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# Investigation of the Antiviral and Antibacterial Potential of Coelomic Fluid from Some Earthworm Species *(Oligochaeta: Lumbricidae)*

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**Abstract**

The coelomic fluid of four earthworm species, *Aporrectodea rosea*, *Eisenia fetida*, *Lumbricus terrestris* and *Octolasion lacteum,* was investigated against Gram-negative bacteria strains: *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas luteola* and human pathogen Herpes simplex virus type 2 (genital herpes). The research revealed that only studied material from *Eisenia fetida* acted selectively on pathogenic bacteria. The greatest growth-inhibiting effect was observed on *Aeromonas hydrophila*. No antiviral potential was demonstrated against Herpes simplex virus. The metabolic components of the coelomic fluid have a strong cytolytic effect, but lack any activity against the tested virus pathogen.
**Key words:** coelomic fluid, Pseudomonas, Aeromonas, Herpes simplex virus, earthworms, Lumbricidae

**Резюме**

Влиянието на целомната течност от четири вида дъждовни червеи: *Aporrectodea rosea*, *Eisenia fetida*, *Lumbricus terrestris* и *Octolasion lacteum* е изследвано върху някои Грамотрицателни бактерии: *Aeromonas hydrophila*, *Pseudomonas fluorescens* и *Pseudomonas luteola*, както и спрямо Herpes simplex virus тип 2 (генитален херпес). Получените резултати показват, че само *Eisenia fetida* има изразен антибатериален ефект върху изследваните микроорганизми като ефекта е най-силен спрямо *Aeromonas hydrophila*. Метаболитните компоненти на целомната течност имат силна цитолитична активност при въздействие на използваната от нас клетъчна линия, но не повлияват репликацията на човешкият херпесен вирус тип 2.

## Introduction

Earthworms live in an environment abundant in pathogens. Some of those pathogens are bacteria and viruses. Earthworms lack true antibodies, hence an adaptive immune response and the coelomic fluid act as an efficient innate immune system to defend them against invading pathogens (Liu *et al.*, 2004).

The coelomic fluid of the earthworm *Eisenia fetida (Oligochaeta: Lumbricidae)*, containing more than 40 proteins, exhibits several biological effects as follows: cytolytic, proteolytic, hemolytic, hemagglutinating, tumorstatic, mitogenic, and bacteriostatic activities (Lange *et al.*, 1997*)*. The cytolytic components secreted by coelomocytes into the coelomic cavity are of particular interest in view of their potential clinical applications. They include factors displaying hemolytic activity accompanied by antibacterial/bacteriostatic effects against pathogenic soil bacteria (Roch *et al.*, 1979; Valembois *et al.*, 1982; Roch *et al.*, 1992). The second group includes factors exerting lytic activity towards other cell types, particularly tumor cells (Bilej *et al.*, 2002).

*Aeromonas hydrophila* can cause gastroenteritis in humans, occurring mostly in young children and people with compromised immune systems. These bacteria are linked to two types of gastroenteritis. The first type is rice-water diarrhea. The other type is dysenteric gastroenteritis, which causes loose stools filled with blood and mucus. *Aeromonas hydrophila* also causes diseases such as myonecrosis and eczema in people with com-

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promised or suppressed immune systems (Janda and Abbott, 1998). In very rare cases it can cause necrotizing fasciitis (Minnaganti *et al.*, 2000). *Aeromonas hydrophila* is proved to be an earthworm pathogenic bacterium, especially to *Eisenia fetida* (Lassegues *et al.*, 1989). *Pseudomonas luteola* is a saprophyte. It is an opportunistic pathogen that particularly affects patients with health disorders. Most reported cases showed septicemia, meningitis, endocarditis or peritonitis (Chihab *et al.*, 2004). Gershman *et al.* (2008) reported that *Pseudomonas fluorescens* is an unusual cause of disease in humans, and usually affects patients with compromised immune systems (e.g., patients on cancer treatment).

