

## Toxicity Studies of Antibiotics in Earthworm, *Eudrilus eugeniae*

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### ABSTRACT

*In the present research work, seven different antibiotics viz., Cyclohexamide, Ampicillin, Cefotaxime, Nystatin, Pyridoxial hydrochloride, Neomycin and Chloramphenicol were used for the analysis of morphological and histopathology studies of earthworm, Eudrilus eugeniae. These seven antibiotics were used in two different methods, one was incorporated into the nutrient agar medium and the other was incorporated on its habitat. In the first method, involving the incorporation into the nutrient agar medium, the earthworms were first incubated on sterile nutrient agar medium containing specific amount of seven different antibiotics. Every single antibiotic was used to prepare nutrient agar plates. Earthworms were then placed on these plates and later checked for its and morphological conditions for every 1 hour up to 24 hours. After 24 hours, it was noted that the earthworm died in the Nystatin added agar plate while other antibiotics incorporated plate with earthworms were observed to be alive.*

*The second method involves the incorporation of earthworms into their habitat. The above mentioned seven antibiotics were taken individually and were then added to 50 ml of sterile water and from that preparation, 1 ml was taken and added directly to the sterilized compost and mixed well. The earthworms were later placed in those seven different antibiotics incorporated compost for 24 hours with an interval observation of every 1 hour in between. After 24 hours, it recorded that all the earthworms inoculated into the sterile compost remained alive.*

*In the present study five different bacterial isolates each from inner as well as the outer regions of the test earthworm were isolated and assayed for the antibiogram studies against the seven different antibiotics. As the Nystatin antibiotic agar medium incubated with earthworm died the particular antibiotic was chosen for further study was treated in earthworm by direct and indirect method. In direct method, the antibiotic was injected in clitellum of the earthworm whereas in indirect method, the earthworm was subjected to nystatin wash. Finally, the nystatin washed earthworms were carried out for histopathological analysis which showed damage in the epidermal and circular muscle layer.*

**Keywords:** Agar-well diffusion, Antibiotics, Earthworm, Haematoxylin and eosin, Nutrient agar medium.

### INTRODUCTION

Earthworms are important regulators of soil structure and dynamics of soil organic matter. They are a major component of soil fauna communities in most ecosystems and comprise a large proportion of macro fauna biomass<sup>1</sup>. They are tube-shaped, segmented animal commonly found in soil which feeds on live and dead organic matter. Its digestive system runs through the length of its body.

It conducts respiration through its skin. Since 1340 AD, earthworms have been used as medicine for various remedies<sup>2</sup> and it has been recognized in oriental medicine as anti-inflammatory, analgesic and antipyretic agent<sup>3</sup>.

The earthworm species, *Eudrilus eugeniae* commonly referred to as the West African night crawler occurs all over the world but mostly in West African regions<sup>4,5</sup>. It is largely found in West Africa from Ghana to Nigeria to West Cameroun and Gabon. It grows well at a temperature of more than 25°C but best at 30°C<sup>6</sup> attaining maximum weight, length and number of segments in about 15 to 20 weeks<sup>7</sup>. It ranges in size from about 10 cm in length to huge specimens of over 12 cm and size may depend on habitat<sup>5</sup>. The total number of segments in *Eudrilus eugeniae* varies from about 80 to over 100 with the location of a thick cylindrical collar, the clitellum between segments 13-20<sup>8</sup>.

Antibiotics are substances which either kill or inhibit the growth of a microorganism. The term antibiotic was first used in 1942 by Selman Waksman and his collaborators in journal articles to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution. Antibiotics are produced by microorganisms or they might be fully or partly prepared by chemical synthesis. They inhibit the growth of micro-organisms in minimal concentrations. Antibiotics may be of microbial origin or purely synthetic or semi synthetic.

The skin flora, more properly referred to as the skin micro biome or skin micro biota are the microorganisms which reside on the skin. Skin flora is usually non-pathogenic and either commensals or mutualistic. The benefits that bacteria can offer includes preventing transient pathogenic organisms from colonizing the skin surface either by competing for nutrients, secreting chemicals against them or stimulating the immune system of the skin<sup>9,10</sup>. A diverse microbial flora is associated with the skin and mucous membranes of every human being from shortly after birth until death. The human body which contains about 10<sup>13</sup> cells routinely harbors about 10<sup>14</sup> bacteria. This bacterial population constitutes the normal microbial flora. The normal microbial flora is relatively stable with specific genera populating various body regions during particular periods in an individual's life.

The clitellum is a part of the reproductive system of clitellates, a subgroup of Annelids which contains Oligochaetes (earthworms) and Hirudineans (leeches). The clitellum is a thick, saddle-like ring found in the epidermis of the worm, usually with a light-colored pigment. To form a cocoon for its eggs, the clitellum secretes a viscous fluid. This organ is used in sexual reproduction of some Annelids. The clitellum becomes apparent in mature annelids, but may be hard to locate visually in younger Annelids. In leeches, it appears seasonally. Its color is usually slightly lighter than that of the body of the Annelid. Occasionally, living segments of the worm will be shed with the clitellum.

