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Comparative Study on the antibacterial activity in the coelomic fluid of Eudrilus eugeniae (Kinberg, 1867) and Polypheretima elongata (Perrier, 1872)

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ABSTRACT

In the present investigation an attempt was made to test the antibacterial activity in the coelomic fluid of the epigeic species of earthworm, *Eudrilus eugeniae* and the anecic species of earthworm, *Polypheretima elongata*. The Eudrilus eugeniae was collected from Selvam Vermicomposting farm, Udumalpet, Tamil Nadu, India. Likewise, the *Polypheretima elongata* was also collected from the soil of National college campus, Tiruchirappalli, Tamil Nadu, India. Both worms were identified by following standard taxonomical key characters. After identification, both worms were cultured under favorable laboratory condition for 30 days providing the food material consisting of 50% predeomposed organic waste and 50% predecomposed cow dung in separate cement tanks. At the end of 30 days a pool of 20 healthy worms was selected from both cultures for the extraction of coelomic fluid. Later on, the fluid was extracted by electric shock method employing 9 volt electrode. The extracted coelomic fluid (100%) was used to test the antibacterial activity against two gram positive bacteria namely *Staphylococcus aureus* and *Pseudomonas aeruginosa* and one gram negative bacterium namely *E.coli*. Among the selected bacterial strains, the coelomic fluid of *Eudrlus eugeniae* and *Polypheretima elongata* showed the maximum inhibition zones 7.01 ± 0.28mm and 2.43 ± 0.38 mm against *Staphylococcus aureus* respectively when compared to inhibition zones 2.35 ± 0.31 mm and 2.28 ± 0.29 mm; 2.66 ± 0.31 mm and 1.35 ± 0.17 mm produced by *Eudrilus eugeniae* and *Polypheretima elongata* against *Pseudomonas aeruginosa* and *E.coli* respectively. Besides that the coelomic fluid of *Eudrilus eugeniae* exhibited more inhibition activity than the zone (3.70 ± 0.13mm) produced by the positive control (standard antibiotic, penicillin) against *Staphylococcus aureus.* But at the same time there is no remarkable difference between the inhibition zone of the coelomic fluid of *Polypheretima elongata* and the positive control experiment against all the bacterial strains tested. However, the control experiment showed more inhibition zone (3.97 ± 0.24 mm) than the inhibition zone produced by the coelomic fluid of Polypheretima elongata against *Pseudomonas aeruginosa*. As for the present study the bacterium *Staphylococcus aureus* activity is highly inhibited by the coelomic fluid of both species of earthworms than the other bacterial strains tested even though there is remarkable difference in the size of zone of inhibition. The data were statistically analyzed at 5.0% level employing ANOVA.

Introduction

Earthworms are macro invertebrates which constitute the most important group of the soil invertebrates biodiversity in terms of their influence on soil formation and maintenance of soil fertility in agro-ecosystems (Edward *et al*., 1995), and also form more than 80% of the invertebrate biomass in world ecosystems (Senepati, 1992). Earthworms are physically aerators, crushers and mixers; chemically decomposers; and biologically stimulators, in the decomposer systems. They effectively harness the beneficial microflora, destroy the soil pathogens and convert the organic wastes into vitamins, enzymes, antibiotics, growth hormones and protein rich casts (Prabha *et al*., 2007). Of late, these organisms have drawn the attention of scientists because of their involvement in organic waste degradation to produce a marketable bio-fertilizer, vermicompost (Edwards and Bohlen, 1996). It is a good substitute for commercial fertilizers and has high level of N, P and K than the normal heap manure (Srivastava and Beohar, 2004). Besides, these organisms are a source of animal protein in the diet of animals such as poultry birds and fish (Harwood,1976; Mekada *et al*.,1979; Sabine,1978; Guerrero,1983; Xu *et al*.,1984;Stafford and Tacon,1988) and indigenous people (Edwards and Lofty,1972;Macdonald,1983). Apart from this, recently earthworms play an important role in the pharmacological studies.

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Earthworms have been used in medicine for various remedies since 1340 AD (Stephenson, 1930). Earthworm has been recognized in oriental medicine as anti-inflammatory, analgesic and anti-pyretic agent (Noda *et al*., 1992). It shows anti-cancer effect by preventing excess glucose uptake (Nagasawa *et al*., 1992). Microorganisms are known to play a major role in soil characteristics; invertebrates are believed to act as regulators of antimicrobial activity. Earthworm surface excreta were found to have potent antimicrobial activity (Oleynik and Byzov, 2008).