Herpes simplex virus type 2 (*HSV-2*) is a sexually transmitted pathogen that infects more than 500 million people worldwide and causes an estimated 23 million new infections each year. It is predominantly associated with genital infection (Johnston *et al.*, 2011).

The aim of our study was to investigate the antiviral and antibacterial potential of the coelomic fluid from earthworms *Aporrectodea rosea*, *Eisenia fetida*, *Lumbricus terrestris* and *Octolasion lacteum* against Gram-negative bacteria strains: *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas luteola*, and the human pathogen Herpes simplex virus *type 2* (genital herpes).

## Materials and methods

*Collection area*

The field investigations were carried out during the spring of 2015 in Leached Vertisols (Bozhurishte, Bulgaria). Earthworms were collected by digging and hand-sorting. Four lumbricid species were found: *Aporrectodea rosea* (Savigny, 1826), *Eisenia fetida* (Savigny, 1826), *Lumbricus terrestris* (Linnaeus, 1758) and *Octolasion lacteum* (Örley, 1881).

*Coelomic fluid sampling*

The earthworms were washed in running distilled water. They were placed on filter paper to remove excess water droplets and were taken into a glass petri dish. The lumbricids were induced to extrude coelomic fluid through the epidermal dorsal pores by 5 V electric stimulation. Then the stimulated earthworms in petri dishes were rinsed with pH 6.8 phosphate-buffered saline (PBS). The collected coelomic fluid containing PBS was centrifuged at 5000 rpm for 10 minutes to sediment coelomocytes and particulate materials. The supernatant was carefully removed and filter sterilized through 0.2 μm (pore size) syringe filter. The filtrate of the coelomic fluid that was free from any suspended cells or coelomocytes (CF) was stored in aliquots at -20°C for subsequent use.

*Antibacterial assay*

The strains used for determining antimicrobial activity included soil pathogen bacteria: *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas luteola* obtained from the Department of Soil Microbiology, Institute of Soil Science, Agrotechnologies and Plant Protection "N. Poushkarov", Sofia, Bulgaria. Bacterial cells were inoculated in nutrient agar with a final concentration of $10^8$ colony-forming units/mL on a 90-mm Petri dish. After the top agar hardened, it was placed on sterilized blotting paper (about 7 mm in diameter), free from any antibacterial activity, and was impregnated with 20 μL of the earthworm coelomic fluid to be tested and placed on agar dishes inoculated with one bacterial strain. The dishes were incubated overnight at 28°C. Control tests were performed with papers impregnated with sterile saline solution (0.9% NaCl). Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 7-mm papers.

*Cells and viruses*

In the study MDBK (Madine and Darby bovine kidney) cells were used, grown in *Dulbecco's* Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (with Gentamycin 8 μg/ml and 10 mM HEPES buffer). The maintenance medium contained fetal calf serum with reduced concentration of 2.5%.

This study employed the BA strain of HSV-2. The virus was propagated in MDBK cells and stored at -70°C until used. The virus titer was determined by cytopathic effect (CPE) assay using the method of Reed and Muench (Reed and Muench, 1938) and plaque assay (Dulbecco, 1952) in MDBK.

*Cell toxicity*

The coelomic fluid of the collected earthworm species was twice diluted in maintenance media just before the experiments (*ex tempore*). The concentration of the coelomic fluid is presented as a percent (%) in each dilution.

Cell toxicity was monitored by determining the effect of the studied materials from the explored earthworm species on cell morphology and cell viability. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, i.e. loss of monolayer, rounding, shrinking of cells, granulation, vacuolisation in the cytoplasm. Cell viability was determined by a colori-

