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Research Article

SYNTHESIS OF NANOFIBRE AND SILVER NANOPARTICLES FROM COELOMIC FLUID OF EARTHWORM, *EUDRILUS EUGENIAE* AND *PONTOSCOLEX CORETHRURUS* AND ITS ANTIMICROBIAL POTENCY

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ABSTRACT

Nanotechnology is also referred to the ability for designing, characterization, production and application of structures. An increasingly common application is the use of silver nanoparticles and nanofibers for antimicrobial coatings, wound dressings, and biomedical devices. In this recent world earthworms are showing the excellent scale in the medical field. In this present paper, we have collected *Eudrilus eugeniae*, and *Pontoscolex corethrurus* to harvest the coelomic fluid from the earthworm for the synthesis of silver nanopaticles and nanofibers. Nanofibers are synthesized using human collagen and poly lactic acid. Coelomic fluid and Ag NPS was investigated for antibacterial activity and haemolytic activity. Haemolytic activity of *Eudrilus eugeniae* was observed even in low quantity of coelomic fluid and haemolysis was observed for *Pontoscolex corethrurus* only in high quantity of fluid. The antimicrobial activity was found high in all types of earthworms. But nanoparticles from coelomic fluid showed higher activity than the coelomic fluid. Nanofibres from coelomic fluid does not showed any bioactivity against pathogens. Ag NPS was confirmed by the colour reduced to form brown and by UV-visible spectrum in the range of 400 to 430nm. Protein profile was investigated by the SDS-PAGE and the molecular weight was determined as 200KDa. From this study we conclude that the coelomic fluid from earthworm can be used as therapeutic agent.

Keywords: Ag NPS, Nanofibers, Haemolysis, UV-visible spectrum, SDS-PAGE.

INTRODUCTION

Nanotechnology is the application of science and technology to control matter at the molecular level. The term nanotechnology was first defined by Norio Taniguchi, Tokyo science University in 1974 [1]. Nanotechnology emerges from the physical, chemical, biological and engineering sciences, where novel techniques are being developed to probe and influenced single atoms, and molecules [2]. Nanoparticles are being viewed as fundamental building blocks of nanotechnology. Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm [3].

Nanoparticles of noble metals such as gold, silver and platinum are widely applied in products that directly come in contact with the human body such as detergent, soaps, cosmetic products, toothpaste besides medical and pharmaceutical applications [4]. The nanoparticles are used in many ways it can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. Recently, there has been growing interest in one dimensional nanomaterial such as nano rods, nanowires, nanofibers and nanotubes of various oxide materials, because of their fundamental scientific interest and also their potential applications in a variety of functional devices [5]. Among them development of nanoparticles and nanofibers has greatly enhanced the scope for fabricating scaffolds that can potentially meet this challenge.

The earthworms body cavity contain coelomic fluid and leukocytes that are as varied as they are in other equally complex invertebrates and they resemble certain vertebrate leukocytes with respect to morphology, cytochemistry and function [6]. Both the leukocytes and the fluid that they synthesize and secrete, affect immunobiological responses [7]. The coelomic fluid of earthworm contains more than 40 proteins. They do by various routes: opsonization, inflammation and phagocytosis, agglutination, mitogenesis, lysis, destruction of experimentally introduced allogeneic, xenogenic but not autogenic transplants invivo and various target cell types invitro. In this present study we selected two types of earthworms i.e., *Eudrilus eugeniae* and *Pontoscolex corethrurus* for the collection of coelomic fluid. The main goal of the present study was to perform antimicrobial activity and haemolytic activity of coelomic fluid of earthworms and to biosynthesize silver nanoparticles and nanofiber from coelomic fluid because the coelomic fluid contains more than 40 proteins and exhibits several biological activities. And final approach was to determine the molecular weight of protein by SDS PAGE.

MATERIALS AND METHODS

Collection of earthworm

Earthworm, *Pontoscolex corethrurus* were collected from the composting area i.e., the place which contains cow dung and the waste materials in Nagercoil. Another earthworm *Eudrilus eugeniae* was purchased from Vivekananda Kendra, Kanyakumari to compare with the isolated worm.