During the course of evolution, earthworm produces certain anti-microbiological substances especially active proteins and enzymes<sup>11</sup>. Earthworm surface excreta are found to have potent antimicrobial activity. *Eudrilus eugeniae* has antibacterial potency which acts on certain plant pathogens<sup>12</sup>. The paste prepared from the earthworm, *E. eugeniae* was tested for its antibacterial potential.

The aim and objectives of the present investigation is to study the effect of antibiotics on earthworm incorporated into its medium, habitat, skin surface, washed on the skin surface of earthworm at different time periods, injected into the earthworm body, antibacterial effect of isolates from exterior and interior of earthworm and to observe the histopathological studies of earthworm.

## MATERIALS AND METHODS

### Collection of earthworms

Earthworms (*Eudrilus eugeniae*) were obtained from the Vermibiotechnology Laboratory of the Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli. They were cultured in the cement tank and acclimatized to the laboratory conditions for about one month for this study<sup>13,14</sup>.

### Gut clearance of earthworms

Earthworms were collected from the culturing cement tank and washed with tap water, the gut clearance of the collected earthworms were performed by introducing the worms into 1% sterile agar medium for about 24 to 48 hours. Then gut cleared worms were washed with sterile distilled water and used for future work.

**Preparation of stock solution for different antibiotics**

The antibiotic sensitivity of *Eudrilus eugeniae* was studied using seven different antibiotics viz., Cyclohexamide, Ampicillin, Cefotaxime, Nystatin, Pyridoxial hydrochloride, Neomycin and Chloramphenicol. About 0.1 gm of each antibiotic was mixed with 50 ml of sterile distilled water separately and used for further studies.

**Effect of antibiotics on earthworm incorporated into the agar medium**

About 1 gm of agar was mixed with 50 ml of distilled water, sterilized and cooled. From the above already prepared stock solutions, 100 µl of each antibiotic were mixed with the previously sterilized agar medium into which the earthworms were introduced and noted for the morphological changes for every 1 hour<sup>15</sup>.

**Effect of antibiotics on earthworm incorporated into its habitat**

About 50 gm of compost was taken in seven beakers separately into which 1 ml of each prepared stock solution of test antibiotic was added respectively. Earthworms were placed in these seven beakers and kept aside and the results were noted in every one hour for a period of 24 hours<sup>16,17</sup>.

**Effect of Nystatin antibiotic washed on the skin surface of *Eudrilus eugeniae***

From the prepared Nystatin stock solution, 1 ml was taken and used to wash the different parts of the earthworm separately. First a set of 10 earthworms were washed from head to tail with Nystatin, in the second set, the earthworms were washed only in their tail portion and the third set of earthworms were washed only in the head region. The fourth set of earthworms was washed from the clitellum to the tail portion. The four sets of earthworms were washed with 1 ml of Nystatin antibiotic for 2 minutes and placed in sterilized agar medium. The results were observed for every one hour and the morphological changes were noted<sup>18</sup>.

**Effect of Nystatin antibiotic washed on the skin surface of *Eudrilus eugeniae* at different time periods**

From the stock solution of Nystatin, 1 ml was taken and the earthworm was washed from head to clitellum. After washing the earthworms were placed in the sterilized agar medium and the results were observed for every one hour to observe the morphological changes<sup>18</sup>.

**Effect of Nystatin antibiotic injected into the clitellum of the earthworm**

Different concentrations of Nystatin stock solution viz., 25µl, 50µl, 1µl, 2µl and 3µl were taken and injected into the clitellum part of the test earthworm<sup>19</sup>. After injection, the earthworms were placed in agar medium and the results were noted for 24 hours with an interval of one hour.

**Isolation of bacterial flora from earthworms *Eudrilus eugeniae***

To the freshly prepared nutrient agar plates, exterior flora from the gut cleared earthworm was swabbed. Similarly, the coelomic fluid from the interior region of the gut cleared earthworm was taken and plated separately for bacterial isolation. These isolated bacteria were used to determine the antibiotic sensitivity towards seven different antibiotics using agar-well diffusion method.

**Antibacterial effect of isolates from exterior and interior of earthworms *Eudrilus eugeniae***

Antibacterial activity of the isolates against all the pathogenic microorganisms was determined by agar-well diffusion method<sup>20,21</sup> under aerobic conditions. Wells of 3 mm diameter were cut into the sterilized nutrient agar medium using sterile cork borer into which 100 µl of each test antibiotic was placed into each well separately which were previously swabbed with the respective isolated bacterial cultures. The inhibitory activity of the antibiotics against the isolated bacteria from the earthworm was noted by incubating the plates at 37°C for 24 hours. The antibacterial activity was determined by measuring the diameter of the inhibition zone around the wells.

**Histopathology study of earthworm**

Longitudinal sections of head to clitellum part of both the control and the test earthworm were subjected for histological examinations. Specimen earthworm was washed with 5 ml of Nystatin antibiotic and subjected for histology. The tissues of the earthworm were separated and fixed in 50 % formaldehyde by standard methods<sup>22</sup> for further analysis to be visualized under fluorescent microscopy.