metric method (MTT assay) (Mosmann, 1983). It is based on the reduction of MTT [3-(4, 5-dimethylthiazol-2ol)-2,5diphenyltetrazolium bromide), Sigma Chem., Co. St. Louis, USA], by the mitochondrial enzyme succinate dehydrogenase of the viable cells, which develops a formazan blue colour product. Confluent monolayers of MDBK cells in 96-well plates were overlaid with 0.1 ml/well maintenance medium supplemented with 2.5% FCS (with Gentamycin 8 µg/ml and 10 mM HEPES buffer), 0.1 ml/well of the dilutions of the obtained coelomic fluid (3 well/dilution) and were incubated at 37°C for 48 h. Minimum 3 wells in the plate were used as controls and were not inoculated (only maintenance medium was added). On the second day, 20 µl of MTT (5 mg/ml in phosphate buffered saline (PBS)) was added to each cell; the monolayers were incubated for 3–4 h at 37°C. The resulting formazan precipitate was dissolved in dimethyl sulfoxide (DMSO). After a few minutes at room temperature to ensure that all crystals were dissolved, the optical densities (OD) were determined by Multiscan MX plate reader with a 540 nm. The percentage of viable treated cells was calculated in relation to the untreated controls:

[(OD)exp.)/(ODcell control)] x 100, where ($OD_{exp.)\ and}$ ($OD_{cell\ control}$) indicate the absorbencies of the test sample and the cell control, respectively. The 50% cytotoxicity concentration ($CC_{50}$) was determined as the test compound concentration required for reduction of cell viability by 50%.

*Virucidal assay*

The direct virus inactivating effect of the studied materials was tested by a direct contact assay. Undiluted stock virus suspensions were treated with equal volumes of the coelomic fluid MNC (maximal nontoxic concentration), prepared in DMEM supplemented with 2.5% FCS and incubated at 37°C for 5', 15', 30', 60', 120', 240' and 360' (in an eppendorf tube). Compound free DMEM, supplemented with 2.5% FCS, was used for viral control. At the end of each time interval, the control and the treated viruses were frozen, and the difference in the biological activities between them was determined on the basis of infectivity. The surviving infectious virus titers were determined using the method of Reed and Muench (Reed and Muench, 1938).

*Cell protection assay*

A modification of an MTT assay developed for screening anti-HSV compounds (Takeuchi *et al.*, 1991*)* wasused. Confluent monolayers in 96-well plates were overlaid with 0.1 ml/well of virus suspension (low multiplicity of infection (MOI) 100 $TCID_{50}$ (tissue culture infectious dose)/well, 0.0039 PFU (plaque forming unit)/cell). The plates were incubated for 1 hour at 37°C for virus adsorption and dilutions of the obtained coelomic fluid. Maintenance media were added for virus control. Uninoculated cells were used for cell control. On the fifth day, 20 µl of MTT (5 mg/ml in PBS) was added to each well and the monolayers were incubated for 3–4 h at 37°C. The medium with MTT was removed and the resulting formazan precipitate was dissolved in DMSO. The extinctions were determined at λ=540 nm. The percentage of protection was calculated by the following formula: $[(OD_{exp.})-(OD_{virus\ control})/(OD_{cell\ control})-(OD_{virus\ control})]$ x100, where ($OD_{exp.}$), ($OD_{virus\ control}$), and ($OD_{cell\ control}$) indicate the absorbencies of the test sample, the virus control and the cell control, respectively.

## Results

*Cellular toxicity*

The coelomic fluid obtained from the collected earthworm species was applied in concentrations ranging from 0.39 % to 25 %. Coelomic fluid from three of the lumbricid species *Aporrectodea rosea* (Savigny, 1826), *Lumbricus terrestris* (Linnaeus, 1758) and *Octolasion lacteum* (Örley, 1881) showed no visual change in the cell monolayer integrity (daily microscopic observation) or any difference in viability of the treated cells and controls, even in the lower dilutions (25%). Unlike them, the studied material from *Eisenia fetida* (Savigny, 1826) behaved in a different way (Fig. 1).