Maintenance of earthworm

Earthworm was maintained in room temperature. Clitellate (sexually mature) earthworms were cultured in a plastic tray (diameter 30 cm, height 20 cm). About 2 kg of cow dung was mixed with half kg of soil. Each worm was maintained in the separate place. The mature worm was used for the study. The earthworms are fed with tissue paper for overnight to clean the gut. After that, they were immersed in clean, cool water to eliminate gastro intestinal metabolites and contaminants. They were then washed with saline solution and dried on whatman filter paper.

Harvesting of coelomic fluid

Mainly there are three major methods to harvest the coelomic fluid from earthworms. They are as follows.,

Ice cold method

The coelomic fluid was collected from the gut cleaned earthworm. In this ice cold method, the earthworm was taken and placed on the petriplate held in standing position. The plate containing earthworm was placed on the beaker with ice cubes to give cold shocks. The coelomic fluid oozed out from the dorsal pores of the earthworm due to shock was collected [8]. The coelomic fluid was collected using sterile dropper into sterile polypropylene tubes. Then the worm was released in their respective compost. After two weeks, again the same worm was used to collect the coelomic fluid. Following harvesting, coelomic fluid was centrifuged immediately at 10,000 rpm for 15 minutes. Cell free supernatant was then passed through a 0.2 µm pore sized millipore membrane filter. The sterile supernatant was stored at -20°C until use.

Electrical shock method

In this method, the gut cleaned earthworms were washed, dried in soft paper, and then excited with a 5V stimulation, which induced them to extrude coelomic fluid through epidermal dorsal pores. Then the stimulated earthworms were washed with pH 6.8 PBS (Phosphate Buffer Saline) twice and dried in soft paper. The coelomic fluid was collected in sterile tubes.

Ethanol extrusion

Coelomic fluid was collected using a non-invasive method. Briefly earthworm was rinsed in saline solution and placed on a paper towel. One-fourth of the posterior part was massaged to expel the content of the lower gut. Then, earthworm was placed for 3 min in a 15 mL polypropylene tube containing 3.0 ml of cold extraction medium. The extraction medium containing 5% ethanol and was adjusted to pH 7.3 with 1M NaOH. After 3 min, the worm was removed and the volume was made up by adding 12 ml of ice-cold saline solution and adjusted to pH 7.3. The cells were recovered by centrifugation at 15,000 rpm at 4ºC [9]. Finally the fluid was stored at -20ºC.

Synthesis of Nano fiber from coelomic fluid

A wide range of polymers had been used to electrospin nanaofibers. In this study, natural polymers such as human collagen and poly lactic acid were used for the synthesis of nanofibers from coelomic fluid.

Using lyophilized human umbilical cord collagen (a natural source) the nanofibre was prepared by electrospray with human collagen. 200 mg of human collagen (isolated from the lab) was dissolved with 5 ml Hexafluroiso propanol (HFIP, Sigma-Aldrich) and was stirred under 4 degrees for 48 hours. 1 ml of coelomic fluid was added to the dissolved collagen and stirred for 2 hours. Finally the dissolved collagen and coelomic fluid was electro sprayed at 25 V with a flow rate of 1 ml/h. The nanofibers were collected in sterile culture plates and cover slips. The nanofiber membrane was prepared by electrospray technique. Using poly lactic acid polymer, the nanofibre was prepared by mixing 400 mg Poly Lactic acid (PLA, Sigma-Aldrich) dissolved with 5 ml Hexafluroiso propanol (HFIP, Sigma-Aldrich) and was stirred under 4 degrees for 48 hours. 1 ml of coelomic fluid was added to the dissolved poly lactic acid polymer and stirred for 2 hours. Finally the dissolved polymer and coelomic fluid was electro sprayed at 25 V with a flow rate of 1 ml/h. The nanofibers were collected in sterile culture plates and cover slips.

Synthesis of silver nanoparticle from coelomic fluid

The 25 ml of coelomic fluid was added into 225 ml of aqueous solution of 1Mm silver nitrate ($AgNO_3$) for reduction of silver nitrate into Ag^+ ions and kept at room temperature. After 3 to 4 days, the colour change was observed. The solution was kept in the dark to avoid other biological changes. Samples showed change in colour from almost colourless to brown, this is a clear indication of the formation of silver nanoparticles in the reaction mixture. In this process silver nanoparticles were produced through reduction of silver ions to metallic silver. Control showed no change in colour of the mixture, when incubated in the same conditions. Then the colour changed solutions were centrifuged in three to four times at 10,000 rpm for 15 minutes. Afterwards the pellet was collected and washed with distilled water. Finally the methanol was used for washing; moreover the pellets were allowed to dry. The dried particle was selected and used for further studies.