## RESULTS AND DISCUSSION

Annappoorani<sup>23</sup> demonstrated that the earthworms are considered as farmer's friend and an indicator of soil quality as they contribute enriched soil to agricultural field. Salokhe *et al.*<sup>13</sup> studied the earthworms are economically important organisms and play a key role in the environmental management. Vermiculture is entirely natural process which maintains the environmental balance. The attempt was made to observe the effect of soil mixed insecticide viz., Fipronil on biological parameters, reproductive physiology and total count of gut microflora of *Eudrilus eugeniae* and the ability of this microflora to produce enzymes.

### **Effect of different antibiotics on earthworm incorporated into its agar medium**

The research work deals with the earthworms *Eudrilus eugeniae* were incubated on sterile agar medium containing specific amount of specific antibiotic compounds. For this purpose, seven different agar plates were prepared which was incorporated with 100 µl of seven different test antibiotics respectively before the solidification of the agar. Earthworms were placed on these plates and checked for its physiological activities and morphological conditions for every 1 hour up to 24 hours (Fig. 1). The worms incubated in Nystatin incorporated agar were found to be dead after 4 hours (Fig. 1 & Plate D). Rest of the earthworms placed in the other antibiotics remained alive even after 24 hours (Fig. 1, Plate A, B, C, E, F and G).

### **Effect of antibiotics on earthworm incorporated into its habitat**

As the second level of investigation, the earthworms were incubated in sterile compost to which the test antibiotics of the same level were incorporated. But in contrast to agar medium incorporated antibiotics, all the earthworms remained alive in the compost (Fig. 2). Vijayan *et al.*<sup>24</sup> studied the earthworm to be a potential contributor in organic waste disposal or vermicomposting and investigated the external features, growth, reproductive morphology and histology.

### **Effect of Nystatin antibiotic washed on the skin surface of *Eudrilus eugeniae***

In another set of experiments, Nystatin was found to act against earthworm leading to its death; this was taken for further study. Nystatin was incorporated to earthworms at different sites. Earthworm was washed with 1 ml Nystatin antibiotic from head to tail, only head and clitellum to tail separately for 2 minutes. Results were observed for every 1 hour up to 24 hours (Fig. 3). Results indicated that after 4 hours, the worms entire body washed with Nystatin and the worms head and clitellum to tail washed with Nystatin were found to be dead (Fig. 3, Plates A, C and D). The earthworm washed with Nystatin on tail portion alone remained alive though it lost its tail portion (Fig. 3 & Plate B).

### **Effect of Nystatin antibiotic washed on the skin surface of *Eudrilus eugeniae* at different time periods**

Since it was shown that Nystatin had a negative effect on earthworms, the time activity was checked and the readings were taken with an interval of every 10 minutes up to 90 minutes after which the time lapse was increased to 60 minutes. After 270 minutes, the results showed that the earthworm's tail was cut off and after 330 minutes, the earthworms were found to be dead (Fig. 4 & Table 1).

### **Effect of Nystatin antibiotic injected into the earthworm region of clitellum**

The earthworms were injected with Nystatin antibiotic in the clitellum region. Nystatin of different volumes viz., 0.25 µl, 0.50 µl, 1 µl, 2 µl and 3 µl were employed for injecting the earthworms. Results were noted for every 1 hour up to 24 hours. Results indicated that all the earthworms remained alive even after 24 hours (Fig. 5). According to previous findings, Oboh *et al.*<sup>25</sup> reported the earthworm, *Eudrilus eugeniae* possessed different concentrations of dump-site soil and petroleum effluents exhibited different morbidity and mortality responses. Lake sediments caused varied fluctuations in weight over a 20 day exposure period. Colour changes and mortality up to 15% were observed in the earthworms cultured in 100% lake sediment, while weight loss, coiling and sluggish movement were observed in 50% lake water.

### **Isolation of bacterial flora from earthworm body**

The bacterial flora were isolated from the interior and exterior part of the earthworm.

The coelomic fluid from the interior region and the bacterial flora from the exterior of the earthworm were swabbed and plated separately.

Five types of bacterial isolates were collected both from the inner and outer region. Esakkiammal *et al.*<sup>26</sup> reported the enumeration of bacterial population in the gut region of *Eudrilus eugeniae* in soils, organic matter. Decomposition and stabilization largely occurs as a result of microbial activity, although when present, earthworms are important drivers of the processes through their interactions with micro flora which begin during organic matter digestion by earthworms.

#### **Antibacterial effect of isolates from exterior of earthworm**

The antibacterial activity of seven different antibiotics was examined using agar-well diffusion method. Antibacterial activities of seven different test antibiotics were tested against five interior and five exterior bacteria isolated from earthworm. The zone of inhibition was expressed in millimeter (mm). The Cefotaximide showed increased inhibitory activity (3 mm) in bacteria 2 from earthworm exterior. Among the bacterial species, the Chloramphenicol and Neomycin showed lesser activity in bacteria exterior 4 and 5 (1 mm). Ampicillin, Pyridoxial hydrochloride, Cyclohexamide and Nystatin rendered no bacterial activity against bacteria exterior 1,2,3,4 and 5 (Fig. 6, Table 2 & Graph 1). From the previous results, Bhorgin and Uma<sup>20</sup> studied the various solvent extracts of dried earthworm powder (*Lampito mauritii*) and subjected to preliminary screening for antimicrobial activity which was determined by disc diffusion method and it was found that 95% ethanol extract of earthworm exhibited potent antibacterial activity against *Aeromonas hydrophila* and antifungal action against *Candida albicans*.

