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An Investigation on Antimicrobial Potency of Coelomic Fluid of Earthworm *Eudrilus eugeniae*

Esaivani C[1*], K.Vasanthi[1] , A.J.A Ranjit Singh[2]
1. Department of Zoology, Sri Parasakthi College for Women, Courtallam, M. S. University, Tirunelveli, Tamilnadu- 627 802, India.
2.Department of Zoology, Sri Paramakalyani College, Alwarkurichi, M. S. University, Tirunelveli, Tamilnadu- 627 412, India

ABSTRACT

Development of microbial resistance to various existing antimicrobial drugs has become a serious public health concern and the search for new classes of antimicrobial agents is a challenging task. The antimicrobial activity of the coelomic fluid (CF) obtained from *Eudrilus eugeniae* was investigated against five bacterial strains as well as four fungal strains using disc diffusion method to verify its claimed pharmaceutical use in the treatment of many infections. 75µl of coelomic fluid exhibited strongest antibacterial activity against *Vibrio parahaemolyticus* and *Klebsiella pneumoniae*, and antifungal activity against *Candida albicans*. This inhibitory effect of CF was compared with the commercial antibiotics like Ciprofloxacin and Fluconazole. For the antimicrobial screening, five species of bacterial isolate and four species of fungal isolates were selected. The bacterial cultures were used for antimicrobial testing maintained on nutrient agar slant and the fungal strains were maintained on Sabouraud dextrose agar slant at 4^{o}C. Minimum inhibitory concentration (MIC) was determined using micro dilution broth method. The MIC results indicated that CF at a dose of 200µl for bacteria and 100µl for fungi was found to be minimum concentration to inhibit the growth of selected pathogenic bacteria and fungi. The results of the present investigation indicates that the CF of *E.eugeniae* has a significant capacity of antibacterial and antifungal activities, which makes them interesting for screening CF of *E.eugeniae* as natural product.

Keywords: *Eudrilus eugeniae, Vibrio parahaemolyticus, Candida albicans* and coelomic fluid, inhibition zone, antimicrobial activity.

*Corresponding Author Email: esaivanispkc2013@gmail.com
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INTRODUCTION

Resistance to antibiotics by bacteria has become a big threat. Thus, the need to search for drugs derived from different sources has increased in recent years. Invertebrate species have developed a variety of defense mechanisms efficiently by recognizing and responding to non-self substances (Little, 2005)[1]. Earthworms have proved to be invertebrate model for immunologists in the early sixties (Cooper, 1969)[2]. *Aeromonas hydrophila* and *Bacillus megaterium* were demonstrated to be sensitive to coelomic fluid of the earthworm, *Eisenia fetida* Andrei (Pan *et al*., 2003)[4]. It also exhibits strong homolytic activity (Du Pasquier and Du-prat, 1968)[14]. Bakti e*t al*., (2003)[3] demonstrated that the extracts of the earthworm, *Pontoscolex corenthrurus* has antimicrobial activity. Aqueous extracts of earthworm *Eudrilus eugeniae* are antimicrobial to plant pathogens, *Xanthomonas campestris* and *Fusarium oxysporum* (Shobha and Kale, 2008)[5]. Thus effectiveness of coelomic fluid of earthworm has been shown to have ability to suppress the proliferation of some of the known plant and animal pathogens. The aim of the present study was to find out the sensitivities of clinical pathogens to coelomic fluid of *Eudrilus eugeniae*.

Earthworms have been living with the aid of their defense system since the early phases of evolution, although they always face the invasion of pathogen microorganisms in their environments (Engelmann *et al*., 2004)[6]. The studies which have been continued for about 50 years showed that earthworms have humoral and cellular immunity mechanisms (Bilej *et al*., 2001 and Field *et al*., 2004)[7,8]. It has been found that coelomic fluid of the earthworms contains more than 40 proteins and exhibits several biological activities as follows: cytolytic, proteolytic, antimicrobial, haemolytic, haemagglutinating, tumorolytic and mitogenic activities (Cooper and Roch, 2003)[9].

