean big watch glass or petriplates for the extraction of coelomic fluid through different methods.

2.3. Extraction and collection of coelomic fluid

There are usually four types of methods used for the extraction and collection of coelomic fluid from earthworms such as

1) Warm water method- Here, 25ml warm water (45°C) is used for the collection of coelomic fluid [18].
2) Electric shock method- Here, electric pulse (5 volts for 30mins) is used for the extraction of 0.5 to 0.25 ml of coelomic fluid in 30mins [19].
3) Cold shock method- Here, ice cubes are used for the extraction of 1.5ml of coelomic fluid with in 30mins.
4) Heat shock method- Here, hot water (55-60°C) is used to extract coelomic fluid [20].

Out of these four methods, we have chosen cold shock method in our study as this method seems to be the safest and easiest method to extract and collect coelomic fluid from earthworms.

2.4. Coelomic fluid extraction by cold shock method

In cold shock method, there is as such no harmful effect on earthworms after placing them in ice cubes witnessed by their activeness, 100% survivability was also noticed every time soon after the collection of coelomic fluid. Growth of these worms was also found to be normal even after three rounds of fluid extraction within a time period of one month and no significant reduction was observed in the volume of the fluid in subsequent collections. The coelomic fluid collected through this method was clear, slightly brownish in colour without any marked debris. Hence, cold shock method was choosen for the extraction and collection of coelomic fluid from three different epigeic earthworms (EE, EF & PE).

2.5. Total coelomocytes count

To determine the total coelomocytes count the coelomic fluid was collected over the cavity slides and was diluted according to method described by Archer [21] with earthworm ringer solution diluting fluid [22] with acetic acid (3%). Diluted coelomic fluid is drawn into the haemocytometer, total coelomocyte counts were made (in triplicate) by keeping haemocytometer slide under the research microscope [23].

2.6. Differential coelomocytes count

For differential coelomocyte count, the smear of coelomic fluid was prepared and air dried. The dried smear was stained with eosin and methylene blue (1:1 ratio). The stained slides were observed under research microscope for the differential count of coelomocytes

(amoebocytes, chloragogen cells, mucocytes and circular cells).

2.7. Statistical Analysis

The significant variations (ANOVA) was analysed with different epigeic earthworms through SPSS program 16.0 version.

3. RESULTS AND DISCUSSION

The data of the total and differential coelomocytes count with respect to three different epigeic earthworms along with their significant values (F&P) were represented in the Table 1 & 2 and Graph 1-5. The captured pictures/ images of different coelomocytes observed in the coelomic fluid of three different epigeic earthworm species were presented in the Plate 1-3.

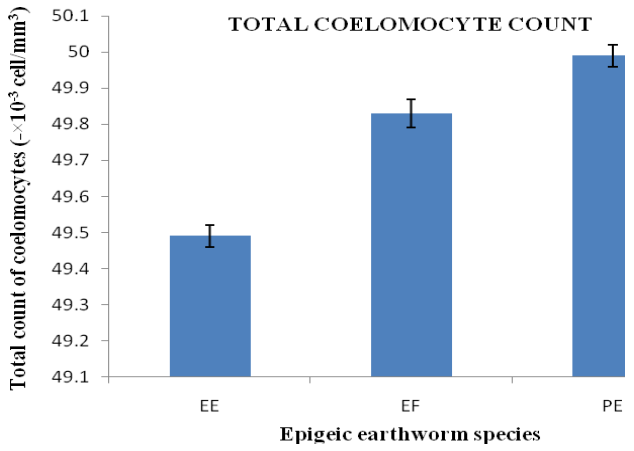
3.1. Total coelomocytes count

The total number of coelomocytes was slightly more in *Perionyx excavatus* (49.99±0.03) followed by *Eisenia fetida* (49.83±0.04) and less in *Eudrilus eugeniae* (49.49±0.03), but there is no significant variation was observed as such in total coelomocyte count among & between three different epigeic earthworms (Table-1).