#### **Antibacterial effect of isolates from interior of earthworm**

The internal bacterial isolate 2 rendered sensitivity towards Cefotaximide (3 mm) to the maximum and the bacterial isolates 4 and 5 exhibited least activity with Ampicillin (0.5 mm). The isolated bacterium 1, 2, 3, 4 and 5 showed resistance towards Pyridoxial hydrochloride, Cyclohexamide and Nystatin<sup>27</sup> (Fig. 7). According to Khomyakov *et al.*<sup>28</sup> the antimicrobial agents of earthworm's digestive fluid are formed in the earthworm body but not by the soil microorganisms entering their digestive tract. (Fig. 7, Table 3 & Graph 2). Nisha *et al.*<sup>29</sup> enumerated the microbial load in earthworm *Eudrilus eugeniae* injected with *Salmonella typhi*.

#### **Histopathology study of earthworm *Eudrilus eugeniae***

Histopathology analysis was done to study the histological changes happening in the test earthworm. The portions of earthworms (head to clitellum) was washed with Nystatin. In control worm, all the layers epidermal, circular muscle and longitudinal cell layers were found healthy without any change. But the earthworms washed with Nystatin showed damage in the epidermal and circular muscle layer of the earthworm. (Fig. 8). The exposure of the test organisms to sub lethal concentrations of the three chemicals resulted in various degrees of histopathological alterations in the clitellum in which the internal cells had different levels of disruption such as inflammation of cells, severe lesion, cellular degeneration, necrosis, malignancy and endothelial degeneration. This result disagrees with the report of Sharma and Satyanarayan<sup>30</sup>. From the previous reports, Oluah *et al.*<sup>31</sup> studied the toxicity and histopathological effects of the herbicide atrazine to the earthworm *Nsukka drilusmbae* under the laboratory conditions. *Nsukka drilusmbae* were exposed to different concentrations of Atrazine (0.0, 0.4, 0.8, 3.0 and 9.0 mg/kg soil) for 96 hours and mortality was recorded in every 24 hours. From the results, in addition, sections of the worms were made after 96 hours for microscopic examination. There was no mortality in the control group but in the experimental groups throughout the study the LC<sub>50</sub> of Atrazine after 24, 48, 72 and 96 hours were 8.60, 7.05, 7.37 and 7.23 respectively. The result of the study confirms that both mortality and histopathology data could be used in environmental risk assessment of Atrazine. Jyothi Gnana Sundarapriya *et al.*<sup>32</sup> have observed the histopathological changes of gut of *Eudrilus eugeniae* in *Leucaena leucocephala*.

**Table 1: Effect of Nystatin antibiotic on the skin surface of *Eudrilus eugeniae***

Earthworm washed from head to clitellum with Nystatin antibiotic	
Time (Minutes)	Earthworm condition (live/dead)
10	Live
20	Live
30	Live
40	Live
50	Live
60	Live
70	Live
80	Live
90	Live
150	Live
210	Live
270	Tail cut off but earthworm alive
330	Decay

**Table 2: Effect of different antibiotics on bacterial isolates from exterior of earthworm, *Eudrilus eugeniae***

Bacterial isolates from exterior of earthworm	Zone of inhibition in diameter (mm)							
	Control	Ampicillin	Chloramphenicol	Pyridoxial	Neomycin	Cyclohexamide	Cefotaximide	Nystatin
01	0	0	2	0	2	0	0	0
02	0	0	2.5	0	2	0	3	0
03	0	0	1.5	0	1.5	0	0	0
04	0	0	1.5	0	1	0	0	0
05	0	0	1	0	2.5	0	0	0

0 – Indicates no zone formation.