It is also having anti-coagulatory and fibrinolytic activity which results in the facilitation of blood circulation (Wang *et al*., 1989). The earthworm has been suspected to contain proteases which dissolve the fibrin clots are anticoagulant which selectively interfere with the intrinsic pathway of blood coagulation cascade (Mannet *et al*., 1990 Davie *et al*., 1991, Leipner *et al*., 1993 Kim *et al*., 1995 and Woo *et al*., 1996). Medicinal properties of earthworm have also been described by authors like Bristow (1932), Ogata and Mori (1938), Ogata *et al* (1939), Carr (1951) and Yegnanarayan *et al* (1987).

Besides that earthworms have largely been used internally and externally as powerful aphrodisiacs (Vohora and khan, 1978). The anti-inflammatory and anti-pyretic activities of biologically isolated extract from whole earthworm, Lampito mauritti was determined (Balamurugan *et al*, 2008). Antimicrobial potency of Eudrilus eugeniae extracts on certain plant pathogens was studied (Shobha and Kale, 2007). Several isolated bacteria strains exhibited at a high pathogenic effect when injected into Eisenia coelomic cavity (Lassbgues *et al*., 1981). Anti-tumor activities of earthworm fibrinolyic enzyme on human hepatoma cells were studied (Hong, 2007). In 1983 it was also reported that very strong and novel fibrinolytic enzymes could be extracted from the earthworm, Lumbricus rubellus. These enzymes were fractionated and purified as six novel fibrinolytic enzymes and named collectively as Lumbrokinase (Mihra *et al*., 1996; Tang *et al*,.2000; Li *et al*,.2008). It was also found that earth worm powder contains two kinds of inhibitory substance for the platelet aggregation induced by collagen and ADP. This novel substance also displays a relaxation effect for the canine saphenous vein induced by prostaglandin F in vitro and an inhibitory effect on the active partial Thromboplastin time (APTT) (Mihara *et al*., 1996). Earthworm powder can be given orally; thus for this reason, earthworm powder has potential application as a thrombolytic and also exerts an inhibitory effect on platelet aggregation, an anticoagulant effect and a relaxation effect for the vascular systems, which are all effective for thrombotic therapy (Kim *et al*., 1998). Earthworm powder represents a very promising agent for the treatment of thrombosis (Mihara *et al*., 1996). Its tonic properties also make it beneficial support for the live and other organ system.

In china, Korea, Vietnam and most of Southeast Asia, Lumbricus has been used for their therapeutic benefits for thousands of years and referred to as earth dragons. It has been found that coelomic fluid of the earthworm contain more than 40 proteins and exhibits several biological activities as follows; cytolytic, proteolytic, antimicrobial, hemolytic, hemagglutinating, tumorolytic, mitogenic activities (Cotuk and Dales 1984; Lange *et al*. 1997; Lange *et al*. 1999; Cooper and Roach 2003; Lassegues *et al*, 1981) found that this hemolytic factor also inhibited the growth of different bacterial species which were isolated from nature. The coelmic fluid of Eisennia fortida Andrei was demonstrated to possess an antimicrobial activity against Aeromonas hydrophila and Bacillus megaterium which are known as earthworm pathogens (Valembois *et al*. 1982 and Pan *et al*. 2003). Afterwards, Milocha *et al*. (1997) obtained two proteins, named Fetidins from dialyzed coelomic fluid of earthworms and confirmed that this antibacterial activity was due to fetidins. Cho *et al*. (1998) found that Lumbricus rubellus also has two antibacterial agents named Lumbricin 1 and Lumbricin 2. Of late, Kathireshwari et.al. (2014) have reported that the antimicrobial activity of the two species of earthworms namely Lampito mauritii and Megascolex konkanesis have revealed the maximum inhibition activity against the bacterial strains such as Aeromonas hydrophilla, Bacillus subtillus and Vibrio para haemolyticus and they also stated that the coelomic fluid of the earthworms namely Darwida impertusa and Darwida lennora showed less inhibition activity against these bacterial strains.