The coelom of earthworms is filled with the coelomic fluid- a milky white alkaline liquid that helps the worm in locomotion, nutrition, excretion, detoxification of tissues, heavy metal accumulation and protects internal organs from external jerks, destroys bacterial attack, prevents dessication, promotes cutaneous respiration and thermal acclimation (Kurek *et al* , 2007 ; Dash, 2012 and Paul, 2014)[10-12]. The coelomic fluid of earthworms and their body extracts were known to have antimicrobial and many medicinal properties since 1340 AD (Hossam *et al*., 2012)[13]. Micro organisms are known to play a major role in soil characteristics and invertebrates are believed to act as regulators of antimicrobial activity. The coelomic fluid of earthworm (*ie*) the surface excreta were found to have potent antimicrobial activity. The coelomic fluid functions as a hydrostatic skeleton and also serves as the circulatory medium. The fluid contains cytolytic, agglutinating and / or antibacterial components, which are involved in the immune systems. Presumably the function of this

system is to destroy membranes of foreign cells, a mechanism that causes cell death by cytosol release and is attributed to the coelomycetes, which secrete humoral effectors into the coelomic fluid. Coelomic fluid is also reported for having anticancer activity. The high concentration of coelomic fluid exhibited toxic effect on HeLa cells, causing the cell lysis and break down into pieces. Antibacterial activity of coelomic fluid is reported to be selective. The coelomic fluid from earthworm is known to contain immunoactive cells and molecules involved in immune defense. Earthworm coelomic fluid is found to contain molecules that bind anti IgA and anti IgG. Elucidation of the earthworm binding sites on anti IgG and anti IgA could make earthworms coelomic fluid a valuable reagent in immunological, chemical and biological research.

Since antibacterial activity of coelomic fluid is reported to be selective. The present laboratory based study; experiment was carried out to know whether the coelomic fluid of earthworm *E.eugeniae* possesses any antimicrobial activities against the selected pathogenic strains.

MATERIALS AND METHOD

Collection of earthworms

Fully matured earthworms, *E.eugeniae* were collected from the stock culture maintained under laboratory condition, using partially decomposed leaf wastes and cowdung as growth medium.

Collection of coelomic fluid from the earthworm

This fluid can be collected by stimulating them in different methods like mild electric shock method, puncturing of coelomic cavity, warm water shock method and cold shock method (using ice packs). The method of collection adopted in the present study is cold shock method.

Collection of coelomic fluid by cold shock method

In this method approximately 15 grams of worms were taken and washed with sterile distilled water. The worms were then dried on a filter paper and placed in a nylon mesh rolled into a ‘cone’ shape to fit into the glass funnel. The funnel was held in a burette clamp on a titration stand. A bag of ice that fits over the funnel was placed above the worms such that the worms could feel the drop in temperature (10 – 15^{o} C) due to the ice pack above. The coelomic fluid was made to release through the dorsal pores of its body due to the ‘cold shock’ method. The fluid was collected in a clean sterile dry beaker that was fit to the end of the funnel. The collection was carried out for 30 minutes and the worms were quickly released into a separate worm - culture trough for relaxation. The collected coelomic fluid was then transferred to sterile test tubes for centrifugation. The collected coelomic fluid was then centrifuged at 5000

rpm for 10 minutes to deposit the debris and the clear straw – coloured supernatant was then filter sterilized through 0.2 µm syringe filter into a clean dry sterile microfuge in a Laminar Air Flow chamber and was stored under - 20^0 C for further study.

Collection of pathogenic bacteria and fungi for antimicrobial studies

Pathogenic organisms were selected based on pathogenesis and drug resistance. The bacterial and fungal strains were obtained from Viveks Laboratory, Nagercoil, India. The pathogenic bacteria tested were *Vibrio parahaemolyticus, Vibrio mimicus , Klebsiella pneumoniae, Pseudomonas aureginosa, Proteus vulgaris* and the fungal strains tested were *Candida albicans, Aspergillus niger, Trichophyton rubrum* and *Trichophyton mentogrophytes.*

Preparation of inoculum

The obtained stock cultures were maintained at 4^o C on slope of nutrient agar. Active cultures for experiments were prepared by transferring a loop-full of cells from stock culture into a test tube of Muller Hinton Broth (MHB) and were incubated without agitation at 37^o C for 24 hours. The cultures were diluted with fresh Muller Hinton Broth to achieve optical densities corresponding to that of colony forming units per ml (CFU).