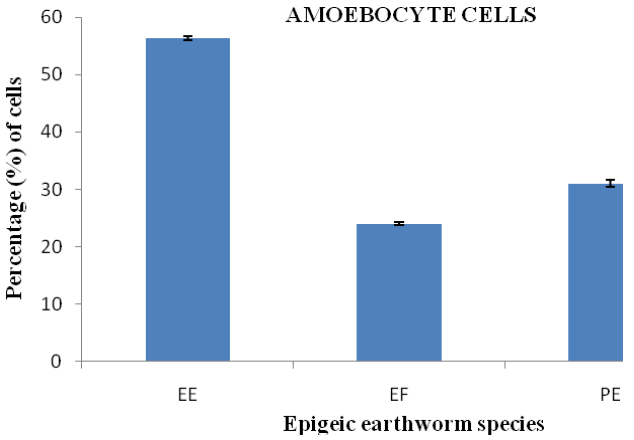
3.2. Differential coelmocytes count

The differential coelomocytes count revealed that amoebocytes, chloragogen and mucocyte cells were more (56.33±0.33, 37.00±0.57 and 3.66±0.33) in *Eudrilus eugeniae* followed by *Perionyx excavatus* (31.00±0.57, 16.33±0.33 and 2.00±0.57) and least in *Eisenia fetida* (24.00±0.32, 8.00±0.21 and 2.00±0.33). Whereas, circular cells were more in *Eisenia fetida* (66.00±0.57) followed by *Perionyx excavatus* (50.66±0.33) and least in *Eudrilus Eugeniae* (2.66±0.33). There was a significant variation noticed differential coelomocyte count among and between three different epigeic earthworms (Table-1 & 2) except between mucocytes and circular cells of EE (Table-2).

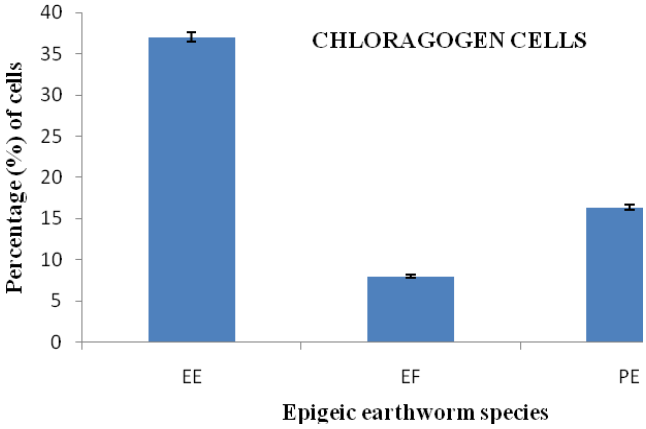
The variations of different coelomocytes count in three epigeic earthworms may be due to species specificity, intrinsic property of earthworms, their habitat and feeding activities etc. Kale and Krishnamoorthy [24] have reported species-specific variations in the total and differential count of coelomocytes in five different earthworm species.



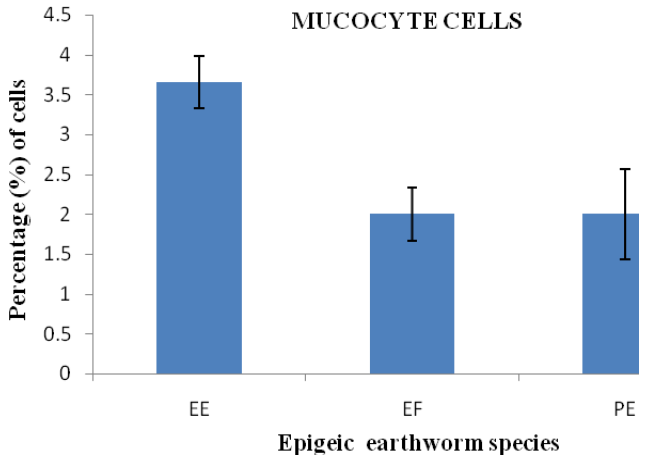
Graph 1: Total coelomocytes count (_ x10³ /Cu/mm) in three different epigeic earthworm species (EE, EF & PE)