Recently, two types of antibacterial factors which include lysozyme-like molecules with hemolytic activity as well as a pattern recognition protein named coelomic cytolytic factor (CCF) have been identified in Eisenia foetida earthworm (Kohlerova *et al*. 2004). Bruhn *et al*. (2006) started that lysenin which was a different protein of Eisenia foetida and lysenin-like proteins had several cytolytic activities which exerted hemolytic, antibacterial and membrane-permeabilizing properties. Many proteins are involved in immunodefense of earthworms, such as lysozyme, lysenin, fetidins and CCF-1 (Cooper *et al*.2002). However, it is not known whether antimicrobial peptides contribute to the innate defense of earthworms. To date only one antimicrobial peptide, lumbricin I, has been isolated from the earthworm Lumbricus rubellus. Lumbricin I, which belongs to proline- rich antimicrobial peptides group, kills both bacteria and fungi without hemolytic activity in vitro (Cho *et al*. 1998). Despite their immense economic importance, the investigation on the antibacterial activity of earthworms is limited to three to four species. Hence, in the present investigation an attempt was made to bring out new antimicrobial agent or components from the coelomic fluid of earthworms, Eudrilus eugeniae and Polypheretima elongata. In addition to that the present study will definitely lead to formulation of new natural antimicrobial agent and this may be found useful in future prospectus for mankind.

Materials and methods

Collection and identification of earthworms

The earthworms were collected with the help of shovel by digging and hand sorting method from the soil of National College Campus Area. The soil was dug up to a depth of 0-100 cm and the worms were hand collected cautiously without any damage and they were washed in clean surface water. Among the collected worms some of them were segregated to study the taxonomical characters. They were allowed in enamel tray half filled with water. They were narcotized by gradually adding ethanol to water. The narcotized worms were fixed in 5%-10% formalin solution depending on the size of the worms for 24 hrs in straightened position. The fixed worms were examined morphologically and anatomically to study the taxonomical characters (Julka and Paliwal, 1993).

Both external and internal characters are considered important in earthworm taxonomy. It is therefore, necessary to dissect the earthworms for their internal observations.

Earthworms were examined in various growth stages. This is indicated in the material examined as 2-4-7, denoting 2 juveniles, 4 aclitellate and 7 clitellate worms (Julka, 1988). Lengths of uniformly contracted worms were measured with a linear scale, and their diameter was measured on segments just anterior to clitellum. The worms were dissected longitudinally slightly left or right side of the mid-dorsal line by using a sharp tissue culture blade. Morphology, location and presence or absences of internal organs were recorded.

A Distance between setae of a worm was measured by an ocular micrometer. Measurement of pineal or copulatory setae was also taken by an ocular micrometer under high magnification. Slides were prepared for studying spermathecae and different kinds of setae. All illustrations were drawn with the help of a camera lucida.

Culturing of earthworms

Among the collected earthworms some of them were segregated to culture under favoural laboratory condition. They were cultured in suitable cement tanks (1m length ×1 meter breadth×50cm height) by providing the feeding material consisting of 25% decomposed organic waste and 25% predecomposed cow dung and 50% garden soil. A pool of 20 healthy matured worms was selected to extract the coelomic fluid for the study of antibacterial activity.

Cleaning and removing of gut content of earthworms

Fully matured earthworms were washed in running tap water in order to remove the sand particles from the surface of earthworms. Then the washed earthworms were soaked in N-saline solution and the solution was exchanged every time after wash till the gut of earthworms was thoroughly cleaned (Abhishek mathur *et al*., 2010).

Extraction of coelomic fluid

Coelomic fluid was extracted by applying a potential difference across a pool of worms, causing them to extrude coelomic fluid. A pool of twenty worms were placed in a large petridish in an electrolyte solution (0.1%Nacl) that was also served to collect the extruded coelomic fluid (ECF), and a potential difference of 9 V was applied across the worms for 20^30 s. The coelomic fluid was collected with the help of micropipette. The collected coelomic fluid was centrifuged for 5 minutes at 350 rpm and the filtrate was used. The fluid was used at the concentration of 100% to study the antibacterial activity (Bundy *et al*., 2001).

Antibacterial assay

Disk method was used to assay the antibacterial activity of coelomic fluid of Eudrilus eugeniae. Bacterial cells were grown overnight in LB medium and inoculated into 5ml of molten 0.6 g/l agar with a final concentration of 106 colony forming units per ml, which was overlaid onto a 90mm petridish containing 10ml of 2g/l LB solidified agar. After the top agar hardened, sterilized blotting paper (about 6.0 mm in diameter), free from antibacterial activity, were impregnated with 20μL of coelomic fluid to be tested and placed on agar dishes inoculated with one bacterial strain. The dishes were incubated over night at 37.0℃ . Control tests were performed with penicilin.