Analysis of silver nanoparticles from coelomic fluid by UV-Vis spectrophotometer

UV-Vis spectrum analysis was done by using UV-Vis spectrophotometer (Double Beam, 1 2902). The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium of sample. The wave length of spectrophotometer was taken between 300-500 nm. The water was used as a blank for UV-Vis spectrum analysis.

Biological activity of coelomic fluid

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

Haemolytic activity

Haemolytic activity was carried out with human red blood cells by agar well diffusion method. The blood agar was prepared and autoclaved, cooled the agar down to about 45-50°C, and 5% v/v human red blood cells were added, mixed well and poured into the sterile petriplates. After solidification, wells were cut out from the agar plates using a sterilized stainless steel borer and filled with the sample (coelomic fluid) in different dilution. Then the plates were incubated in 25°C for 24 hours. After incubation, the diameters of haemolytic zones were measured.

Antimicrobial activity of coelomic fluid by agar well diffusion method

The bacterial pathogens such as *Staphylococcus* sp., *Proteus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *E.coli* were collected from Vivek laboratory, Nagercoil. During the bioactivity method, the Muller Hinton Agar media was prepared and they were sterilized using autoclave. After sterilization, the media was poured in the sterile petriplates. After solidification the overnight culture such as *Staphylococcus* sp., *Proteus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *E.coli* were swabbed in sterile condition. Then the wells were cut out (8mm diameter) from the agar plates using a sterilized stainless steel borer. The coelomic fluid was diluted in the ratio of 1:1, 1:2, 1:4, and 1:16 and concentrated coelomic fluid was also used. Each well was loaded with 30µl of the sample. In the case of silver nanoparticles, only 10 µl of the sample was loaded and the plates were incubated at 37°C for 24 hours. After incubation, the diameters of inhibition zones were measured.

Antimicrobial activity of Nano fiber coated with coelomic fluid

In this method, the Muller Hinton Agar plates were prepared and swabbed with the test organisms. Then the nanofiber was removed from the cover slip and placed over the inoculums. The plates were incubated at 37°C for 24 hours. After incubation the results were observed by measuring the zone of inhibition.

Molecular mass determination of coelomic fluid by SDS-PAGE

The coelomic fluid was protein in nature. It is large in size and can be isolated and the size of the protein was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were detected by Coomassive blue staining and then destained using a mixture of methanol, glacial acetic acid and distilled water. The separated protein molecules were analyzed by comparing it with standard protein [10].

RESULT

Identification of the earthworms

The earthworms were collected from the composting area. The worms were identified based on their morphological and physiological characteristic features.

Based on the various characteristic features, the earthworms were identified as *Pontoscolex corethrurus* (Fig. 1). The other worm i.e., *Eudrilus eugeniae* (Fig.2) was purchased from Vivenkanandha Kendra, Kanyakumari.

Figure1: *Pontoscolex corethrurus*

Figure 2: *Eudrilus eugeniae*

Harvesting of coelomic fluid

The ice cold method, ethanol extrusion and electrical shock methods were used for harvesting the coelomic fluid. In Ice cold method, the yield of fluid was higher and the mortality rate was less. In electrical shock method, the mortality rate was high and the yield of fluid was less. In ethanol extrusion method, the 100% mortality rate was occurred and yield of fluid was very high. Among all these three methods, the ice cold method was very efficient, because the mortality rate was low and the yield of fluid was also high. So the ice cold method was used in this study to collect the coelomic fluid. The nature of the *Eudrilus eugeniae* fluid was colorless thick jelly and the fluid of *Pontoscolex corethrurus* was light coloured and slightly jelly in nature.

Synthesis of Nano fiber from coelomic fluid

The *Eudrilus eugeniae* and *Pontoscolex corethrurus* fluids were used for the nanofiber synthesis. In this method, the natural human collagen and poly lactic acid was used for the synthesis of nanofiber. The microscopic examination of nanofibers was given in Fig.3. The polymeric (natural or synthetic) form of the nanofiber was prepared using electro spinning or electro spraying technique. We have achieved the synthesis of good nanofibers from the coelomic fluid.