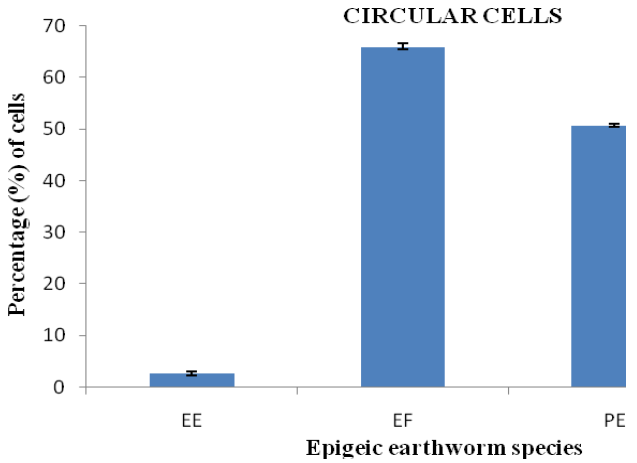


Graph 2: Percentage of differential count of amoebocytes found in three different epigeic earthworm species (EE, EF and PE)



Graph 3: Percentage of differential count of chloragogen cells found in three different epigeic earthworm species (EE, EF and PE)





Graph 4: Percentage of differential count of mucocyte cells found in three different epigeic earthworm species (EE, EF and PE)

Graph 5: Percentage of differential count of circular cells found in three different epigeic earthworm species (EE, EF and PE)

Table 1: Total and differential coelomocytes count of three different epigeic earthworms species (EE, EF &PE) and their significance F &P values (P< 0.05)

Sl. No	Coelomocytes	*Eudrilus eugeniae*	*Eisenia fetida*	*Perionyx excavatus*
1	Total cell count (--×10^3/ Cu/mm of coelomic fluid	49.49±0.03	49.83±0.04	49.99±0.03
2	Differential count of coelomocytes*			
a	Amoebocyte cells (%)	56.33±0.33	24.00±0.32	31.00±0.57
b	Chloragogen cells (%)	37.00±0.57	08.00±0.21	16.33±0.33
c	Mucocyte cells (%)	03.66±0.33	02.00±0.33	02.00±0.57
d	Circular cells (%)	02.66±0.33	66.00±0.57	50.66±0.33
3	F- value	4.076	1.000	1.948
4	P- value	0.00	0.00	0.00

Data are in Mean ± SE

Table 2: Significant variations (P<0.05) in different coelomocyte count between three different epigeic earthworm species (EE, EF &PE)

Earthworm species		EE				EF				PE			
		Am	Ch	Mu	Ci	Am	Ch	Mu	Ci	Am	Ch	Mu	Ci
EE	Am	-	0.00	0.00	0.00	-	-	-	-	-	-	-	-
	Ch	0.00	-	0.00	0.00	-	-	-	-	-	-	-	-
	Mu	0.00	0.00	-	**0.122**	-	-	-	-	-	-	-	-
	Ci	0.00	0.00	**0.122**	-	-	-	-	-				-
EF	Am	-	-	-	-	-	0.00	0.00	0.00	-	-	-	-
	Ch	-	-	-	-	0.00	-	0.00	0.00	-	-	-	-
	Mu	-	-	-	-	0.00	0.00	-	0.00	-	-	-	-
	Ci	-	-	-	-	0.00	0.00	0.00	-	-	-	-	-
PE	Am	-	-	-	-	-	-	-	-	-	0.00	0.00	0.00
	Ch	-	-	-	-	-	-	-	-	0.00	-	0.00	0.00
	Mu	-	-	-	-	-	-	-	-	0.00	0.00	-	0.00
	Ci	-	-	-	-	-	-	-	-	0.00	0.00	0.00	-

EE- Eudrilus eugeniae, EF- Eisenia fetida and PE- Perionyx excavatus

Am- Amoebocytes, Ch- Chloragogen cells, Mu- Mucocytes and Ci- Circular cells

The coelomocytes present in three different epigeic earthworms have their own morphology and different functions such as desiccation, promoting cutaneous respiration or producing protective measures against predators, regeneration, nutritive circulation and they are also involved in defensive mechanism in protection of earthworms through cell mediated and humoral immunity.