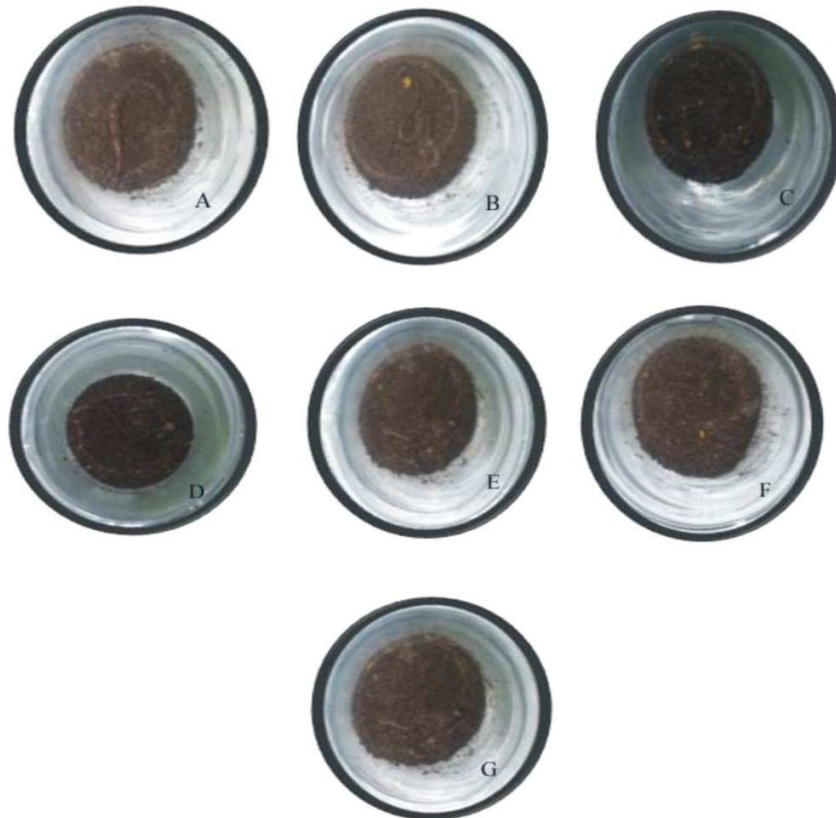
**Table 3: Antibacterial effect of isolates from interior of earthworm, *Eudrilus eugeniae***

Bacterial isolates from interior of earthworm	Zone of inhibition in diameter (mm)							
	Control	Ampicillin	Chloramphenicol	Pyridoxial	Neomycin	Cyclohexamide	Cefotaximide	Nystatin
1	0	0	0	0	2	0	2.5	0
2	0	0	2	0	0	0	3	0
3	0	0	0	0	1.5	0	2	0
4	0	0	1.5	0	1	0	1.5	0
5	0	0.5	1	0	1.5	0	2	0

0 - Indicates no zone formation

**Fig. 1: Indicating the earthworm placed in agar plates incorporated with different antibiotics**

A, B, C, E, F and G: Agar plate incorporated with Neomycin, Chloramphenicol, Ampicillin, Cyclohexamide, Cefotaximide and Pyridoxial hydrochloride (live). D: Agar plate incorporated with Nystatin (died). H: Control.

**Fig. 2: Earthworm placed in compost incorporated with different antibiotics**

A, B, C, D, E, F and G: Compost incorporated with Neomycin, Chloramphenicol, Ampicillin, Cyclohexamide, Cefotaximide, Nystatin and Pyridoxial hydrochloride (live).

**Fig. 3: Nystatin activity in earthworm, *Eudrilus eugeniae***

A: Earthworm washed from head to tail (died).  
 B: Earthworm washed only in tail portion (tail cut off but earthworm alive).  
 C: Earthworm washed at head (died).  
 D: Earthworm washed at clitellum to tail (died).  
 E: Control.



Fig. 4: Earthworm washed with Nystatin antibiotic (head to clitellum)



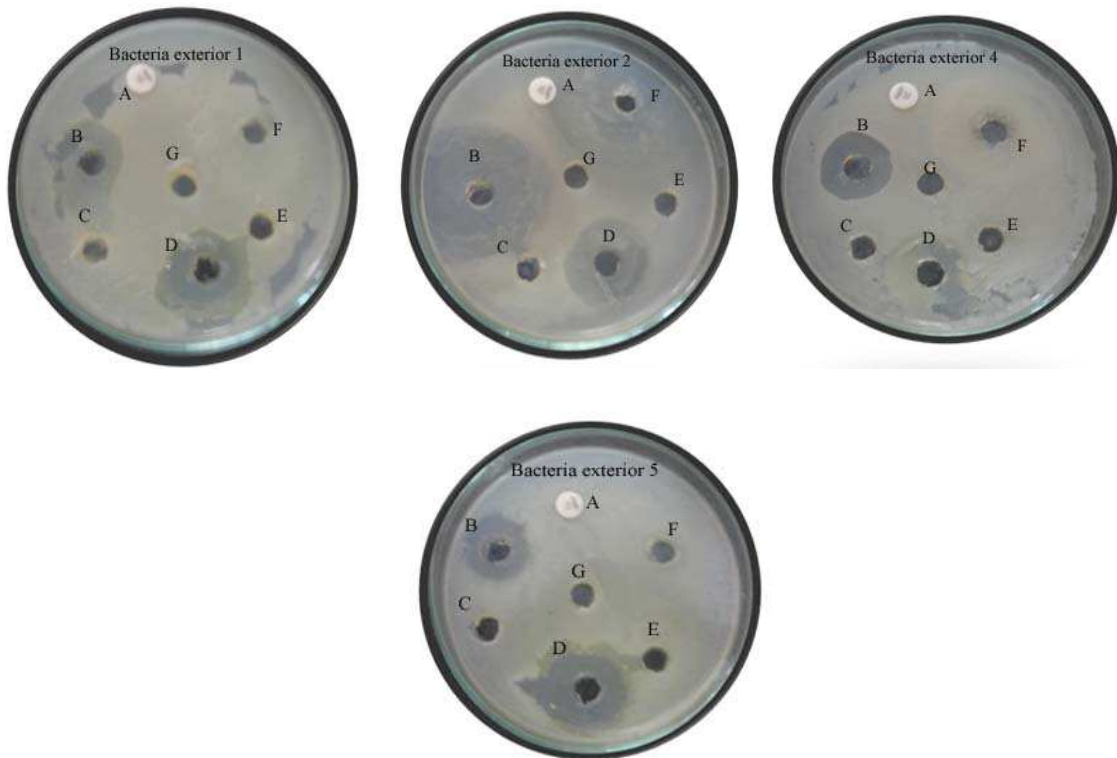
In 10-210 minutes: Earthworm remained alive, In 270 minutes: Earthworm tail was cut but still found alive, After 330 minutes: The earthworm started to decay, Control: Earthworm alive