Figure 3: Microscopic observations of nanofiber from coelomic fluid

Synthesis of silver nanoparticles from coelomic fluid

The coelomic fluid of earthworm i.e. *Eudrilus eugeniae* and *Pontoscolex corethrurus* was selected for the biosynthesis of silver nanoparticles. After the incubation the silver ions were reduced to form brown colour and it was verified by UV-Vis spectrophotometer. A strong peak was observed at 410 nm and 430 nm for nanoparticles from *Eudrilus eugeniae* and *Pontoscolex corethrurus* respectively

Biological activity of coelomic fluid

Hemolytic activity

The hemolytic activity was carried out for coelomic fluid of *Eudrilus eugeniae* and *Pontoscolex corethrurus* against human erythrocytes. The 5% erythrocytes were used. After the incubation period the zone of hemolysis were measured.

The coelomic fluid of earthworm showed the zone of hemolysis as follows: 12 mm in 10 µl**,** 14mm in 20µl and 16mm in 100 µl for *Eudrilus eugeniae* and hemolysis was observed for *Pontoscolex corethrurus* only in 100 µl loaded well. The hemolytic activities of the coelomic fluid against human erythrocytes were given in the Fig. 4 and 5.

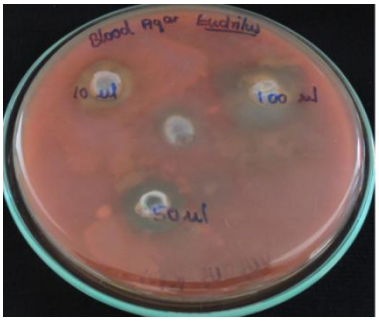

Figure 4: Haemolytic activity of *Eudrilus eugeniae*

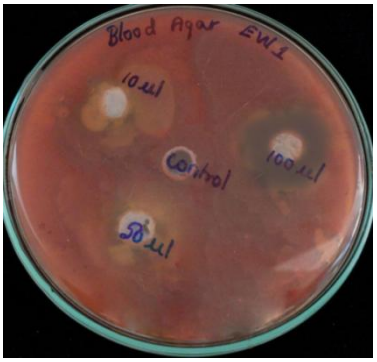

Figure 5: Haemolytic activity of *Pontoscolex corethrurus*

Antimicrobial activity of the coelomic fluid

The antimicrobial activity of coelomic fluid of earthworms was performed against gram positive and gram negative bacteria. The activity was limited in *E.coli* (9mm), *Pseudomonas* sp. and *Klebsiella* sp. (10mm), *Proteus* sp. (13mm) and *Staphylococcus* sp. (16mm) in 1.16 ratio. The concentration of coelomic fluid increases; the antimicrobial activity was also increases. The concentrated coelomic fluid displayed broad spectrum of antibacterial activity. The diluted fluids (1:16) also inhibit the pathogens but not as effective as in concentrated fluid. In this bioactivity, the *Staphylococcus* sp. was highly sensitive i.e. 20 mm in diameter of inhibition zone. The following species were inhibited in the range of 17 mm for *E.coli*, 18 mm for *Proteus* sp., 15 mm for *Pseudomonas* sp. and 10 mm for *Klebsiella* sp. The antimicrobial activity of the coelomic fluid of *Eudrilus eugeniae* against pathogens was shown in table 1 and fig. 5 and 6.

Table 1: Antimicrobial activity of coelomic fluid of *Eudrilus eugeniae* against human pathgens

S.No	Organisms	1:1	1:2	1:4	1:16	concentrated
1	*Staphylococcus* sp.	18 mm	17 mm	17 mm	16 mm	20 mm
2	*Proteus* sp.	18 mm	16 mm	15 mm	13 mm	18 mm
3	*E.coli*	13 mm	11 mm	10 mm	9 mm	17 mm

4	*Klebsiella* sp.	11 mm	11 mm	10 mm	10 mm	15 mm
5	*Pseudomonas* sp.	13 mm	13 mm	12 mm	10 mm	15 mm