There are four types of coelomocytes namely amoebocytes, chloragogen cells, mucocytes and circular cells were observed in all the three epigeic earthworms in our study (Plate-1-3). The structure and functions of various coelomocytes are as follows.

a. Amoebocytes

These cells are usually large in size, slightly irregular in shape, mostly helps in removing harmful bacteria from earthworms through phagocytosis. These cells are also known as granulocytes or phagocytes.

b. Chloragogen cells

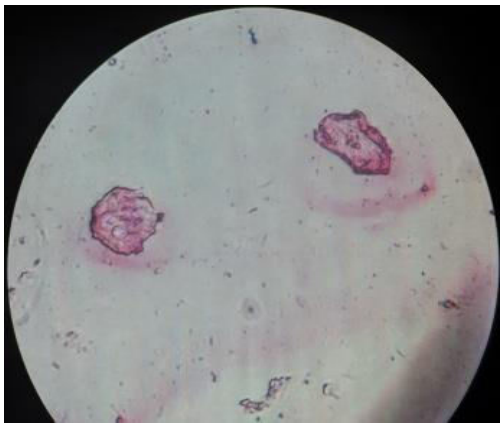
These cells are star shaped and are usually known as y-cells derived from inner coelomic epithelium. These cells functions as like liver organ in case of vertebrates, stores glycogen, neutralize toxins and helps in excretory functions. They look yellowish in colour due to the presence of yellow granules called as chloragosomes. These cells are sometimes called as elocytes too, as they have characteristic vesicular bulging, which stores and transports substances like glycogen and nitrogenous wastes, they take part in the deamination of amino acids and also in the synthesis of urea.

c. Mucocytes

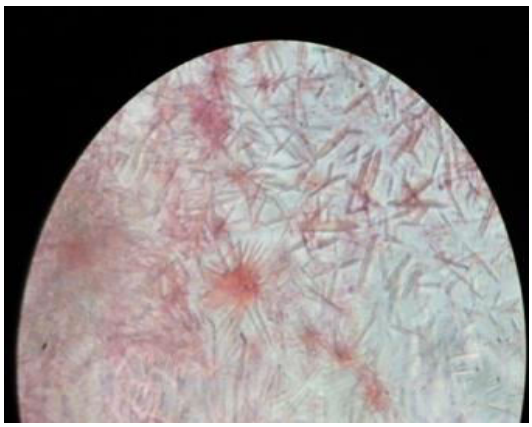
These cells are elongated with a nucleus at one side and expanded fan like structures at the other end. These cells secrete mucous substances so as to keep skin always moist for respiration and other physiological functions in the earthworms.

d. Circular cells

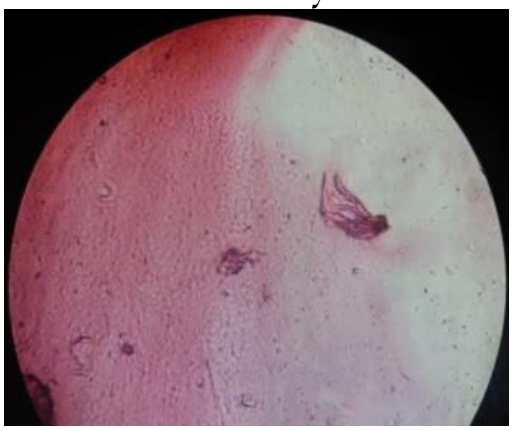
These cells are circular in shape, nucleated and have characteristic markings. The functions of these cells are not known exactly, but may be helps in the transportation of nutrients and other gases by circulation throughout the body.