**Fig. 5: Nystatin antibiotics injected into the clitellum of the earthworm**

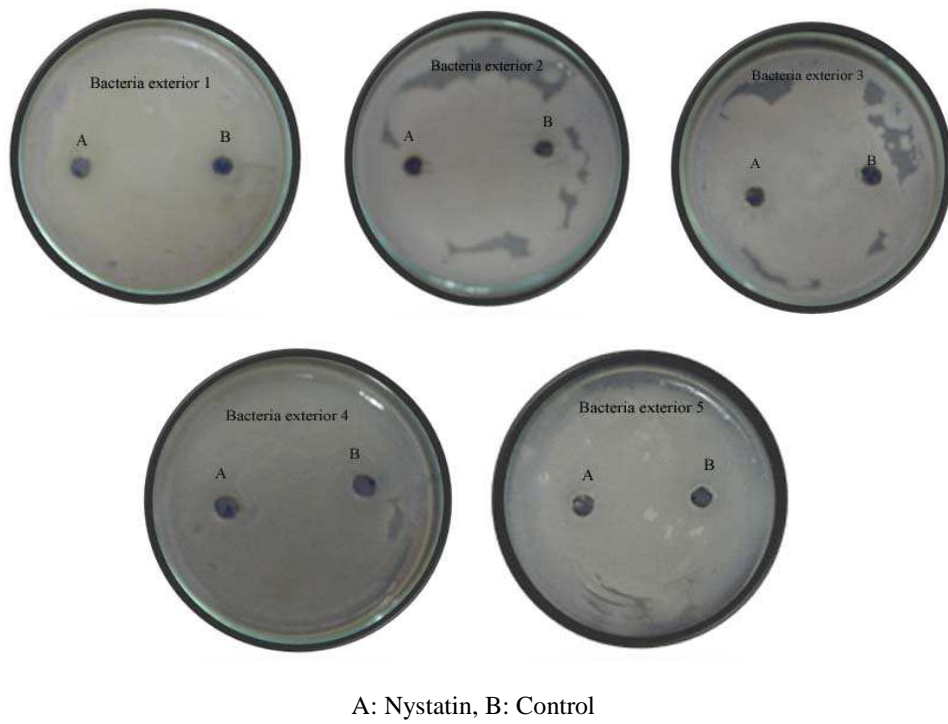


A, B, C, D and E: Earthworm injected at clitellum in different volumes (0.25µl, 0.50µl, 1µl, 2µl and 3µl) (Live). F: Control.

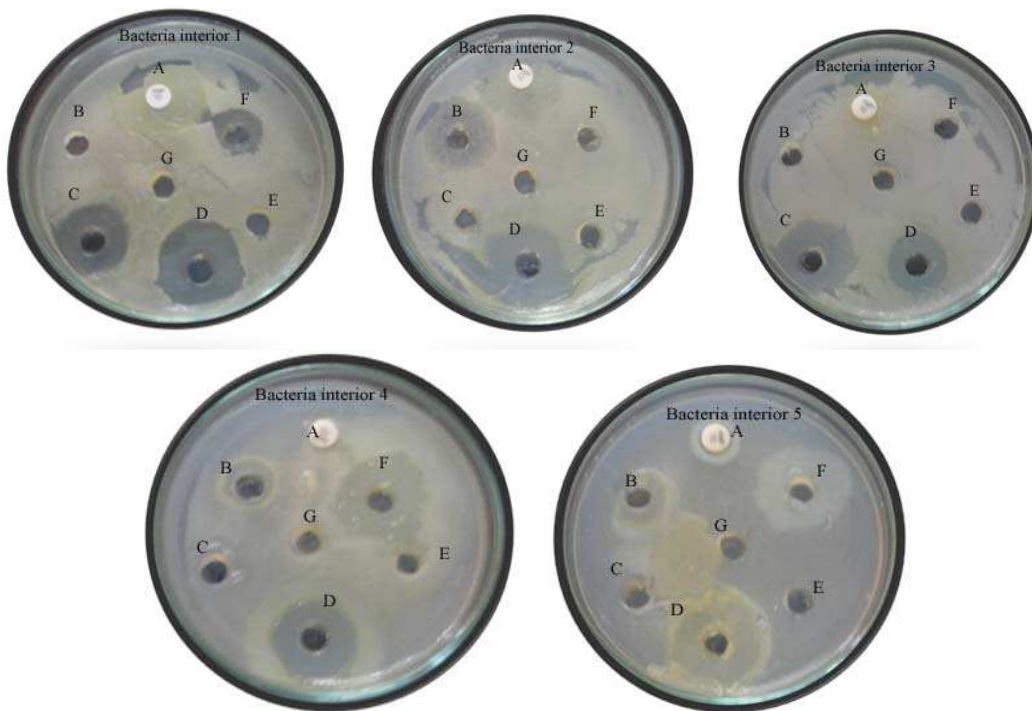
**Fig. 6: Antibacterial effect of isolates from exterior of earthworm**