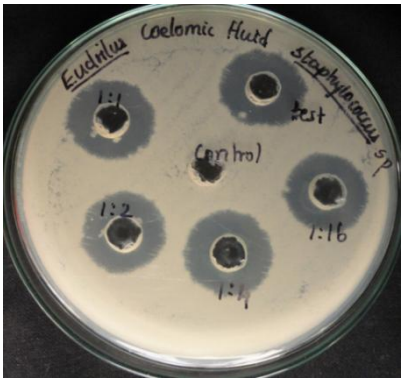

Figure 5: *Eudrilus eugeniae* coelomic fluid fluid against *Proteus* sp

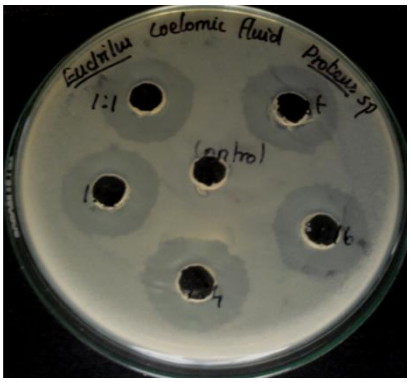

Figure 6: *Eudrilus eugeniae* coelomic against *Staphylococcus* sp

The antimicrobial activity of coelomic fluid of *Pontoscolex coerthrurus* was limited in *Klebsiella* sp. (11mm), *Staphylococcus* sp. (12mm), *Proteus* sp. (14mm), *E.coli* (15mm) and *Pseudomonas* sp. (16mm) in 1.16 ratio. The concentrated fluid displayed broad spectrum of antibacterial activity. The diluted fluids (1:16) also inhibit the pathogens but not as effective as in concentrated fluid. The coelomic fluid of *Pontoscolex corethrurus* inhibits *Staphylococcus* sp. and *E.coli* effectively and the zone of inhibition was 18 mm and 17 mm respectively for concentrated fluid. The *Pseudomonas* sp. was inhibited only a limited level i.e., 12 mm in diameter and for *Proteus* sp. 14 mm in diameter and for *Klebsiella* sp. , 13 mm in diameter (Table 2 and Fig 7, 8 and 9).

Table 2: Antimicrobial activity of coelomic fluid of *Pontoscolex corethrurus* against human pathgens

S.No	Organisms	1:1	1:2	1:4	1:16	concentrated
1	*Staphylococcus* sp.	15 mm	16 mm	17 mm	12 mm	18 mm
2	*Proteus* sp.	11 mm	12 mm	14 mm	14 mm	14 mm
3	*E.coli*	12 mm	16 mm	16 mm	15 mm	17 mm
4	*Klebsiella* sp.	12 mm	11 mm	11 mm	11 mm	13 mm
5	*Pseudomonas* sp.	15 mm	15 mm	16 mm	16 mm	16 mm

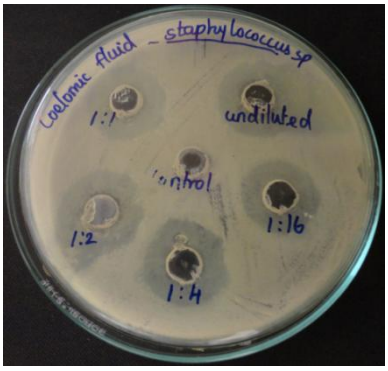

Figure 7: *Pontoscolex corethrurus* coelomic fluid coelomic fluid against *Proteus* sp