EE- Amoebocytes



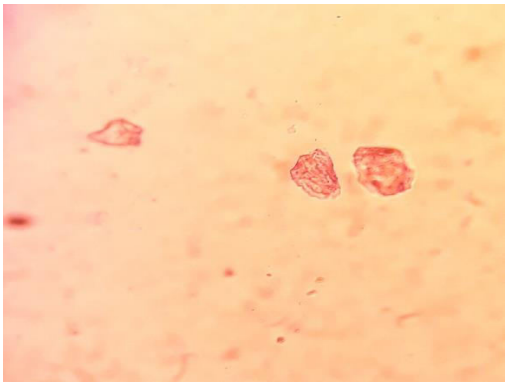
EE-Chloragogen cells



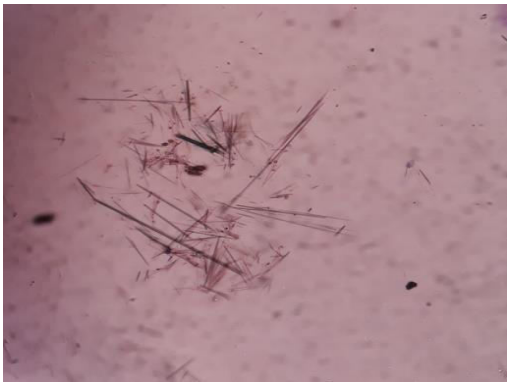
EE- Mucocytes

EE-Circular cells

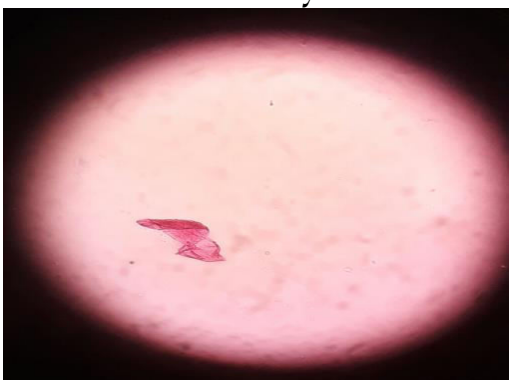
Plate 1: Images of different coelomocytes (amoebocytes, chloragogen cells, mucocytes and circular cells) observed in the coelomic fluid of the epigeic earthworm, *Eudrilus eugeniae* (EE)