Preparation of dried filter paper discs

Whatman filter paper no .1 was used to prepare discs approximately 6mm in diameter, the discs were placed in a petridish and were sterilized in a hot air oven. The discs were then loaded with 25, 50 and 75 µl of earthworm coelomic fluid (ECF) per disc.

Antibiotic discs

Two commercial antibiotic discs namely Ciprofloxacin (Cf) and Fluconazole (Fu) were purchased from HIMEDIA, Mumbai and were used as standards against bacterial and fungal pathogen's selected for the experiment.

Antibacterial assay

The invitro antimicrobial activity was screened against *Vibrio parahaemolyticus, Vibrio mimicus, Klebsiella pneumoniae, Pseudomonas aureginosa, Proteus vulgaris* by using Muller Hinton Agar (MHA). The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes. About 0.1 % inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. The different concentrations of earthworm's coelomic fluid (25, 50 and 75 µl / disc) were loaded on 6mm sterile discs. The loaded disc was placed on the surface of the medium and the compound was allowed to diffuse for 5 minutes. The plates were then kept for incubation at 37^o C for 24 hours. At the end of incubation, inhibition zones formed around the discs were measured with zone reader scale in mm. The studies were performed in triplicates. The inoculate absorbance was established between 0.08 to 0.10AU (equivalent to 0.5 McFarland

10^8 CFU /ml) adding sterile nutrient broth, before incorporating bacteria (ʎ= 625 nm). A disc of Ciprofloxacin (Cf) (30 µg / disc) was used as a positive control.

Antifungal assay

Antifungal activity was carried out against *Candida albicans, Aspergillus niger, Trichophyton rubrum* and *Trichophyton mentogrophytes*. The microorganisms were inoculated on Sabouraud dextrose broth (HIMEDIA, Bombay) during 24 h at 25^o C. The inoculate absorbance was established between 0.08 and 0.10 AU (equivalent to 0.5 McFarland 10^8 CFU/ml) adding sterile Sabouraud dextrose broth, before incorporating fungi (ʎ = 530 nm). Fungal strains were seeded agar Sabouraud dextrose agar with 4% glucose. The sterile discs were impregnated in the seeded agar. The discs were then loaded with coelomic fluid at a concentration of 25, 50 and 75 µl / disc. Fluconazole (Fu) (5µg/disc) an antifungal agent was used as positive control. The plates were incubated at 25^o C for 48 hrs. All the assays were carried out in triplicate. The diameter (mm) of the growth inhibition zone was measured with standard zone reader scale (HIMEDIA, Bombay) and recorded a mean diameter.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was determined using micro dilution broth method. The earthworm coelomic fluid were diluted to different concentrations (50, 100, 150, 200 µl /ml) for bacteria whereas for fungi the dilutions prepared were (40, 60, 80 and 100 µl / ml). To find out the MIC three strains of bacteria and fungi were used. The bacterial strains *Vibrio parahaemolyticus, Klebsiella pneumoniae, Proteus vulgaris* and the fungal strains *Candida albicans, Trichophyton rubrum, Aspergillus niger* were prepared in broth. The broth culture suspension of each isolates (0.5 ml) with an optical density of McFarland 0.5 × 10^7 - 10^8 CFU / ml was added to test tubes containing different concentrations of earthworm's coelomic fluid (ECF). To the control test tubes the ECF was not added instead only sterile distilled water was added. The inoculated test tubes were incubated at 37^o C under aerobic conditions. After 24 hr the turbidity was evaluated. The MIC is the lowest concentration of ECF that inhibited the growth completely. For all the tests including control, triplicates were maintained.