Bacterial strains used for determining antibacterial activity included Escherichia coli, *Staphylococcus aureus* and *Pseudomonas aeruginosa* which were obtained from Clab cultural laboratory, Trichirappalli, Tamilnadu, India. Antibacterial activity was determined by determining the Relative Magnitude of Inhibition (RMI). The data were statistically analyzed at 5.0% level employing ANOVA.

$$RMI = \frac{[Area\ defined\ by\ zone\ of\ inhibition\ (including\ disc)]}{Area\ defined\ by\ the\ disc}$$

Results and Discussion

Table 1 shows the antibacterial activity of the coelomic fluid of the epigeic species of earthworm *Eudrilus eugeniae* and the anecic species of earthworm *Polypheretima elongata* against two strains of gram positive bacteria namely *Staphylococcus aureus* and *Pseudomonas aeruginosa* and one strain of gram negative bacterium namely *E. coli*. Among the selected bacterial strains the maximum inhibition zone of coelomic fluid of *Eudrilus eugeniae* (7.01 ± 0.28mm) and *Polypheretima elongata* (2.43 ± 0.80mm) was noticed against the same species of bacterium *Staphylococcus aureus* when compared with the inhibition zones produced by other bacterial strains tested in the present study. The maximum antibacterial activity in the coelomic fluid may be due to the presence of the range of humoral defence factor including agglutinins (e.g. lectin), lysosomal enzymes (e.g. acid phosphatase) and bactericidins (Cooper, 2005). But at the same time the coelomic fluid of *Polypheretima elongata* exhibited the minimum inhibition zones (2.43 ± 0.80mm, 1.35 ± 0.19mm and 2.28 ± 0.29mm) against all the bacterial strains tested than the inhibition zone produced by the coelomc fluid of *Eudrilus eugeniae* against these strains (Plate 1, 2,3,4,5 and 6).

Besides, the antibacterial activity in the coelomic fluid of both species of earthworms against *Pseudomonas aeruginosa* did not make much difference in the size of zone of inhibition. As for *E.coli Eudrilus eugeniae'*s coelomic fluid showed higher inhibition zone against this bacterium than the zone produced by the ceolomic fluid of *Polypheretima elongata*. According to control experiment the maximum and minimum inhibition zones were noticed against *Pseudomonas aeruginosa* and *Escherichia coli* respectively. However, the coelomic fluid of *Eudrilus eugeniae* showed high inhibition zone against *Staphylococcus aureus* and *Escherichia coli* than the control experiment whereas the control test revealed high inhibition activity against *Pseudomonas aeruginosa* than the coelomic fluid of *Eudrilus eugeniae*. But at the same time the control experiment revealed high inhibition zone against all the bacterial strains than inhibition zone of coelomic fluid of *Polypheretima elongata*. Dhanam *et al*. (2016) also suggested that the antimicrobial function of earthworm skin extraction to *S. marcescens* exposure was highest and the diameter of the growth of inhibition was 3mm whereas *S. aureus, K. pneumonia, B. aryabhattai, B. megaterium, P. putida* did not have the activity. Moreover, in the present investigation it has been proved that on the whole *Staphylococcus aureus*`s activity is highly inhibited by the coelomic fluid of both species of earthworms than the other bacterial strains tested.

The earthworm is a first terrestrial invaded organism and it is living in the soil with millions of microorganisms. During the microbial infection the immune system in the earthworms has evolved to protect them from pathogens, microbes, viruses and infected cell. Popovic *et. al*. (1996) have reported that the antibacterial activity in the coelomic fluid of earthworm may be due to the presence of innate immune mechanism and detect the microorganisms by recognizing conserved molecular pattern. The present study corroborate with those of Ramasamy *et al*.(2008) and Zasloff (2002), who stated that anti-microbial peptides constitute a

very important component of the innate immune system of *Eudrlus eugeniae* and *E.foetida* respectively.

Invertebrates in general and earthworm in particular apparently lack lymphoid organs, lymphocytes and immunoglobulins but they have very efficient host defence system, because the body fluids contain a range of humoral defence factors including agglutinins (e.g. lectin), lysosomal enzymes (e.g. acid phosphatase) and bactericidins (Cooper, 2005). Earthworms have primitive non-specific recognition systems, and they are mediating through innate immune responses. Earthworms have extremely effective physiochemical barriers and are acting as first line of defence against pathogens. During infection, group of leucocytes, free within blood vessels, or occupying fluid filled body cavities known as coelom, are ejected out through dorsal pores, bind to microbes, engulf and then kill them. The coelomocytes of earthworm otherwise known as leucocytes (monocytes, macrophages polymorphonuclear and neutrophils) are responsible for innate immune functions such as phagocytosis and encapsulation against pathogens and parasites. With this effect the worms are able to survey the danger exposures necessitated by their more sluggish habit (Renganathan.2006).