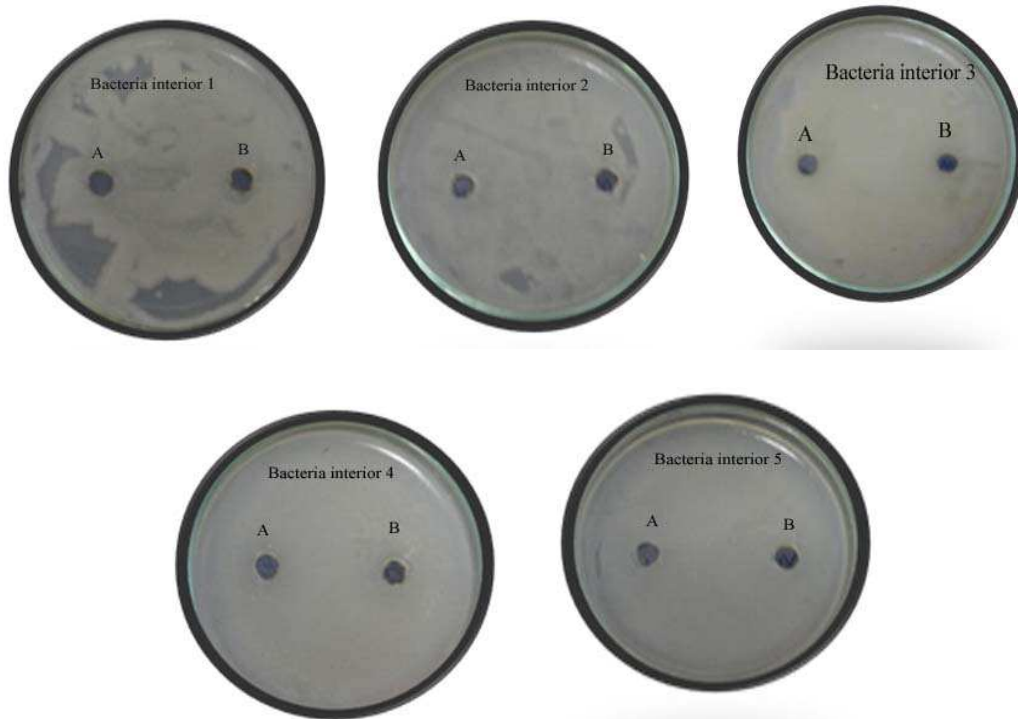
A: Ampicillin, B: Chloramphenicol, C: Pyridoxial, D: Neomycin, E: Cyclohexamide, F: Cefotaximide, G: Control



**Fig. 7: Antibacterial effect of isolates from interior of earthworm, *Eudrilus eugeniae***

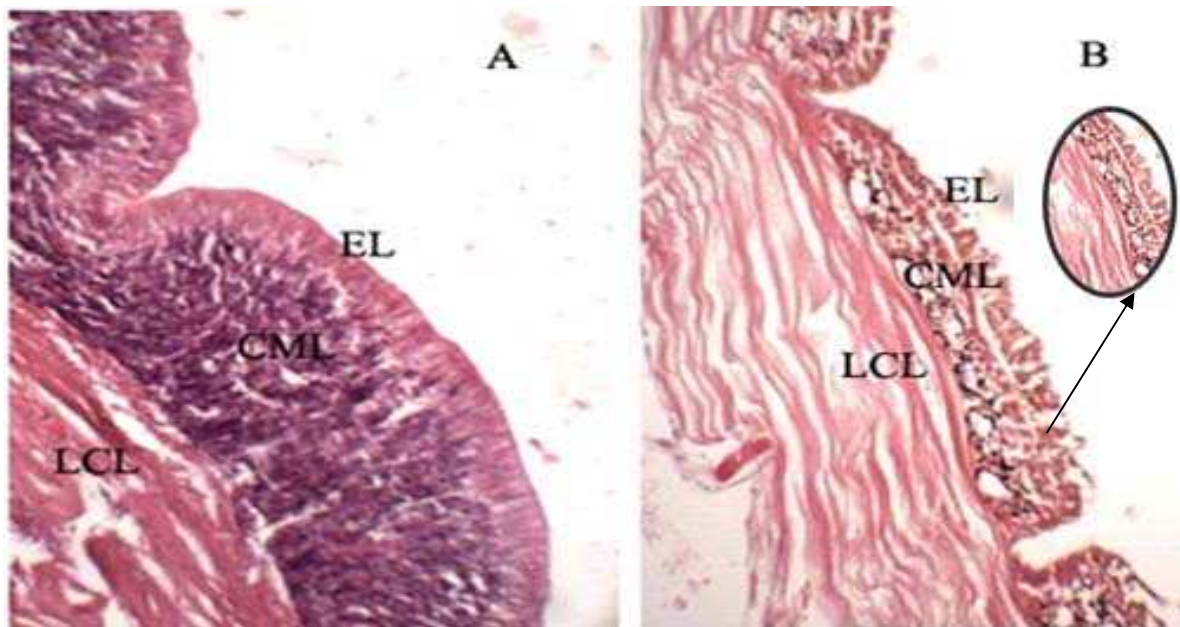


A: Ampicillin, B: Chloramphenicol, C: Neomycin, D: Cefotaximide, E: Cyclohexamide, F: Pyridoxial, G: Control