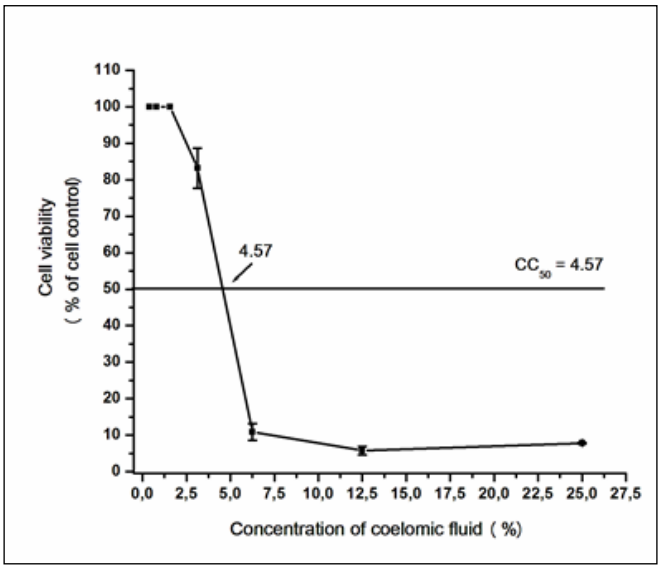


**Fig. 1.** Toxicity of coelomic fluid from *Eisenia fetida* against MDBK cell line

It shows toxicity against MDBK cell line in a dose-dependent manner. According to the MTT test, cell viability was very low in a concentration range between 25 % and 6.25 %. Cells reached 50 % viability (cytotoxic concentration 50 ($CC_{50}$) at a concentration of 4.57% of coelomic fluid.

*Antiviral activity of coelomic fluid*

*Virucidal activity*

For more complete understanding, we tested the direct virus inactivating effect against HSV-2, strain BA of the obtained coelomic fluid from all collected earthworm species by direct contact assay. Undiluted stock virus suspensions were treated with equal volumes of studied materials in MNC for different time intervals. Unfortunately, we obtained for none of the coelomic fluid from each earthworm species change in titer of the treated virus on virus control (no virucidal activity for any of the time intervals).

*Cell protection activity*

The antiviral potential of the coelomic fluids against the replication of HSV-2 strain BA was tested. The obtained coelomic fluid from three of the earthworm species *Aporrectodea rosea* (Savigny, 1826), *Lumbricus terrestris* (Linnaeus, 1758) and *Octolasion lacteum* (Örley, 1881) was applied in concentrations ranging from 25 % (corresponding to their MNC) to 0.39 %. The obtained materials from the forth of the earthworm species *Eisenia fetida* (Savigny, 1826) was applied in concentrations ranging from 1.56 % (corresponding to its MNC) to 0.097 %. Unfortunately, no antiviral activity was found according to the modified MTT assay (no improvement of cell viability of infected and treated cells in comparison with virus controls was measured).

*Antibacterial function of coelomic fluid*

The observations with coelomic fluid of earthworm species: *Aporrectodea rosea*, *Lumbricus terrestris* and *Octolasion lacteum* showed that they do not have cytolytic or antibacterial activity. Only the coelomic fluid of *Eisenia fetida* affects microbial growth. Three bacteria strains were involved in the experiment. The inhibitory effect against *Aeromonas hydrophila* was the largest, with a 17 mm average diameter. Similar results were obtained for bacteria strains from genus *Pseudomonas. Pseudomonas luteola* has inhibition zone of 11.66 mm and *Pseudomonas fluorescens* with 11 mm (Fig. 2). The *Eisenia fetida* coelomic fluid has a bacteriostatic effect on the studied pathogenic microorganisms.