Earthworms kill microorganism by recognizing conserved molecular patterns (lipopolysaccharides (LPS) or peptidoglycans from bacterial cell walls, β-1, 3-glucan of fungal cell walls, and double stranded RNA of viruses) on pathogen's body surface. Recognition of molecules for foreign material has been named as pattern-recognition proteins (PRPs) (Medzhitov and Janeway, 1997) because the host's primitive effecter cells would recognize molecular patterns rather than particular structures of the invading microorganisms. When bacteria invade into the coelomic cavity of earthworm, the coelomocytes starts to connect with each other by their adhesive structures around the bacteria and form "brown bodies" (Valembios, *et al*., 1982). At the same time the coelomocytes intensively synthesize and secrete proteins that adhere to the bacteria, forming aggregations and may inhibit their further proliferation. These proteins attach to the lectin like monosaccharide of the cellular membrane of the bacteria. Different proteins and peptides of different species of earthworms have been extensively studied and different mechanisms of actions have been proposed (Marta *et al.*, 2010). Hence, it is very difficult to define which molecule and mechanism of coelomic fluid or extract of earthworms is responsible for its antibacterial activity.

Milochau *et.al* (1997) revealed the anti-bacterial activity of dialyzed coelomic fluid of *Lumbricid, Eisenia foetida Anderi.* The antibacterial l activity of coelomic fluid of *Esienia foetida* was proposed by Valem *et al (1982). Liu et.al., (2004)* identified and purified a novel antibacterial peptide namely OE3121 which is responsible for the efficient immune response of earthworm. It has been reported that the coelomic cytofactor I isolated from the coelomic fluid is involved in the activation at prophenoloxidase carcade via recognition at gram negative bacterial cell wall molecules, such as glucan and lipopolysaccharide (Hrzenjak *et al*. 1992). The present investigation states that it is difficult to define which molecules of coelomic fluid are responsible for its antibacterial activity. Hence, the further detail study is needed for screening out the exact molecules which are responsible for its antibacterial activity.

Plate 1. Inhibition zone of coelomic fluid of earthworm, Eudrilus eugeniae against Staphylococcus aureus

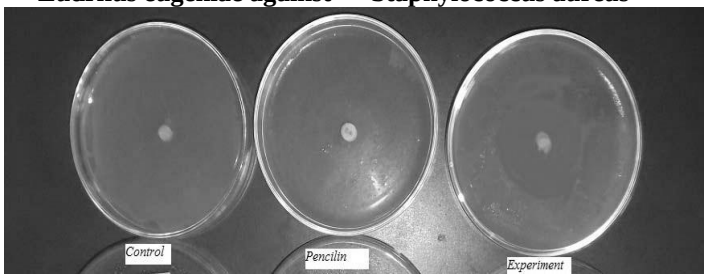

Plate 2. Inhibition zone of coelomic fluid of earthworm, Eudrilus eugeniae against Pseudomonas aeruginosa

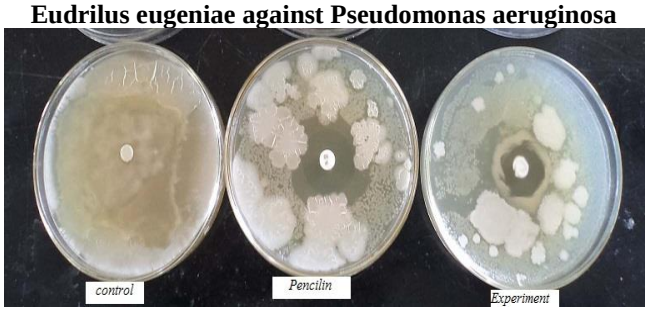

Plate 3. Inhibition zone of coelomic fluid of earthworm, Eudrilus eugeniae against E.coli.