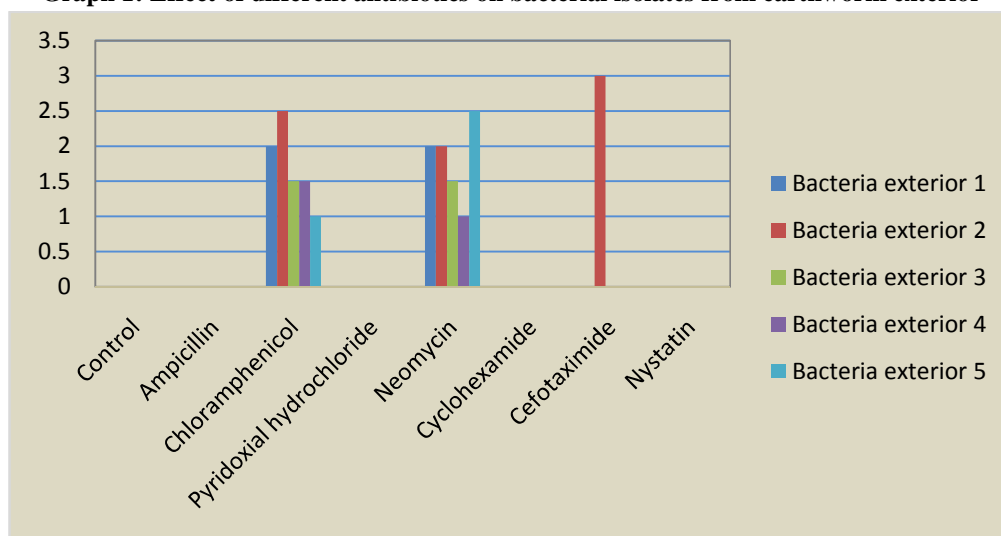
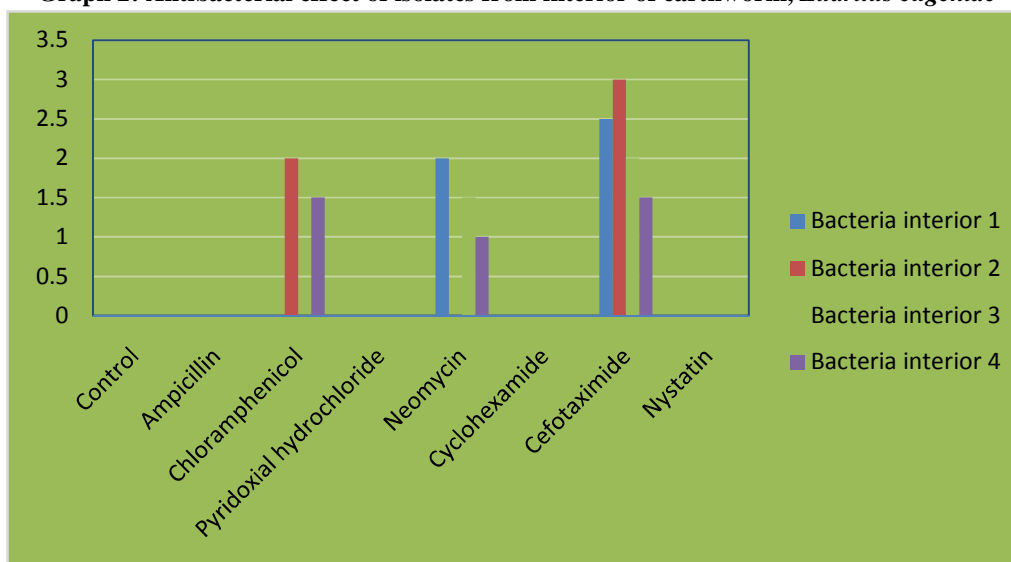
A: Nystatin, B: Water

Fig. 8: Histopathology studies of earthworm, *Eudrilus eugeniae*



A: Normal tissue section of earthworm, B: Nystatin washed earthworm (head to clitellum) showing skin damage.

(EL: Epidermal layer, CML: Circular muscle layer, LCL: Longitudinal cell layer)

**Graph 1: Effect of different antibiotics on bacterial isolates from earthworm exterior****Graph 2: Antibacterial effect of isolates from interior of earthworm, *Eudrilus eugeniae***

## CONCLUSION

It could be finally concluded that the earthworms on subjection to adverse conditions showed damage especially in the epidermal and circular muscle layer. The exact reason for the death of the earthworm with its tail amputated is yet to be researched.

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