RESULTS AND DISCUSSION

The extracted coelomic fluid of *Eudrilus eugeniae* was tested against five bacterial strains *V. parahaemolyticus, V.mimicus, K.pneumoniae, P.aureginosa* and *P.vulgaris* and four fungal strains like *C.albicans, A.niger, T.rubrum* and *T.mentogrophytes*. The observed results are summarized in Table 1 and 2.

Table 1: Antimicrobial activity of Coelomic fluid against bacterial pathogens

Microorganisms	Diameter of inhibition zone (mm)			
	25µl	50µl	75µl	Standard antibiotics Ciprofloxacin (Cf) (30µg/ disc)
Vibrio parahaemolyticus	13.66±0.33	15.66±0.66	21.00±0.57	22.33±0.33
Vibrio mimicus	14.00±0.57	16.33±0.33	21.33±0.66	23.66±0.33
Klebsiella pneumoniae	15.33±0.33	17.00±0.57	19.66±0.66	21.00±0.57
Pseudomonas aeruginosa	12.66±0.33	16.00±0.57	19.00±0.57	22.33±0.66
Proteus vulgaris	10.11±0.57	10.33±0.33	14.33±0.33	19.00±0.57

Table 2: Antimicrobial activity of Coelomic fluid against fungal pathogens

Microorganisms	Diameter of inhibition zone (mm)			
	25µl	50µl	75µl	Fluconazole (Fu) (5µg/ disc)
Candida albicans (MDR)	10.66±0.66	14.00±0.57	16.66±0.33	17.00±0.57
Aspergillus niger	08.33±0.33	10.21±0.33	12.00±0.57	17.63±0.57
Trichophyton rubrum	08.66±0.57	11.36±0.66	12.66±0.66	16.33±0.66
Trichophyton mentogrophytes	09.00±0.66	12.30±0.33	13.33±0.66	16.66±0.33

The influence of coelomic fluid of earthworm *E.eugeniae* on the growth of bacterial and fungal cultures was evaluated against selected pathogenic bacterial and fungal strains. The best inhibitory effect of CF of *E.eugeniae* on the growth of *Vibrio parahaemolytic* and *Klebsiella pneumonia* was seen to be 21.00 ± 0.57 mm to 19.66 ± 0.66 mm zone of inhibition. The inhibitory effect is comparatively less against *Pseudomonas aureginosa* and *Proteus vulgaris* exhibiting 19.00 ± 0.57 mm and 14.33 ± 0.33 mm inhibitory zones. All the five bacterial isolates responded to the coelomic fluid of *E.eugeniae*. Maximum antibacterial activity 21.00 ± 0.57 mm was found to be with *V. parahaemolyticus* which was almost closer (22.33 ± 0.33 mm) to the standard antibiotic Ciprofloxacin. Minimum antibacterial activity (14.33 ± 0.33 mm) was obtained with the case of *P.vulgaris*.

As with antibacterial activity antifungal activity was also evaluated in triplicate by measuring the zone of inhibition on SDA plate after 48 hrs incubation at 25^{o} C by disc diffusion method. The results are also tabulated in Table 1. Similar to antibacterial activity the coelomic fluid of *E.eugeniae* also exhibited antifungal activity against all the pathogenic fungi tested by disc diffusion method. The fungal strains were inhibited by CF in a dose dependent manner. The high dose of CF (75 µl) showed highest anti fungal potential. Of the four strains tested; the growth of *Candida albicans* was much inhibited (16.66 ± 0.33 mm). The inhibitory potential of CF was less in case of *Aspergillus niger* (12.00 ± 0.57 mm). To find out the minimum inhibitory concentration (MIC) of the earthworm CF three bacterial as well as three fungal strains were selected. Bacterial strains like *V.parahaemolyticus, K.pneumoniae, P.vulgaris*

whereas fungal strains like *C.albicans, T.rubrum* and *A.niger* were used. The results indicated that CF at a dose of 200µl inhibited the growth of bacteria (Table 3).