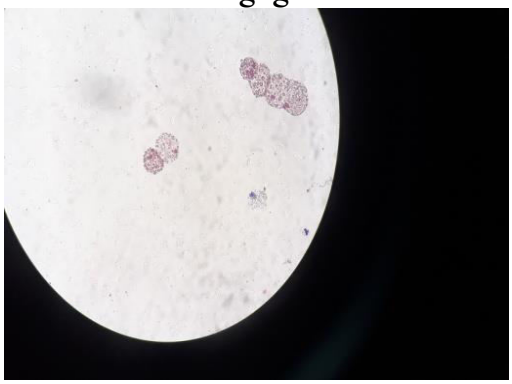
EF- Amoebocytes



EF- Cholragogen cells

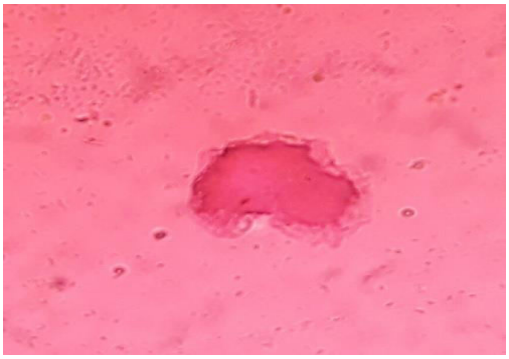


EF- Mucocytes

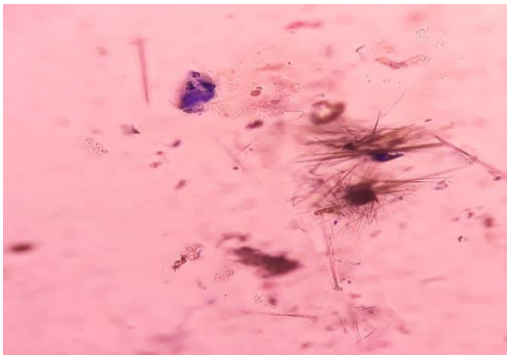


EF- Circular cells

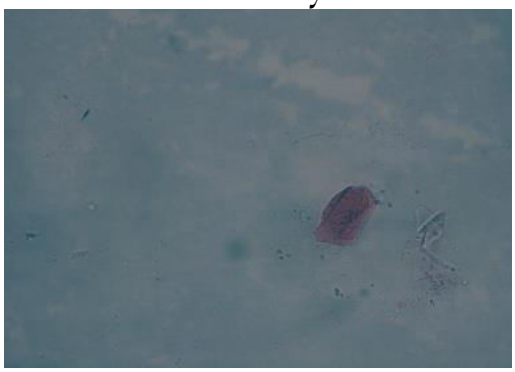
Plate 2: Images of different coelomocytes (amoebocytes, chloragogen cells, mucocytes and circular cells) observed in the coelomic fluid of the epigeic earthworm, *Eisenia fetida* (EF)



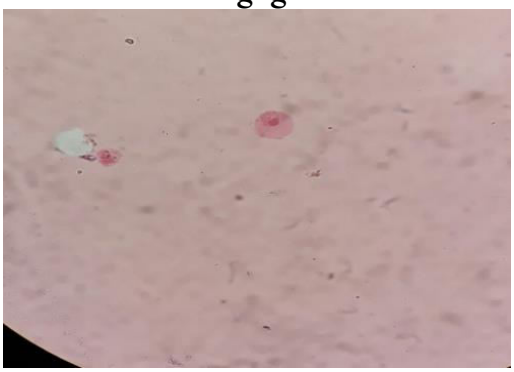
PE- Amoebocytes



PE- Chloragogen cells



PE- Mucocytes



PE- Circular cells

Plate 3: Images of different coelomocytes (amoebocytes, chloragogen cells, mucocytes and circular cells) observed in the coelomic fluid of the epigeic earthworm, *Perionyx excavatus* (PE)

Result of the present study revealed that the structure and number of particular coelomocytes varies among different earthworms species. However in general, all four types of coelomocytes differed slightly in structure and morphology with respect to all three earthworm species. Among various coelomocytes witnessed in our study, amoebocytes, chloragogen cells and mucocytes were more in EE followed by PE and less in EF, whereas circular cells were more in EF followed by PE and less in EE. The presence of more number of amoebocytes in all three epigeic earthworm species are generally known/regarded as immune cells involved in the process of phagocytosis and encapsulation [25]. The number and composition of different coelomocytes present in earthworms depends on exogenous (environmental factors) factors and endogenous factors (biotic and life cycle) [14]. A uniform classification of coelomocytes of among various earthworm species is very much difficult as they exists in different habitat, various functional states and different stages of maturation [2].

Several authors have demonstrated that the earthworm's innate immunity depends on different types of coelomocytes and their secretions as humoral antimicrobial molecules such as lyenin, fetidin, coelomic cytotoxic factor-1 [26]. However, the exact functional role by each of the coelomocytes played in the earthworm remains yet to be fully understood. Studies on the population and morphology of different coelomocytes present in the earthworms have their own significance, since these cells can be used as a model system in the analysis of mechanism of invertebrate immunity. The characterization of each and every coelomocytes is essential in analyzing the way in which these earthworms respond to the changing environmental factors/ conditions. This may also help in the utilization of various types of earthworms in monitoring the environmental quality as bioengineers.

4. CONCLUSION

The results of the present study suggests that cold shock method is the most advantageous and beneficial technique for the extraction and collection of coelomic fluid from earthworms over other methods. Even this is also a suitable method for earthworm survivability, growth and reproduction. The total coelomocytes count was sightly more in PE followed by EF and EE with no significant difference. Whereas there is a significant difference in differential coelomocytes count was noticed among and between different epigeic earthworms. Each of the coelomocytes are having their own morphology and functions in different earthworm species primarly as immune cells in the defensive mechanism, respiration, regeneration, circulation, and providing protection from predators and preventing from desiccation. The secretions of these cells also have various biological activities such as antimicrobial, antitumor, anti-inflammatory antioxidant etc, to tackle with many more diseases.

5. ACKNOWLEDGEMENT

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Conflict of Interest

The authors report no financial or any other conflicts of interest in this work.

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None declared

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