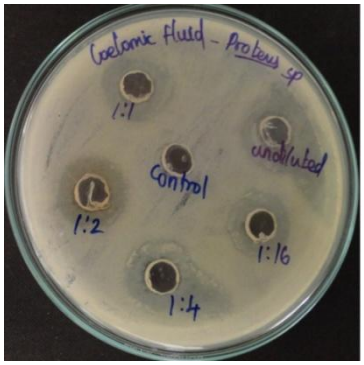

Figure 8: *Pontoscolex corethrurus* against *Staphylococcus* sp

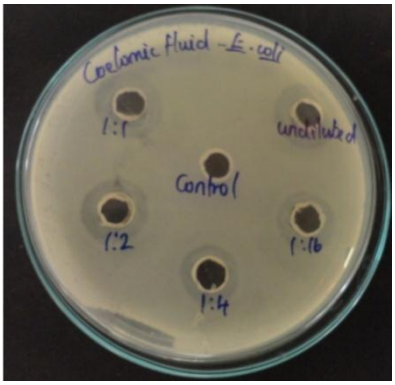

Figure 9: *Pontoscolex corethrurus* coelomic fluid against *E.coli*

Antimicrobial activity of Nano fiber from coelomic fluid

The nanofibers synthesized from coelomic fluid of two different earthworms were coated in the cover slip by spraying. These coated nanofibers were used for the testing of antimicrobial activity. In this method, no zone of inhibition was observed for both *Pontoscolex corethrurus* and *Eudrilus eugeniae*. This may be due to the application of high voltage during electro spraying in the nanofiber synthesis. So the coelomic fluid which was rich in protein may get denatured in this high voltage. Hence the zone of inhibition doesn't occur in the nanofibers synthesized from two different earthworms by two different methods.

Antimicrobial activity of silver nanoparticle from coelomic fluid

The *Staphylococcus* sp. were inhibited in high level i.e. 40 mm followed by *Pseudomonas* sp. (19mm), *E.coli* (20mm), *Klebsiella* sp. (21mm) and *Proteus* sp. (16mm) were found (Table 3) in the nanoparticles from coelomic fluid of earthworm, *Eudrilus eugeniae*. Ag nano showed better result than the bioactivity pattern of normal coelomic fluid.

Table 3: Antimicrobial activity of silver nanoparticles from *Eudrilus eugeniae*

S.No	Organisms	Zone of inhibition (mm)
1	*Staphylococcus* sp.	40
2	*Proteus* sp.	16
3	*E.coli*	20
4	*Klebsiella* sp.	21
5	*Pseudomonas* sp	19

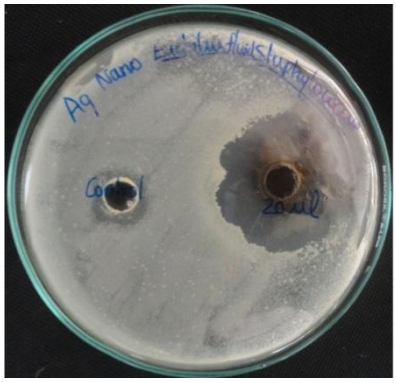

Figure 10: Silver nanoparticles from *Eudrilus eugeniae* against *Staphylococcus* sp.

The Ag nano from *Pontoscolex corethrurus* were used for the antimicrobial activity and shown in Table 4. The *Staphylococcus* sp. were inhibited in high level i.e. 19 mm followed by *Proteus* sp. (17 mm), *Pseudomonas* sp. (7mm), *Klebsiella* sp. and *E.coli* (8mm). *Klebsiella* sp., *E.coli*, and *Pseudomonas* sp. were found to be less sensitive when compared to *Staphylococcus* sp. and *Proteus* sp. Ag nano showed better result than the bioactivity pattern of normal coelomic fluid. It showed best activity even at the low quantity of nanoparticles (Fig 11).

Table 4: Antimicrobial activity of silver nanoparticles from *Pontoscolex corethrurus*

S.No	Organisms	Zone of inhibition (mm)
1	*Staphylococcus* sp.	19
2	*Proteus* sp.	17
3	*E.coli*	8
4	*Klebsiella* sp.	8
5	*Pseudomonas* sp.	7

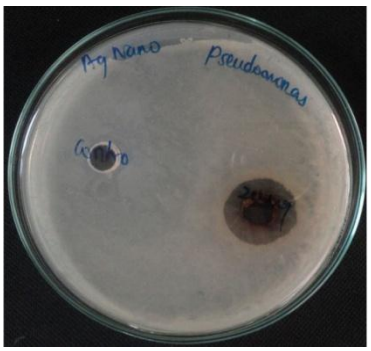

Figure 11: Silver nano particles from coelomic fluid of *Pontoscolex corethrurus* against *Pseudomonas* sp.

Determination of molecular weight of coelomic fluid by SDS-PAGE

The molecular weight of the coelomic fluid was determined by SDS-PAGE. The size of the protein in the fluid was determined as more than 205 KDa in both samples. It showed that the protein was very large in size (Fig.12).