Table 3: Minimum Inhibitory Concentration (MIC) of Coelomic fluid using optical density method against bacterial pathogens

Microorganisms	Concentration of Coelomic Fluid in (µl)	Optical Density (OD)
Vibrio parahaemolyticus	Control	16.66±0.57
	50 (µl)	14.53±0.61
	100 (µl)	13.21±0.52
	150 (µl)	10.11±0.48
	200 (µl)	7.08±0.36
Klebsiella pneumoniae	Control	17.18±0.68
	50 (µl)	16.54±0.62
	100 (µl)	15.04±0.32
	150 (µl)	12.13±0.51
	200 (µl)	10.32±0.48
Proteus vulgaris	Control	19.44±0.66
	50 (µl)	18.11±0.58
	100 (µl)	17.20±0.53
	150 (µl)	15.43±0.63
	200 (µl)	14.20±0.51

Table 4 Minimum Inhibitory Concentration (MIC) of Coelomic fluid using optical density method against fungal pathogens

Microorganisms	Concentration of Coelomic Fluid in (µl)	Optical Density (OD)
Candida albicans	Control	19.01±0.63
	40 (µl)	18.11±0.61
	60 (µl)	16.40±0.54
	80 (µl)	15.61±0.46
	100 (µl)	12.23±0.42
Trichophyton rubrum	Control	21.22±0.74
	40 (µl)	21.01±0.52
	60 (µl)	20.43±0.50
	80 (µl)	17.21±0.51
	100 (µl)	14.03±0.43
Aspergillus niger	Control	23.03±0.56
	40 (µl)	22.00±0.48
	60 (µl)	21.68±0.61
	80 (µl)	19.83±0.58
	100 (µl)	17.14±0.53

Similarly, the fungal growth was found to be inhibited at a dose of 100 µl. The optical density (OD) was less at these concentrations. This indicated that CF at a dose of 200µl for bacteria and 100 µl in case of fungi was found to be minimum concentration to inhibit the growth of the selected pathogenic bacteria and fungi.

Our study was in accordance with (Ramasamy *et al*., 2008)[19] who has reported that the coelomic fluid of *E.eugeniae* has maximum antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae.* (Milochau *et al*., 1997) [16] showed that the coelomic fluid of the lumbricid, *Eisenia fetida* andrei possesses antibacterial activity. And also, (Oleynik and Byzov,2008)[17] have found earthworm surface excreta to have potent antimicrobial activity. According to Zasloff (2002)[18] the antimicrobial peptides constitute a very important component of the innate immune system of *E.fetida.*

The coelomic fluid of *E.eugeniae* possesses maximum antifungal activity against *C.albicans* the measurement of inhibition zones revealed that CF was less effective against *A.niger*. The diameter of zone of inhibition observed was (16.66±0.33mm) for *C.albicans* which is followed by *A.niger* (12.00±0.57mm). Fluconazole (5µg/disc) was used as positive control and possessed maximum antifungal activity in comparison to CF in each fungal tested. Our study was supported by (Vasanthi *et al*., 2013)[21] who reported that earthworm paste showed antifungal activity against *C.albican, Aspergillus niger, Aspergillus flavus, Penicillium notatum* and *Trichophytum rubrum*. Our study was also in contrary with (Mathur *et al*., 2011)[15] who reported that the petroleum ether extract of earthworm powder was found to possess maximum antifungal activity against *A.niger* in comparison to *C.albicans.* (Prakash, 2013)[20] have reported that earthworm powder was against various ailments in indigenous system of medicine which was found to be fruitful against microorganism.

According to the obtained results it can be concluded that coelomic fluid of *E.eugeniae* can be used to formulate a new natural antimicrobial product for controlling infection of multidrug resistant bacteria and fungi where treatment is very difficult as the drug of choice for treating the infection doesn't work. The present study reports that earthworm's can be used not only in environmental monitoring but also in the acquisition of novel molecules for human therapeutic purposes. This study may lead to formulation of new natural antimicrobial agent and thus may found to be beneficial in future prospects for mankind.

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