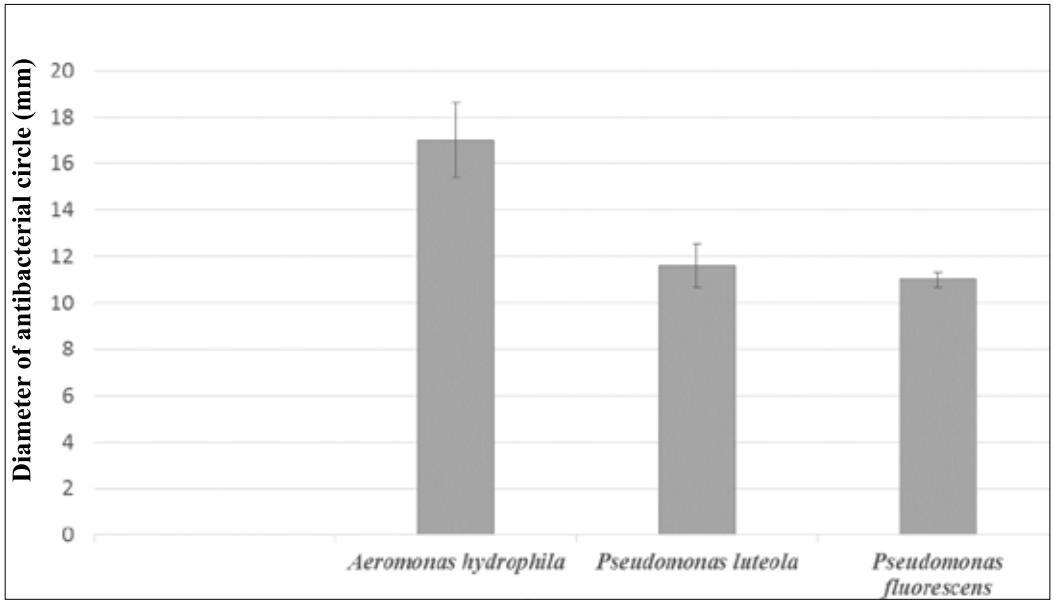


**Fig. 2.** Antibacterial activity of coelomic fluid of *Eisenia fetida*

## Discussion

Few studies have revealed antibacterial activity of earthworm extract to *Pseudomonas aeruginosa* and *Pseudomonas pyocyanea* (Liu *et al.*, 2004; Wang *et al.*, 2007). To our knowledge, the current paper is the first exploration of the effect of earthworm extract against Gram-negative bacteria *Pseudomonas fluorescens*. The coelomic fluid of the earthworm *Eisenia fetida (Oligochaeta, Lumbricidae)* was demonstrated to possess antimicrobial activity directed against earthworm pathogenic bacteria *Aeromonas hydrophila* (Lassegues *et al.*, 1989; Valembois *et al.*, 1982). Antibacterial effect of *Dendrobaena veneta (Oligochaeta, Lumbrici-*

*dae)* on *Pseudomonas luteola* was observed by Arslan-Aydoğdu and Çotuk (2008). Until now, only few research papers regarding the antiviral activity of coelomic fluid from earthworm have been published. Lui *et al.* (2008; 2012) demonstrated antiviral effect against influenza and adeno viruses.

Our research revealed that only the coelomic fluid secreted from the dorsal pores of epigeic earthworm *Eisenia fetida* has bactericidal activities. The results indicated that the studied materials can affect the growth of Gram-negative bacteria from genus *Aeromonas* and *Pseudomonas* and the effect is bacteriostatic.

The antiviral experiments involved investigation of the cellular toxicity of the coelomic fluid of four earthworm species against the cell line used. Only the studied material from *Eisenia fetida* affected cell growth. Microscopic observation of the cell monolayer one hour after adding the dilutions of the coelomic fluid revealed changes in the monolayer integrity and cell morphology. This means that toxicity develops quickly and is probably connected with the disturbance of the cell membrane integrity and functions. Presumably, this quickly developing toxicity is due to proteins capable of forming pores into the membrane, such as lysenin (Sekizawa *et al.*, 1996) and eiseniapore (Lange *et al.*, 1997). Unfortunately, no activity against the replication of Herpes simplex virus type 2 (genital herpes) or its extracellular forms (virucidal activity) was observed.

Unlike vertebrates, earthworms lack real anti-bodies and efficient innate immune systems to defend themselves against invading foreign materials (Wang *et al.*, 2007). So, the coelomic fluid of *Eisenia fetida* plays a very important role in antibacterial defense (Valembois *et al.*, 1982). Coelomic fliud has various metabolic complements, which inhibit the growth of potent worm pathogenic bacteria (Lassegues *et al.*, 1989).

Further studies are required to estimate the mechanism of action of coelomic fluid on bacteria, viruses and eucariotic cells. Proper utilization of active peptide components of coelomic fluid may lead to discovery of a new way to manage bacterial or tumor cells growth.

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