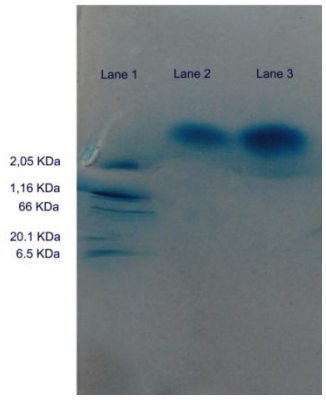

Figure 12: SDS PAGE of coelomic fluid of Earthworms

Lane 1 Protein marker
Lane 2 Coelomic fluid of *E. Eugeniae*
Lane 3 Coelomic fluid of *P. corethrurus*

DISCUSSION

Earthworm's immunity consists of humoral and cellular components and works as specific and non-specific mechanisms which occur in coelomic fluid [11]. The coelomic fluid was harvested in the various methods. Prochazkova et al., [12] says that the earthworms were punctured in the post clitellum segments of the coelomic cavity for the release of fluid. In the present study we selected ice cold treatment. In this method the earthworm doesn't die and the worm was alive. So we selected this particular method for this study. Earthworms contain amazing antimicrobial activity. Cotuk and Dales [13] showed that coelomic fluid of *Eisenia fetida* was ineffective against this bacterium. These differences in results support to compare with our result. In this present study the coelomic fluid of *Pontoscolex corethrurus* and *Eudrilus eugeniae* earthworm was effective against all the tested pathogens. In the hemolytic study of Prochazkova et al., [12], the coelomic fluid of *E.fetida* earthworm have two main hemolytic proteins fetidin and lysenin, although it has not yet been convincingly shown whether these proteins are isoforms or encoded by two independent genes. The earthworm fluid was distributed in four groups based on hemolytic patterns; the fourth group was good agreement with the very low hemolytic activity assessed in suspension. Roch [8] found six different hemolytic patterns based on the presence of four isoforms of the 40 k Da hemolytic. In our study, we used the two different earthworms coelomic fluid and to check the hemolytic activity against human erythrocytes. The coelomic fluid of *Eudrilus eugeniae* lysed the blood cells very effectively even at low concentration, but the *Pontoscolex corethrurus* coelomic fluid lysed the human erythrocytes only in high concentration. Also the bioactivity of the silver nanoparticles synthesized from coelomic fluid was better than the natural coelomic fluid. The low amount of sample was used for this study but the activity was found high compared to the activity of normal coelomic fluid. Among the five different organisms, the *Staphylococcus* sp. was highly inhibited than another organism. The zone of inhibition found for *Pontoscolex corethrurus* was 19 mm in diameter and the fluid of *Eudrilus eugeniae* showed 40 mm zone of inhibition. The nanofiber from coelomic fluid showed no antimicrobial activity, because high voltage is used to synthesise the nanofibre, this might denatured the bioactive molecules in the coelomic fluid. In this study, we selected the silver nanoparticles synthesized from coelomic fluid. The colour change occurs in the *Boswella ovalifoliolatus* within 10 min, the visual colour change in broccoli was observed within 2 minutes in microwave oven condition, within 30 minutes at 34^{o}C, 45 minutes at 27^{o}C [14], and in *Morinta tinctoria* was observed within 10 minutes [15] but in the coelomic fluid, the colour change was appeared after 2 days. After colour change the silver nanoparticles had been confirmed by measuring the UV-Vis spectrum of the reaction media. The synthesized coelomic fluid was then used for further antimicrobial studies. The molecular size analysis was determined as more than 210 KDa by SDS- PAGE. The study of Hanusova et al., [16] supports our study that they also reported the protein of more than 200 KDa.

Perionyx excavates contains high protein, nitrogen and fat content [17] and his study also supported this research.

CONCLUSION

To optimize this drug delivery system, greater understanding of the different mechanism of biological interaction, and particle engineering, is still required. Further advantages are needed in order to turn the concept of nanoparticle technology into a realistic practical application as the next generation of drug delivery system. Nanofibers matrices are currently being explored as musculoskeletal tissue engineering (including bone, cartilage, ligament and skeletal muscle), skin tissue engineering, and control delivery of drugs, proteins, DNA. If low concentrations of coelomic fluid are effective against bacteria but not on vertebrate erythrocytes, it can be used as alternative drug. Moreover, earthworms have been used to treat upper respiratory tract infection, typhoid and diarrheal pathogenic bacteria.

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Conflict of Interest

None

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