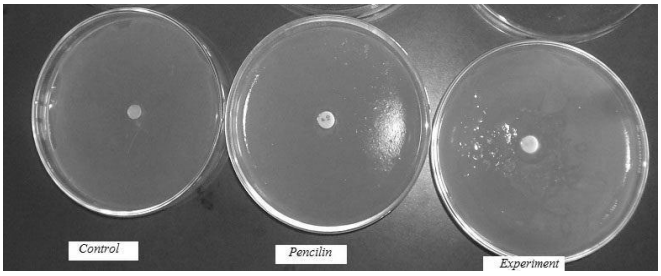

Plate 4. Inhibition zone of coelomic fluid of earthworm, Polypheretima elongata against Staphylococcus aureus

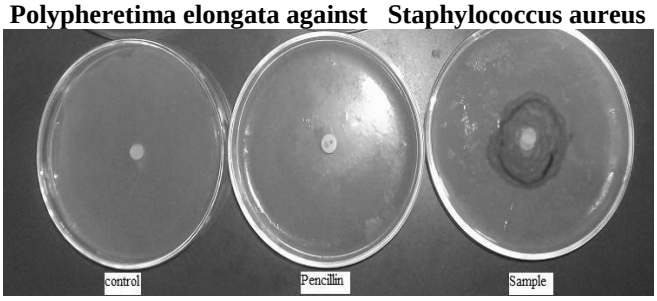

Plate 5. Inhibition zone of coelomic fluid of earthworm, Polypheretima elongata against Pseudomonas aeruginosa

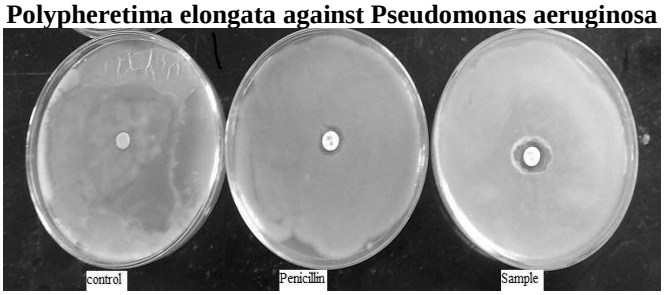

Plate 6.Inhibition zone of coelomic fluid of earthworm, Polypheretima elongata against E.coli.

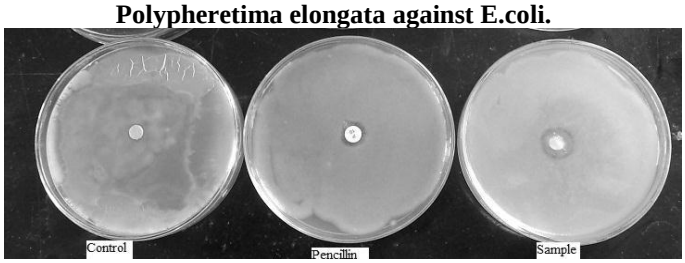

Table 1. Comparative study on antibacterial activity in the coelomic fluid of *Eudrilus eugeniae* and Polypheretima elongata against different species of bacteria. Size (mm) of the zone of inhibition produced by 6mm disc on bacterial lawn.

Species of bacteria	Inhibition zone (mm) of coelomic fluid of *Eudrilus eugeniae*			Inhibition zone (mm) of coelomic fluid of Polypheretima elongata		
	A1	A2	A1/A2=RMI	A1	A2	A1/A2=RMI
Control	0	0	0	0	0	0
I Gram- positive -*Staphylococcus aureus* -*Pseudomonas aureus*	 42.60 14.10	 6.0 6.0	 7.01 2.35	 14.60 13.60	 6.0 6.0	 2.43 2.28
II Gram-negative - E.coli	16.0	6.0	2.66	8.10	6.0	1.35
III Antibiotic (Penicillin) -*Staphylococcus aureus* -*Pseudomonas aureus* - *E.coli*	Zone of inhibition(mm) of antibiotic(Penicillin)					
	A1		A2	A1/A2 = RMI		
	21.0 21.4 6.6		6.0 6.0 6.0	3.70 3.97 1.77		

A1- Area defined by zone of inhibition (including disc) A2- Area defined by the disc RMI- Relative magnitude of inhibition

Statistical analysis for table 1

S.No	Species of bacteria	Zone of inhibition(mm) of coelomic fluid of *Eudrilus eugeniae*	Zone of inhibition(mm) of coelomic fluid of *Polypheretima elongata*
1.	I- Gram positive - *Staphylococcus aureus* - *Pseudomonas aureus*	 7.01 ± 0.28 2.35 ± 0.31	 2.43 ± 0.38 2.28 ± 0.29
2.	II- Gram negative - E.coli	 3.66 ± 0.31	 1.35 ± 0.19
3.	III- Antibiotic (Penicillin) -*Staphylococcus aureus* -*Pseudomonas aureus* - *E.coli*	 3.70 ± 0.13 3.97 ± 0.24 1.77 ± 0.44	

Values are explained by mean ± SD of six samples - significant at 5% level

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